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Uptake of pharmaceuticals and personal care products in *Lactuca sativa*: Stress responses in lettuce and on the plant-associated bacterial community composition

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Summary

As severe and long-lasting drought events and water stress occur more frequently in many regions in the world, alternative water sources are becoming increasingly relevant. Treated wastewater can therefore be an alternative water source for the irrigation of crops to reduce the pressure on our water reserves. However, treated wastewater can also contain residual organic microcontaminants such as pharmaceuticals and personal care products (PPCPs), which are applied through the irrigation water to agricultural soils and can even be taken up and translocated in crops. Lately, several studies have been published studying the effects of these compounds on plants and soil organisms. Nevertheless, there is still a lack of knowledge when it comes to the influence of environmentally relevant concentrations of PPCPs on the expression of plant stress related genes and on the composition and diversity of plant-associated bacterial communities in combination with the analysis of the plant uptake and metabolization of the compounds.

To investigate whether different PPCPs can trigger various stress responses in plants a hydroponic experiment was conducted where lettuce plants (*Lactuca sativa* L.) were individually treated with either diclofenac or lamotrigine at environmentally relevant concentrations for 48 hours. Both compounds were detected in lettuce roots and lamotrigine was furthermore translocated to lettuce leaves. After 6 hours the diclofenac phase I metabolite 4'-hydroxydiclofenac was quantified and after 24 hours the concentration of the metabolite was even higher than that of the parent compound. Furthermore, the expression of several stress related genes was transiently reduced in lettuce roots treated with diclofenac. With decreasing concentrations of this compound, the observed influence on the gene expression was reduced. In contrast, lamotrigine was shown to putatively trigger a phase shift in the diurnal expression of stress related genes and might act as a *zeitgeber*. Comparing the stress enzyme activities, only the guaiacol-peroxidase activity was influenced by lamotrigine exclusively in lettuce roots. The tested concentration of diclofenac did not affect the activity of any of the selected stress enzymes in lettuce roots or leaves.

Besides the changes triggered by PPCPs on the plant itself, also the plants' associated bacterial communities can be influenced. A greenhouse experiment in soil-filled pots was performed on the one hand to analyze the uptake and translocation of PPCPs in lettuce and on the other hand to study the influence on the lettuce root-associated bacterial communities using 16S rRNA amplicon sequencing after the exposure to a complex mixture of fourteen spiked PPCPs or to real or spiked treated wastewater. Additionally, to test for the accumulation of PPCPs, a consecutive, second cultivation campaign was performed in the same soil after the harvest of the first experiment. In this second experiment, translocation to the lettuce leaves

of thirteen of the fourteen spiked compounds was detected. Furthermore, higher concentrations of PPCPs were detected in roots, when lettuce plants were irrigated with spiked treated wastewater compared to spiked tap water. Interestingly, the results of the present thesis also revealed a more pronounced effect of treated wastewater compared to PPCPs at the tested concentrations of 10 and 100 µg/L on the lettuce-associated bacterial community composition and diversity. In this regard, a reduced alpha diversity at the end of the second cultivation campaign and a changed beta diversity and community composition at the end of both campaigns was monitored in lettuce roots when plants were irrigated with treated wastewater. Moreover, an influence of the PPCP concentration on the beta diversity and a changed community composition after the treatment with PPCPs in the worst-case scenario at the end of the second cultivation campaign was also observed. Four genera were significantly affected by the irrigation with treated wastewater and two by PPCPs at a concentration of 100 µg/L. The relative abundance of the taxonomic clade *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was increased, and the abundance of *Haliangium* which had previously been published to be susceptible to veterinary antibiotics decreased in response to the PPCPs.

In the further context of the present thesis, during a data analysis of 53 published ISI scientific articles and one technical report a great importance was identified of collecting appropriate meta data such as growth media parameters or environmental conditions during the experimental phase of experiments for studying the uptake and translocation of PPCPs in plants. This information can be useful to reduce potential misinterpretations of results and to make studies more comparable.

Finally, it can be summarized that the exposure to PPCPs at environmentally relevant concentrations can trigger significant changes in the plant stress gene expression even though the uptake rates were relatively low. Moreover, the lettuce root-associated bacterial community composition and diversity was influenced by treated wastewater but also in response to PPCPs in a worst-case scenario. As a conclusion of the present thesis, the influence of environmentally relevant concentrations of PPCPs on the crops' biochemistry and physiology and also on its associated bacterial communities should not be underestimated. Due to these complex interactions, it might be still too early to exclude negative environmental and economic consequences completely.

Zusammenfassung

Durch immer häufiger auftretende Dürreereignisse und Wassermangel in vielen Regionen der Welt gewinnen alternative Wasserquellen zunehmend an Bedeutung. Behandelte Abwässer können hierbei eine alternative Wasserquelle für die Bewässerung von Nutzpflanzen darstellen, um so den Druck auf unsere Frischwasserreserven zu verringern. Jedoch kann das behandelte Abwasser noch Rückstände von organischen Kontaminationen wie Pharmazeutika und Körperpflegeprodukte (auf Englisch: Pharmaceuticals and personal care products; kurz: PPCPs) enthalten, welche durch die Bewässerung in landwirtschaftliche Böden eingetragen und sogar von Nutzpflanzen aufgenommen und in der Pflanze verteilt werden können. In den letzten Jahren wurden mehrere Studien veröffentlicht, welche die Auswirkungen dieser Verbindungen auf Pflanzen und Bodenorganismen untersucht haben. Gleichwohl besteht immer noch ein Wissensdefizit, wenn es um den Einfluss von PPCPs in umweltrelevanten Konzentrationen auf die Expression von pflanzlichen Stressgenen und auf die Zusammensetzung und Diversität der pflanzenassoziierten bakteriellen Gemeinschaften in Kombination mit der Analyse der Aufnahme und Metabolisierung der Fremdstoffe in Pflanzen geht.

Um zu untersuchen ob verschiedene PPCPs unterschiedliche Stressreaktionen in Salat auslösen können, wurden Salatpflanzen (*Lactuca sativa* L.) in einem hydroponischen Experiment entweder mit Diclofenac oder Lamotrigin in umweltrelevanten Konzentrationen für 48 Stunden behandelt. Beide Verbindungen konnten in den Salatwurzeln festgestellt werden, und Lamotrigin wurde zusätzlich noch in den Salatblättern detektiert. Bereits nach 6 Stunden konnte der Diclofenac-Metabolit 4’ Hydroxydiclofenac gemessen werden und nach 24 Stunden war dessen Konzentration sogar höher als die der Muttersubstanz. Die Expression mehrerer Stressgene war nach der Behandlung mit Diclofenac in Salatwurzeln transient reduziert, wobei bei einer geringer werdenden Konzentration der Muttersubstanz Diclofenac ein reduzierter Einfluss auf die Stressgenexpression festgestellt werden konnte. Im Gegensatz dazu konnte gezeigt werden, dass Lamotrigin potenziell die circadiane Rhythmik der Expression von Stressgenen beeinflusst und als eine Art Signal oder *Zeitgeber* fungieren könnte. Wenn man nun die Enzymaktivitäten von pflanzlichen Stressenzymen vergleicht, hat lediglich Lamotrigin zu einer veränderten Aktivität der Guajacol-Peroxidase geführt und das auch nur in den Salatwurzeln. Die Diclofenac-Konzentration war vermutlich zu gering, um Unterschiede in der Aktivität der getesteten Stressenzyme auszulösen.

PPCPs können jedoch nicht nur einen Einfluss auf die Pflanzen selbst, sondern auch auf die pflanzenassoziierten bakteriellen Gemeinschaften haben. Ein Gewächshausexperiment wurde durchgeführt, um einerseits die Aufnahme und Verteilung von PPCPs in Salatpflanzen

zu analysieren und um andererseits den Einfluss auf die salatassoziierte bakterielle Gemeinschaft mittels 16S rRNA Amplikon-Sequenzierung nach der Behandlung mit 14 verschiedenen PPCPs bzw. mit normalem behandeltem Abwasser oder mit solchem, das mit den 14 PPCPs angereichert wurde, zu untersuchen. Die Salatpflanzen wurden hierfür in mit Erde gefüllten Töpfen kultiviert. Zusätzlich, und um die Anreicherung von PPCPs im Boden zu testen, wurde eine zweite, konsekutive Kulivierungskampagne nach der Ernte der ersten Pflanzen durchgeführt. Dabei konnten dreizehn der vierzehn angereicherten Substanzen in Salatblättern nachgewiesen werden. Außerdem konnten höhere Konzentrationen der PPCPs in den Wurzeln von denjenigen Salatpflanzen gefunden werden, welche mit angereichertem, behandeltem Abwasser anstatt mit angereichertem Wasser gegossen worden waren. Interessanterweise zeigten die Ergebnisse der vorliegenden Arbeit ebenfalls einen stärkeren Effekt des behandelten Abwassers auf die Zusammensetzung und Diversität der salatassoziierten bakteriellen Gemeinschaft im Vergleich zu der Behandlung mit 14 PPCPs bei einer Konzentration von 10 and 100 µg/L. Dabei konnte eine reduzierte Alpha-Diversität am Ende der zweiten Kulivierungskampagne sowie eine veränderte Beta-Diversität und Zusammensetzung der bakteriellen Gemeinschaft am Ende von beiden Kulivierungskampagnen nach der Verwendung von behandeltem Abwasser identifiziert werden. Ungeachtet dessen hatten auch die PPCPs einen Einfluss auf die Beta-Diversität, und auch die Zusammensetzung der bakteriellen Gemeinschaft wurde durch die PPCP-Exposition in einem Worst-Case-Szenario am Ende der zweiten Kulivierungskampagne verändert. Vier taxonomische Genera wurden durch die Behandlung mit behandeltem Abwasser, und zwei Genera durch direkte Applikation der PPCPs in Wasser und in behandeltem Abwasser bei einer Konzentration von 100 µg/L beeinflusst. Dabei war die relative Abundanz von *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* erhöht, wohingegen die von *Haliangium* durch den Einfluss von PPCPs verringert war.

Im weiteren Kontext der vorliegenden Arbeit wurde im Rahmen einer Datenanalyse von 53 publizierten wissenschaftlichen ISI-Artikeln und eines technischen Berichts die hohe Relevanz der Sammlung von Metadaten wie beispielsweise von Umwelt- und Substratbedingungen während der Durchführung von Experimenten, die die Aufnahme und Verteilung von PPCPs untersuchen sollen, festgestellt. Diese Informationen sind nützlich, um potenzielle Missinterpretation der Ergebnisse zu verringern, und um eine verbesserte Vergleichbarkeit von Studien zu erzielen.

Abschließend lässt sich zusammenfassen, dass schon die PPCP-Exposition bei umweltrelevanten Konzentrationen signifikante Änderungen der Expression pflanzlicher Stressgene in Salat auslösen kann, selbst dann, wenn die Aufnahmeraten relativ gering sind. Darüber hinaus kann die Zusammensetzung und Diversität der salatassoziierten bakteriellen

Gemeinschaft durch die Bewässerung mit behandeltem Abwasser aber auch als eine Reaktion auf PPCPs in einem Worst-Case-Szenario beeinflusst werden. Deshalb sollte der Einfluss von umweltrelevanten Konzentrationen von PPCPs auf die Biochemie und Physiologie von Nutzpflanzen und auch auf die pflanzenassoziierten bakteriellen Gemeinschaften nicht unterschätzt werden, denn es könnte beim gegenwärtigen Wissensstand zu früh sein, negative ökologische und ökonomische Konsequenzen komplett auszuschließen.

I. List of Abbreviations

%	Percentage
µg	Microgram
µl	Microliter
µM	Micromolar
A. thaliana	*Arabidopsis thaliana*
A. hydrophila	*Aeromonas hydrophila*
ABC transporter	ATP binding cassette transporters
ACN	Acetonitrile
ADI	Acceptable daily intake
ANOVA	Analysis of variance
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistance gene
ASV	Amplicon sequence variant
AWARE	Project: Assessing the fate of pesticides and waterborne contaminants in agricultural crops and their environmental risks
BCF	Bioconcentration factor
bp	Base pair
BPA	Bisphenol A
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CDNB	1-chloro-2,4-dinitrobenzene
CDPKs	Calcium dependent protein kinases
CIP	Ciprofloxacin
cm	Centimeter
COD	Chemical oxygen demand
CYP	Cytochrome P450
DCF	Diclofenac
DNA	Deoxyribonucleic acid
DTE	Dithioerythritol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
$FeSO_4$	Iron(II) sulfate
g	Gram
GAPDH	Glyceraldehyde-3-dehydrogenase
GSH	Reduced glutathione
GST	Glutathione-*S*-transferase
h	Hours
H^+	Hydrogen ion

H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HESI-MS/MS	Heated electrospray ionization-tandem mass spectrometry
HR culture	Hairy root culture
IBU	Ibuprofen
IRSTEA	Institut national de recherche en sciences et technologies pour l'environnement et l'agriculture
IS	Internal standard
IVCW	Integrated vertical-flow constructed wetlands
kg	Kilogram
L	Liter
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
$log\ D_{OW}$	Logarithmic distribution coefficient
$log\ K_{OW}$	Logarithmic octanol-water partition coefficient
LOQ	Limit of quantification
M	Molar
m/z	Mass-to-charge ratio
MATE transporter	Multidrug and toxic compound extrusion transporters
meq/100g	Milliequivalents per 100 grams of soil
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
MRP	Multidrug resistance proteins
MS	Mass spectrometry
ng	Nanogram
NGS	Next generation sequencing
NH_4^+	Ammonium
nm	Nanometer
NMDS	Non-metric multidimensional scaling
NMR	Nuclear magnetic resonance
NO_3^-	Nitrate
NPFs	Nitrate transporter 1/Peptide transporter family
NSAIDs	Non-steroidal anti-inflammatory drugs
O_2^-	Superoxide radical
OCT	Organic cation transporters
OH^-	Hydroxyl radical
PCDDs	Polychlorinated dibenzo-p-dioxins

PCDFs	Polychlorinated dibenzofurans
PCR	Polymerase chain reaction
pKa	Acid dissociation constant
pmol	Picomole
PPCP	Pharmaceuticals and personal care products
psi	Pounds per square inch
qPCR	Quantitative real-time PCR
QTOF	Quadrupole time-of-flight
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RSD	Relative standard derivation
sec	Seconds
SMRM	Scheduled multiple-reaction-monitoring
SMX	Sulfamethoxazole
SOM	Soil organic matter
sp.	Singular species
spp.	Species pluralis
TF	Translocation factor
Tris	Tris(hydroxymethyl)aminomethane
TRR	Total radioactive residue
TTC	Threshold of toxicological concern
UHPLC	Ultra High Performance Liquid Chromatography
V	Volt
WWTPs	Wastewater treatment plants

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1. Introduction

1.1 Entry of pharmaceuticals and personal care products into agroecosystems

Pharmaceuticals and personal care products (PPCPs) are a diverse group of chemicals including pharmaceutically active ingredients with benefits for human or animal health, or non-medicinal substances for cosmetic applications such as fragrances in lotions and soaps. Pharmaceuticals are used for the prevention, treatment or diagnosis of diseases and can be divided into different classes based on the organ or system on which they act, on their specific mechanism of action, or on their pharmacological and therapeutic properties. In this regard, the most prescribed drug class of the 200 most prescribed drugs in the United States in 2018 was “cardiovascular drugs” which accounted for 24%, followed by pharmaceuticals acting on the central nervous– (21%), on the endocrine– (15%) and on the musculoskeletal system (9.5%) (Fuentes et al., 2018). Since the mid-90s, due to the technical development in the field of analytical chemistry and the more accurate detection of low concentrations (ng/L and µg/L range) of compounds, PPCPs have gained remarkable growing scientific attention as these anthropogenic chemicals have frequently been found in the environment as unwanted pollutants (Mezzelani et al., 2018). Nowadays, the constant increase in the global population, due to reasons such as a higher life expectancy or better access to medical care, is associated to an increased consumption of pharmaceuticals. Moreover, the usage of veterinary drugs for the treatment and prevention of diseases in livestock results in a higher consumption of these substances, especially of antibiotics, antiparasitic drugs and steroidal hormones. In this context, millions of tons of pharmaceuticals are consumed every year in human and veterinary medication, which can be released to the environment, and especially agroecosystems, via different routes.

1.1.1 Routes of PPCPs: From human consumption to agricultural fields

After PPCPs enter the body by oral intake, injection or dermal absorption, substances can be assimilated and excreted as the original compound (30-90%) or as their corresponding metabolites through feces and urine (Christou et al., 2017; Patel et al., 2019). Pharmaceutical residues originating from humans can thus enter the domestic and hospital sewage systems and wastewater treatment plants (WWTPs) by contaminated excreta or by incorrect disposal of unused and expired medication through the toilet. As conventional WWTPs are shown to be only moderately effective in removing the relatively stable substance class of PPCPs from wastewater, complex mixtures of these substances and their metabolites are frequently detected in wastewater effluents. These WWTP effluents are discharged directly into rivers and surface waters or are reused for agricultural irrigation practices and can still contain

residues of the PPCPs in concentrations between ng/L to low µg/L (Bartelt-Hunt et al., 2009; Roberts & Thomas, 2006; Vanderford & Snyder, 2006). Furthermore, besides their release into agroecosystems by the reuse of treated wastewater, residual PPCPs can also reach agricultural fields through the application of sewage sludge from WWTPs or livestock manure used as organic fertilizers on agricultural soils in some countries.

1.1.2 Reuse of treated wastewater for agricultural practices

The demand for freshwater in agriculture is enormous. Agricultural practices, especially the irrigation of crops accounts for 70% of the global freshwater withdrawals (FAO, 2017). Using treated wastewater for the irrigation of crops can provide a reliable water supply especially in areas experiencing high water stress or severe and long-lasting droughts, which might become more frequently due to climate change. Treated wastewater as an alternative water source potentially reduces the risk of crop failures, famines and income losses and thus has significant environmental, social and economic benefits (Drewes et al., 2017; European Commission, 2022, Climate-ADAPT, 2022). Even more, the irrigation with wastewater can also provide nutrients to crops and therefore reduces the consumption of synthetic fertilizers. A significantly increased average fruit weight and fruit yield of cucumbers (Qaryouti et al., 2015) and an increased diameter and weight of tomato fruits (Al-Lahham et al., 2003) were detected after the crops were irrigated with raw wastewater from food processing industry or with treated wastewater, respectively. This corresponding recovery of the resources water and nutrients is therefore an interesting contribution to the promotion of a circular economy.

Numerous countries all over the world are using treated wastewater for different types of uses and to varying degrees for several years now. Already since 1912 water is reused for the irrigation of parks in California (USA). Furthermore, the irrigation of crops with reused water in Israel goes back to the 1950s and reclaimed wastewater is now covering 50% of the country’s current agricultural water demand (Helmecke et al., 2020; Tal, 2006). In Europe, several southern countries like Spain, Portugal, Greece, Cyprus, Italy and France, but also central and northern countries like Belgium, Sweden, Germany and the UK, are using reclaimed wastewater nowadays under certain conditions. In Spain 71% of the wastewater volume is used for the irrigation of crops (Sato et al., 2013), whereas in Germany reclaimed wastewater is only utilized in two cities, Braunschweig and Wolfsburg. One of the reasons for this limited use in Germany might be the fact that, besides its numerous advantages, reclaimed wastewater can also possess several risks for consumers and the environment.

Conventional WWTPs consisting of two or three treatment stages are only moderately effective in removing emerging contaminants such as PPCPs or per- and polyfluorinated alkyl substances (PFAS). After the irrigation of crops with treated wastewater or the application of biosolids from these WWTPs, the substances can possibly leach to surface- and groundwater bodies with a potential risk for the drinking water or accumulate in soils and even be taken up in plants and agricultural crops (Topp et al., 2008; Lapen et al., 2008; Wu et al., 2023; Shenker et al., 2011; Christou et al., 2017). These mentioned chemical risks therefore depend on different factors such as the composition of wastewater, its treatment, how it is applied to soils/crops, but also on the soil conditions and the climate (Helmecke et al., 2020). Carbamazepine, diclofenac, ibuprofen, gemfibrozil, atenolol, propranolol, and several antibiotics like sulfamethoxazole belong to the most frequently detected pharmaceuticals in the aquatic environment and in wastewaters (Fatta-Kassinos et al., 2011b) with reported concentrations up to several µg/L (Loos et al., 2009; Hirsch et al., 1999; Bendz et al., 2005). As these compounds are discharged and distributed continuously to the environment, they can be considered as “pseudo-persistent” even though they are present in relatively low concentrations (Ebele et al., 2017), leading to chronic exposure with unknown consequences. PPCPs can be present simultaneously with various other foreign substances as a complex mixture in the treated wastewater which may vary between days, seasons or years in their composition and concentration (Diwan et al., 2013; Veach & Bernot, 2011; Pan et al., 2020; Singh & Suthar, 2021; Thomas et al., 2012). The potential risk of complex mixtures of different organic contaminants, as well as their metabolites, can be even more critical than the single molecules per se. However, a cumulative risk assessment of these mixtures is almost impossible as the possibilities of composition and the complexity in the wastewater are innumerable.

Besides the mentioned chemical risks, the use of reclaimed wastewater for agricultural irrigation can also possess biological risks. On the one hand, plant and human pathogens are possibly applied through the reused wastewater on soils and crops (Cui et al., 2020). Relevant human pathogens in this context are not only bacteria such as *Escherichia coli* or *Salmonella spp*., but also intestinal protozoans (e.g. *Giardia* and *Cryptosporidium*), helminths or waterborne viruses (e.g. rota- and adenoviruses) (Gatta et al., 2020). Even though WWTPs can reduce the load of *E. coli* and other bacteria by 1 to 4 log units and several viruses by 1 to 2 log units (Helmecke et al., 2020), a full elimination of these pathogens is almost not possible. On the other hand, the presence of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARG) are an additional known biological risk, which should not be underestimated. Conventional WWTPs are reported to be an important source for the spread and selection of ARB, as well as for the transfer of ARGs. The presence of antibiotic residues

may exert selective pressure on the plethora of bacteria, which are also present in the wastewater leading to an increased emergence and propagation of ARGs, a severe global threat for modern medicine (Wang et al., 2019; Fatta-Kassinos et al., 2011a; Christou et al., 2017).
Another disadvantage and environmental risk of reclaimed wastewater can be the nutrient load. Depending on the function of the WWTP, reused water can contain high amounts of nutrients, especially nitrogen and phosphorous. These nutrients can have ecological and economic benefits when they are used for the substitution of synthetic fertilizers but the concentrations in the reused water should always be considered carefully for the nutrient balance. If too many nutrients are continuously applied, a complete uptake by plants is not possible and excess nutrients might leach into the groundwater, or reach surface waters leading to eutrophication.
All of these mentioned risks and potential negative aspects of reused wastewater can be minimized by using advanced treatment processes downstream of conventional WWTPs. Removal efficacies of emerging contaminants such as antibiotics were shown to be significantly improved by e.g. membrane filtration, reverse osmosis or activated carbon adsorption (Rizzo et al., 2020; Michael et al., 2013). However, these additional treatment stages are time- and cost- intensive and many conventional WWTPs would need an extensive upgrade before implementing such solutions (Helmecke et al., 2020).

1.1.3 Fate of PPCPs in agricultural soils

PPCPs can potentially have ecotoxicological effects on the macro-, meso- and microfauna and on various other functions of the soil (Caracciolo et al., 2015; Gallego et al., 2021; Römbke et al., 2010; Becerra-Castro et al., 2015). The total number of juvenile earthworms was reduced after the application of 30 mg/kg doxycycline (Litskas et al., 2019), however the same antibiotic did not affect the earthworm mortality rates in soil amended with spiked pig slurry (75 or 7500 mg/L) (Fernández et al., 2004). Oxytetracycline is an antibiotic frequently used in livestock and poultry treatment and can thus be often found in soils fertilized with manure. Residual oxytetracycline did not only affect the structure of the soil microbiome as well as soil microbial enzyme activities (e.g. phosphatase and dehydrogenase), it was also observed that the abundance of ARG were significantly increased in soil and lettuce tissues in presence of this antibiotic (De Liguoro et al., 2003; Boleas et al., 2005; Qian et al., 2016). Furthermore, the frequently detected antiepileptic drug carbamazepine was reported to enhance the horizontal transfer of multi-antibiotic resistance genes within and across bacterial genera at environmentally relevant concentrations (Wang et al., 2019), pinpointing the importance of studying the accumulation and distribution behavior of PPCPs in soils.

The fate of pharmaceuticals in arable soils mainly depends on the physico-chemical properties of the molecules as well as on transformation and uptake processes in combination with the soil characteristics and the pedoclimatic conditions. The soil sorption capacity plays an important role for the bioavailability of active substances which can interact with the organic matter and mineral composition of soils by surface adsorption, ion exchange, hydrogen bonding or complexation (Gallego & Martin-Laurent, 2020). Kodešová et al. (2016) indicated that the sorption and degradation of seven tested pharmaceuticals was mainly dependent on the soil type conditions instead of the compound sorption affinity. Furthermore, several studies summarized a dependency on the compound's characteristics and environmental factors (Hiller & Šebesta, 2017; Xu et al., 2021). The sorption of various PPCPs was shown to be positively correlated with the clay or organic carbon content of soils mainly as a result of their high ion exchange capacity (Kodešová et al., 2015). A moderate or strong sorption to soil components was detected for triclosan (Yu et al., 2013), tetracyclines (Hamscher et al., 2005) and paracetamol (Li et al., 2014), whereas a low sorption and therefore a relatively high mobility was observed for diclofenac, ibuprofen, sulfamethoxazole (Lin & Gan, 2011) as well as for carbamazepine (Yu et al., 2013). As a consequence, these mobile compounds can leach to the groundwater or are available for plant uptake (Yu et al., 2013; Boxall et al., 2006; Fatta-Kassinos et al., 2011a).

In addition to the sorption to soil constituents, PPCPs can undergo different biotic and abiotic transformation processes in soils such as photodegradation, hydrolysis or microbial degradation. The photolysis of diclofenac and naproxen under sunlight was direct and rapid whereas ibuprofen was only slowly photodegraded in aquatic media (Packer et al., 2003; Vulava et al., 2016). However, direct photodegradation in soils is very limited due to the reduction of the light intensity underneath the canopy or in the first cm of soil. Only on the surface of pig slurry applied soils photodegradation of sulfonamides and tetracyclines was considerably enhanced but still in lower rates than in water (Thiele-Brun & Peters, 2007). Besides their bulk density and chemical complexity, soils also harbor a high diversity of microbes especially in the area of the rhizosphere. Biotic transformation processes are mainly performed by an ensemble of different non-specific and specific enzymes of bacteria and fungi leading to the partial or complete metabolization and dissipation of pharmaceuticals (Gallego & Martin-Laurent, 2020).

1.2 Occurrence of PPCPs in plants

PPCPs as an important group of wastewater contaminants can be taken up by plants as demonstrated by several studies (Malchi et al., 2014; Goldstein et al., 2014; Boxall et al., 2006). The majority of plant uptake studies were performed on the accumulation of PPCPs in edible crops but also on plants used for the phytoremediation of these contaminants e.g. in constructed wetlands. Typically, these studies were either performed in hydroponic systems to minimize possible interactions with the substrate, and to focus on the plant-substance interaction, or in potted soil to reflect a more realistic real-life scenario. Thus, uptake studies in soil can be performed in phytochambers, in the greenhouse or on larger scales such as under field conditions. Even though Wu and colleagues (2014) reported relatively low concentrations of PPCPs under real field conditions in eight tested vegetables irrigated with treated wastewater (ranging from 0.01 to 3.87 ng/g dry weight), the analysis of the edible tissues showed a detection frequency of 64% and 91%, after the irrigation with treated wastewater or PPCP-fortified water in all vegetables. Moreover, the irrigation of ten vegetable species (i.a. carrot, lettuce, potato and zucchini) under field conditions with real treated municipal wastewater resulted in the detection of 12 micropollutants in all of the samples in concentrations between 1.7 and 216 ng/g of dry weight (Riemenschneider et al., 2016).

1.2.1 Plant uptake and translocation of PPCPs from soil

The uptake and translocation of PPCPs in plants does not only differ across plant species but also between plant tissues and is furthermore dependent on the physico-chemical properties of the compounds, the composition of the soil and on environmental conditions (Eggen et al., 2011; Wu et al., 2012; Nason et al., 2018; Dodgen et al., 2015).

Only organic substances which are dissolved in soil pore water are considered for the possible uptake by plants. The sorption to soil organic matter (SOM) by chemical interactions such as hydrogen bonding, cation-/anion-exchange or surface complexation plays an important role for the bioavailability of PPCPs. Increased SOM contents consequently lead to higher sorption and reduced uptake rates of the contaminants. Litz and colleagues (2007) for example detected an increased uptake of polycyclic musks by carrot roots from soils with decreased SOM contents. However, by the secretion of root exudates such as organic acids or by H^+ and OH^- ions, plants can also induce changes in the rhizosphere close to the plant roots (~2-3 mm) and therefore alter the bioavailability of organic substances directly or indirectly by increasing the microbial mineralization (Hinsinger et al., 2003).

In general, substances can enter the plant roots through the epidermis of the growing root tips such as the root hairs, as more mature parts of the roots might be covered by a relatively impermeable exodermis. If they do not accumulate in the roots and tubers directly in contact

with the soil, once having passed the epidermis, solutes have to cross the cortex and the endodermis before reaching the vascular system, i.e. the xylem and phloem, to be transferred to the aboveground tissues.

For the transport within the roots two different pathways exist; the symplastic pathway from the cytosol of one cell to the other through interconnecting plasmodesmata or membrane permeation, and the apoplastic pathway along cell walls within the intercellular space. For the apoplastic pathway, the casparian strip which is a lignin and suberin containing impregnation in the center of the endodermis than stops the transfer of solutes as a hydrophobic barrier and compounds must cross at least one lipid bilayer to enter the vascular bundle or they tend to accumulate in the roots (Miller et al., 2016; Bigott et al., 2020). In contrast, solutes which are transported via the symplastic pathway can cross the casparian strip, access the xylem and can therefore move to the aboveground tissues through the transpiration flow.

The distribution of compounds among different plant tissues is often not dependent on only one parameter, but on the interaction of various ones. The interaction of PPCPs with each other or of PPCPs with heavy metals, metalloids or micro- and macroelements has already been published in several studies (Goldstein et al., 2018; Papaioannou et al., 2019; Papaioannou et al., 2020). Synergistic effects were detected in cucumber plants (*Cucumis sativus*) when lamotrigine and carbamazepine were present under hydroponic conditions with enhanced uptake rates for lamotrigine but unaffected concentrations of carbamazepine (Goldstein et al., 2018). Furthermore, a study focusing on the interaction of PPCPs and heavy metals in beet root (*Beta vulgaris* L.) revealed effects of an increased concentration of sulfamethoxazole but a decreased accumulation of metoprolol with increasing concentrations of a mixture of heavy metals. However, the concentration of most of the PPCPs was not influenced by the presence of the heavy metals (Papaioannou et al., 2019).

The molar mass of the compounds is one of the important parameters which influences their uptake and translocation within the plant. Small molecules (≤300 g/mol) can in general enter the roots through the epidermis (Chuang et al., 2019), where further physico-chemical properties of the molecules such as the lipophilicity or the charge plays a crucial role (Figure 1). Lipophilic substances are potentially adsorbed to root lipids like membranes (lipid bilayers) and storage lipids. Therefore, the lipophilicity of the compounds as well as the lipid content of the plant tissues are key drivers for the accumulation of PPCPs in roots. For neutral chemicals the octanol/water partition coefficient (log K_{OW}) is an important predictor for the uptake of xenobiotics by plants (Briggs et al., 1982), however for charged compounds the pH-dependent log D_{OW} seems more appropriate (Xing & Glen, 2002). The charge of a molecule can on the one hand lead to the interaction with plant structures such as the negative surface potential of

the cell membranes and on the other hand also cause its trapping within cells or vacuoles and impede their translocation within the plant. In this context, it is relevant to mention that many PPCPs are ionizable and can exist in neutral and charged forms depending on the molecules p*K*a and the different pH conditions of the plant tissues. Substances which are neutral at pH 4−6 might be mobile in the apoplast but if they ionize at pH 7−7.5 a trapping and accumulation within the cells can occur (Miller et al., 2016).

Besides these parameters as well as the concentration of the compounds, the time of exposure or the protein content of the plant tissues, transporter proteins are an interesting field of research to study the uptake of PPCPs. Plants possess a variety of transporter proteins for the uptake and distribution of different structurally diverse endogenous and foreign substances like plant hormones, secondary metabolites (e.g. glucosinolates), glutathione and glutathione-conjugates or metals (Chiba et al., 2015; Tal et al., 2016; Nour-Eldin et al., 2012; Zhang et al., 2004; Korshunova et al., 1999). However, compared to the research of drug transporters in mammals there are not many studies focusing on this topic in plants. Nevertheless, Cui et al. (2015) as well as Eggen and Lillo (2016) postulated that similar to transporter proteins in mammalian cells, cationic and zwitterionic PPCPs like metformin can be possibly transported by organic cation transporters (OCTs) in plants. In this regard, Khalaf et al. (2022) also published findings on a putative involvement of plantal OCTs in the uptake and transport of tramadol. Besides the cation- and zwitterion-specific OCTs, further transporter families such as nitrate and peptide transporters (e.g. NPFs; Nitrate transporter 1/Peptide transporter family) or multidrug and toxic compound extrusion (MATE) transporters in the cell membrane or tonoplast can be relevant in this context (Eggen & Lillo, 2016).

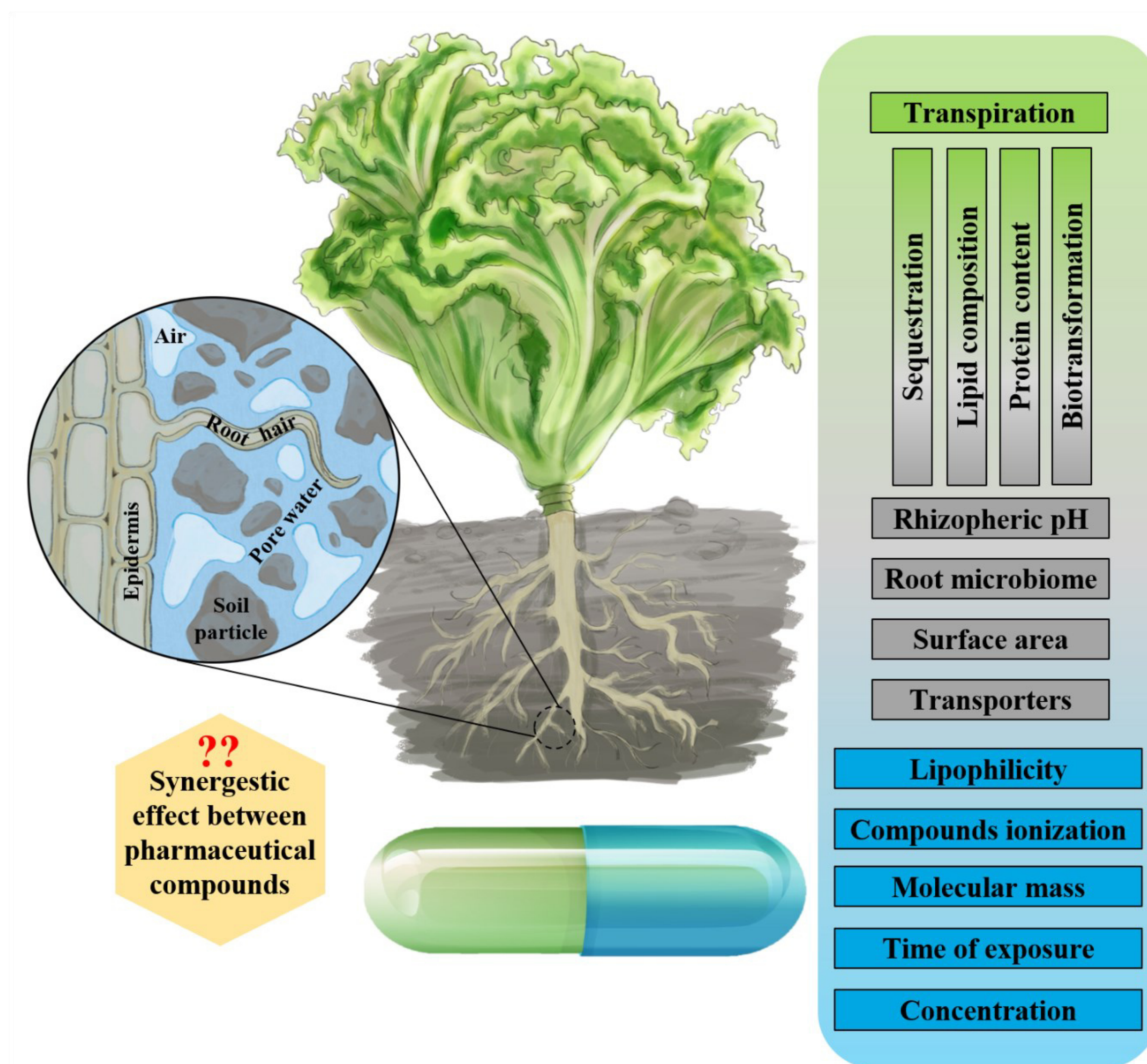

Figure 1: Parameters influencing the uptake and translocation of pharmaceuticals. Reprinted with permission from "*Bigott, Y., Khalaf, D. M., Schröder, P., Schröder, P. M., & Cruzeiro, C. (2020). Uptake and translocation of pharmaceuticals in plants: principles and data analysis. In S. Pérez Solsona, N. Montemurro, S. Chiron, & D. Barceló (Eds.), Interaction and Fate of Pharmaceuticals in Soil-Crop Systems. The Handbook of Environmental Chemistry (Vol. 103, pp. 103-140). Springer, Cham. https://doi.org/10.1007/698_2020_622*". Copyright 2023, Springer Nature.

Once having entered the vascular system, the transpiration flow is one of the most important factors influencing the translocation of PPCPs to the aboveground tissues (Nason et al., 2019). As the transpiration flow is dependent on the transpiration pull and root pressure, it is also dependent on environmental conditions like high light intensity (increased photosynthesis rates and therefore gas exchange), warm temperatures (increased water saturation pressure) and dry air/wind. A well-studied pharmaceutical which is frequently detected in higher

concentrations in aboveground tissues rather than in roots is carbamazepine. Carbamazepine was even present in xylem sap as well as in transpiration waters (Goldstein et al., 2018; Tanoue et al., 2012) most probably because it is a neutral compound with intermediate hydrophobicity (log K_{OW} 3.64). For the translocation of PPCPs moderately lipophilic uncharged substances (log K_{OW} 1-3.5) are preferred, however exceptions exist. These exceptions can be dependent on the compounds physico-chemistry but also on the plant species. Although it was not studied specifically for pharmaceuticals, zucchini was shown to have an enhanced ability to take up and translocate highly hydrophobic polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) to the aboveground tissues (Hülster & Marschner, 1994; Campanella et al., 2002) possibly because of binding substances in the root exudates and leaf extracts which form a complex with the substance to increase its apparent aqueous solubility (Campanella & Paul, 2000). Furthermore, comparable to the uptake of PPCPs by plant roots, the physico-chemical properties of the compounds and the environmental conditions are also highly relevant for the translocation to the aboveground tissues of the plant. Dodgen et al. (2015) reported that the charge of the molecules is important, as a higher accumulation of cationic and neutral PPCPs and much lower concentrations of anionic substances were shown in leaves of carrot, lettuce, and tomato plants. The pH of the nutrient media is an environmental factor additionally influencing the translocation of PPCPs. An altered pH in the rhizosphere changed the accumulation of ionizable lamotrigine but not of carbamazepine in durum wheat. Moreover, an increased translocation of lamotrigine to the leaves was detected when higher concentrations of the neutral species of the compound in porewater were present (Nason et al., 2018).

1.2.2 Metabolism of PPCPs in plants

PPCPs as a class of xenobiotics can be structurally similar to naturally occurring compounds. During millions of years of evolution plants have developed specific mechanisms to metabolize foreign substances and thereby aim to reduce their toxicity. As the processes involved in the plantal metabolization have several similarities with the detoxification of xenobiotics in the liver of animals, Sandermann (1994) proposed a “green liver concept” of metabolization. Like in animals, these metabolization processes of xenobiotics in plants can be divided into three phases: activation (phase I), conjugation (phase II) and compartmentation/sequestration (phase III) as firstly described by Shimabukuro (1976). During the phase I, reactive sites are created on the molecules by e.g. hydrolysis or oxidation reactions. Enzymes involved in the phase I are e.g. cytochrome P450s (CYPs), laccases or hydrolases (Zhang & Yang, 2021). Besides their involvement in the detoxification of xenobiotics, the mentioned CYP enzymes are furthermore known to be involved in various processes such as defense and stress

response mechanisms or the synthesis of pigments and steroids (Pandian et al., 2020; Hatlestad et al., 2012; Ohnishi et al., 2009; Xu et al., 2015). Some of the emerging free radical intermediates or metabolites from phase I reactions can cause oxidative stress themselves or are even more toxic than the parent compound itself such as the carbamazepine metabolite 10,11-epoxycarbamazepine (Malchi et al., 2014).

Activated molecules as a product of phase I reactions can later be conjugated to single amino acids, glutathione, glycosides or other endogenous compounds like sulfonates during the phase II reaction. One of the most relevant enzymes in this context is the glutathione-*S*-transferase (GST) which catalyzes the conjugation of the electrophilic sites of the xenobiotic to the tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine). Many GST isoforms exist that are capable to use a broad range of xenobiotics as substrate. However, GSTs as a multifunctional family of enzymes are also involved in other processes than conjugation such as in normal plant development or in other stress responses (Dixon et al., 2002). As especially studied for agrochemicals, the conjugated xenobiotics resulting from phase II reactions can be metabolized, further conjugated (e.g. malonylation of glucosides), sequestered into the vacuole or the apoplast or incorporated into the cell wall during phase III. Glucose–conjugated xenobiotics have been detected to be more water soluble and less toxic than their corresponding parent compound (Bártíková et al., 2015; Taguchi et al., 2010; Laurent et al., 2007). On the one hand, these glucoside metabolites can be relatively easy reconverted by glucosidases present in the cytoplasm under specific conditions (Bártíková et al., 2015) but on the other hand their stability can be increased by additional malonylation of the molecule. Furthermore, the malonylation of glucosides was suggested to be a key reaction or a “tag” for vacuolar compartmentation (Taguchi et al., 2010; Theodoulou, 2000). Therefore, conjugated metabolites are potentially translocated into the vacuole through transport mediating proteins in the tonoplast such as multidrug and toxic compound extrusion (MATE) transporters or ATP binding cassette (ABC) transporters like multidrug resistance proteins (MRP) (Morita et al., 2009; Theodoulou, 2000; Schröder et al., 2007). In this regard, a plant efflux carrier was also discovered in the plasmalemma mediating the sequestration of plant-derived alkaloids, antibiotics and heavy metals from the cytoplasm into the apoplast (Li et al., 2002).

In general, metabolization processes of PPCPs within the plant can lead to a variety of different metabolites and transformation products (Riemenschneider et al., 2017; Reichl et al., 2018; Emhofer et al., 2018; He et al., 2017) which can be even more toxic and persistent than the corresponding parent compound (Malchi et al., 2014). However, the accumulation of the toxic metabolite 10,11-epoxycarbamazepine in spinach was reduced when plants were co-exposed

to fluoxetine or amitriptyline besides carbamazepine possibly because of transcriptional or enzymatic modifications (Nason et al., 2019).

1.3 PPCP-induced plant stress

PPCPs can trigger stress responses in plants. Some of these responses are clearly visible by looking at the plants, others are manifested on the cellular or molecular level. Phytotoxic effects were reported by a significantly decreased root and shoot elongation after the application of chloramphenicol, tetracycline, sulfamethazine, erythromycin and norfloxacin in carrot, cucumber, lettuce and tomato in most cases already at a concentration of 10 µg/L (Pan & Chu, 2016). Furthermore, impacts on enzyme activities such as the inhibition of the dihydropteroate synthase involved in the folate synthesis pathway by sulfamethoxazole (Brain et al., 2008) or on the plant hormone synthesis by a promoted increase of abscisic acid production by erythromycin and tetracycline (Pomati et al., 2004) were detected. However, a seed germination test with sweet oat, rice and cucumber on antibiotic saturated filter papers showed that this phytotoxic effect depends not only on the contaminant but also on the plant species itself (Liu et al., 2009). This dependency of the phytotoxic effects on the substance properties and the plant species, as well as on the concentration of the xenobiotic and on the plants' tissue and physiological stage has also been described in several articles (Hillis et al., 2011; Migliore et al., 2010b; Grossmann, 2010). Moreover, when pharmaceuticals were applied in a mixture (diclofenac, sulfamethoxazole, trimethoprim and 17a-ethinylestradiol), negative effects on the plants like an increased lipid peroxidation level which is an indicator for cell membrane damage were more pronounced compared to the treatment with only the single substances in alfalfa (Christou et al., 2016).

One of the first responses to various biotic and abiotic stressors is the production of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-) and hydroxyl radicals (OH^-) (Mittler, 2002). Depending on their concentration they can play an important role in signaling or even induce a hormetic/priming effect at low concentrations or lead to phytotoxic effects at high concentrations (Migliore et al., 2003; Wen et al., 2012). ROS can act as secondary messengers and trigger further downstream signaling and stress-responses. As one of the initial reactions, H_2O_2 is formed and released e.g. by NADPH oxidases in the plasma membrane. As a consequence, ROS accumulate in the apoplast, where an activation of ion channels and an influx of calcium ions (Ca^{2+}) into the cytoplasm can occur. These Ca^{2+} ions can then induce the production of apoplastic ROS and therefore enhance the ROS-calcium signaling pathway under stress conditions and enable cell-to-cell communication even on longer distances (Steinhorst & Kudla, 2013, 2019; Lamers et al., 2020). Within the cell, Ca^{2+} sensors (e.g. calcium dependent protein kinases (CDPKs)) can afterwards decode these

stress-specific signatures and the signals can be further transmitted by phosphorylation cascades leading i.a. to changes in the gene expression to control plant acclimation during biotic and abiotic stresses (Gorovits et al., 2020; Choudhury et al., 2017). In the context of the present thesis, the pharmaceutical lamotrigine was identified recently as a putative signal to affect the circadian expression pattern of different stress related genes in lettuce (Bigott et al., 2021). In contrast, high concentrations of ROS can result in membrane lipid peroxidation, protein oxidation or DNA and RNA alterations leading to an irreversible oxidative damage of plant membranes and organelles such as chloroplasts (Mittler, 2002). In this regard, a loose chloroplast thylakoid layer structure and severe chloroplast damages were observed after the treatment of the green algae *Scenedesmus obliquus* with different non-steroidal anti-inflammatory drugs (NSAIDs) (Wang et al., 2020). To prevent plants from these oxidative damages, ROS scavenging enzymes are crucial to keep the balance between the production and the degradation of ROS and to stabilize a redox homeostasis. Superoxide radicals can be converted to hydrogen peroxide (H_2O_2), which is less reactive than the starting molecule, by the activity of the superoxide dismutase. Afterwards, H_2O_2 can then be transformed to water (H_2O) by catalases, peroxidases or ascorbate peroxidases (Mittler, 2002). In addition, antioxidant molecules such as glutathione and ascorbic acid are key factors in the ascorbate-glutathione cycle and are therefore also important in the defense against oxidative stress in plants (Noctor & Foyer, 1998). As reported previously, PPCPs were detected to trigger the accumulation of ROS (Chen et al., 2018; Sun et al., 2018) as well as the induction of ROS detoxification enzymes (Chen et al., 2017; Pierattini et al., 2018; Wen et al., 2012; Dordio et al., 2011) and of enzymes involved in phase II reactions like GSTs (Bartha et al., 2014).

1.4 Plant-associated bacterial communities exposed to PPCPs

Plants can host diverse bacterial communities which are inhabiting the rhizosphere (narrow region of soil directly influenced by root exudates), the phyllosphere (surface of leaves) and the endosphere (inside the plant). These plant-associated bacteria can be potentially beneficial for the plant by stimulating the plant health and growth, enhancing the uptake of different nutrients, inducing systemic resistance or by increasing the stress tolerance against various biotic and abiotic stresses (Aeron et al., 2020; Vargas et al., 2017; Compant et al., 2005; Kumar et al., 2020). In return, plants can release root exudates such as organic acids or secondary metabolites which can provide a carbon and energy source for microbes or induce different processes like the expression of specific genes involved in the metabolization of xenobiotics (Patra et al., 2021; Tan et al., 2013; Chen & Aitken, 1999). Thus, plant-associated bacteria and especially endophytes can be a promising strategy to improve the phytoremediation efficiency of the host plant by increasing the degradation of different organic contaminants and

by reducing their phytotoxicity (Afzal et al., 2014). Furthermore, the involvement of plant-associated bacteria in the detoxification of PPCPs (Li et al., 2016; Sauvêtre & Schröder, 2015) and even the activation of specific degradation pathways for the metabolization of carbamazepine in hairy root (HR) cultures of horseradish (*Armoracia rusticana*) by endophytic bacteria was published previously (Sauvêtre et al., 2018). In this regard, Sauvêtre et al. (2018) also detected increased carbamazepine removal rates from the spiked liquid media when HRs were inoculated with *Rhizobium radiobacter* (21%) or *Diaphorobacter nitroreducens* (10%) compared to the non-inoculated plants (5%). Moreover, some bacterial strains originating from roots and rhizomes of *Miscanthus* x *giganteus* were identified which are able to degrade diclofenac (DCF) and sulfamethoxazole (SMX) and possess further plant-growth promoting traits in vitro (Sauvêtre et al., 2020a).

As genes coding for xenobiotic metabolizing enzymes are enriched in plant-associated bacterial communities in contaminated areas, it can be proposed that plants are able to influence the abundance of specific microbes and reveal a selective control over these organisms (Hartman & Tringe, 2019; Sauvêtre et al., 2020b). This selection of beneficial microbial communities is potentially driven by specific root exudates and rhizodeposition as suggested by Zhao et al. (2015), but the communities can also be impacted by the type and concentration of PPCPs. In this regard, most of the studies performed were testing the influence of different antibiotics but also of other frequently occurring PPCPs like DCF or ibuprofen (IBU) or even of complex PPCP mixtures. Several of these studies reported a decreased diversity of bacterial communities associated with plants growing in constructed wetlands after the treatment with sulfonamides, triclosan, IBU or a mixture of metal pollution in combination with bisphenol A (BPA), SMX and ciprofloxacin (CIP) (Man et al., 2020; Zhao et al., 2015; Zhang et al., 2016; Syranidou et al., 2018). Additionally, for bacterial communities associated with a crop plant, the irrigation with a mixture of 8 antibiotics together with 3 PPCPs also resulted in a reduced α-diversity in lettuce roots, shoots and soil (Shen et al., 2019). In contrast, two studies revealed an increased alpha diversity while studying the effects of sulfonamides or fluoroquinolone antibiotics on wetland plant-associated bacterial communities (Chen et al., 2020; Li et al., 2020).

Interestingly, while looking at the plant-associated bacterial community composition, several authors detected an increased abundance of bacterial taxa in PPCP-exposed environments which had previously been shown to have plant growth promotion capabilities (Jaiswal et al., 2021; Sah et al., 2021; Dimkić et al., 2022; Asaf et al., 2020; Wemheuer et al., 2017; Ulrich et al., 2022). On the one hand, Zheng et al. (2021) identified an increased abundance of *Pseudomonas*, *Bradyrhizobium, Sphingomonas* and *Luteimonas* in response to the chronic exposure to SMX in integrated vertical-flow constructed wetlands (IVCW) planted with

Phragmites australis. On the other hand, a reduction of the order Rhizobiales (new name: Hyphomicrobiales) in lettuce roots exposed to a mixture of ofloxacin, SMX and trimethoprim (Cerqueira et al., 2020) and of the family *Rhizobiaceae* in roots and rhizomes of *Miscanthus* × *giganteus* plants after the application of DCF and SMX (Sauvêtre et al., 2020a) was shown, although representative species of these taxa are well described plant growth promoting bacteria (Jaiswal et al., 2021; Vargas et al., 2017). Furthermore, Man et al. (2020) detected an inhibition of bacterial groups related to nitrogen and sulfur cycles, but at the same time a significantly higher abundance of methylotrophs in response to the exposure to sulfonamides in the rhizosphere of wetland plants. These enriched methylotrophs such as *Methylosinus*, *Methylotenera*, *Methylocaldum* and *Methylomonas* can potentially biodegrade sulfonamides (Man et al., 2020) which might underline the previously mentioned hypothesis of a specific selection of beneficial bacteria by the plant, or it could be the result of selection processes because of the antibiotic pressure. In this regard, some plant-associated bacterial groups were observed in several studies to be significantly increased after the application of different antibiotics or other pharmaceuticals like DCF. Representatives of the taxonomic family of *Sphingomonadaceae* showed an enriched abundance in plant-associated bacterial communities of *Juncus acutus* after the exposure to a mixed metal pollution in combination with BPA, SMX and CIP (Syranidou et al., 2018), of *Phragmites australis* after chronic contamination with SMX (Zheng et al., 2021) and of *Miscanthus* × *giganteus* plants treated with SMX and DCF (Sauvêtre et al., 2020a). Also, the genus *Luteimonas* was significantly increased in bacterial communities associated with different wetland plants after the exposure to SMX or to SMX and DCF (Zheng et al., 2021; Sauvêtre et al., 2020a). As these mentioned bacterial taxa include several representatives which are on the one hand known to have plant growth promoting capabilities but on the other hand to be resistant against, or even to degrade antibiotics (Asaf et al., 2020; Ulrich et al., 2022; Verma et al., 2016; Liu et al., 2021) the risk of a potential contamination of food and feed commodities with antibiotic resistant bacteria as well as with human- and plant pathogens should not be underestimated.

1.5 Aims and Hypotheses

This thesis is embedded in the AWARE project, which was supported and funded by the Joint Programming Initiative on “Water challenges for a changing world” (Water-JPI) of the European Research Area (ERA-NET). The AWARE project aimed to investigate the fate and metabolism of relevant wastewater-derived pollutants in different crop species and soils and to evaluate their impact on soil- and plant-associated organisms to support the development of novel irrigation strategies together with stakeholders from different countries reflecting various socio-environmental settings from the northern and southern European countries (Water-JPI/AWARE, 2022).

Within AWARE, the present thesis focusses (**I**) on studying the uptake and translocation of PPCPs and their metabolites in lettuce roots and shoots, and (**II**) on determining changes of the antioxidant enzyme system and stress gene expression in lettuce as well as (**III**) of the composition of lettuce-associated bacterial communities in response to PPCPs and treated wastewater.

Hypotheses:

Hypothesis 1: *The uptake and translocation of PPCPs in lettuce differs between the different substances because of their individual physico-chemical properties.*

Hypothesis 1 was mainly tested by quantifying the parent substances and several metabolites in lettuce samples in a hydroponic experiment with lamotrigine and diclofenac (Publication I), in a greenhouse experiment where plants were exposed to a mixture of 14 PPCPs (Publication II), as well as by performing a data analysis of 53 published ISI scientific articles and one technical report (Publication III).

Hypothesis 2: *The uptake of PPCPs in lettuce is increased when plants are irrigated with spiked treated wastewater compared to spiked tap water as the uptake of PPCPs follows the uptake of other substances in the treated wastewater.*

This hypothesis was mainly examined by the quantification of the parent substances as well as their main metabolites in roots and leaves of greenhouse-grown lettuce irrigated with tap water and treated wastewater spiked with a mixture of 14 PPCPs (Publication II).

Hypothesis 3: *PPCP-induced plant stress responses differ in their type and intensity between different substances as the PPCPs are translocated and metabolized differently within the plant. Stress responses are only detected in the corresponding plant part if the PPCP is present.*

Hypothesis 3 was examined by measuring stress enzyme activities and the expression of stress genes in lettuce roots and leaves after the treatment with lamotrigine and diclofenac at environmentally relevant concentrations during a hydroponic experiment (Publication I).

Hypothesis 4: *In the presence of PPCPs, the lettuce-associated bacterial diversity will decrease and the community composition will change. Bacterial groups which had been shown to be resistant to PPCPs or other abiotic stressors are enriched.*

Hypothesis 4 was tested by bacterial community analyses using 16S rRNA gene amplicon sequencing of bacteria associated with PPCP-exposed lettuce in a greenhouse experiment (Publication II).

2. Materials and Methods

2.1 Experimental setup

2.1.1 Hydroponic experiment

A hydroponic experiment was performed to study the effects of the pharmaceuticals lamotrigine and diclofenac on lettuce (*Lactuca sativa* var. capitata cv. 'Tizian', Syngenta, Bad Salzuflen, Germany). After the germination of seeds in petri dishes on tap water-soaked filter papers, single lettuce plantlets were transferred into pots filled with sterilized perlite in hydroponic systems filled with nutrient solution (modified 0.5 × Johnson`s solution pH 5.4 containing 20 µM $FeSO_4 \times 7 H_2O$). After an initial growing phase of 21 days in a phytochamber under controlled conditions (16/8 h light/dark cycle at 20/15°C, average humidity of 50%) the nutrient media was renewed and either pure ethanol (control), lamotrigine (final concentration: 60 µg/L) or diclofenac (final concentration: 20 µg/L) was added. At harvest, plant leaves and roots were separated and perlite was carefully removed from the roots at time points 0, 6, 12, 24, 30, 36 and 48 hours (n = 3). Afterwards, samples were directly frozen in liquid nitrogen and stored at – 80°C until further processing. Shortly after, the deep-frozen samples were ground into fine powder with liquid nitrogen cooled mortars and pestles for either H_2O_2, cytosolic enzyme or RNA extraction. The cultivation campaign was repeated under the same conditions to gain enough plant material and samples were collected at 0, 6, 12, 24 and 48 hours after treatment and lyophilized for the analytical measurements.

2.1.2 Greenhouse experiment

A greenhouse experiment (Figure 2) in soil-filled pots was conducted to study the effects of a complex mixture of PPCPs and treated wastewater on the bacterial root community composition in lettuce (*Lactuca sativa* var. capitata cv. 'Tizian'). The soil experiment was repeated for a consecutive second campaign in the same soil to test for an accumulation of PPCPs and the corresponding influence on the root microbiome. Lettuce seeds were germinated and plantlets were initially grown in peat soil before single plants were transferred after 4 weeks to 3 L pots for the treatment under greenhouse conditions (20 ± 5°C, 16/8 h light/dark). Pots contained 2 kg dry weight (1st campaign) and 1.1 kg dry weight (2nd campaign) of soil (loamy soil, pH 8.2, soil organic matter 3.68%, total organic carbon 2.13%, total nitrogen 0.201%) which was collected from the experimental fields of the IRSTEA institute in Montpellier (Lavalette, France, 43.64682 N, 3.87418 E). The soil water holding capacity was kept at 50% by adding tap water daily for 4 weeks before the actual experiment. The secondary treated domestic wastewater used (pH 7.1, COD 200 mg/L, total suspended solids 58 mg/L,

total organic carbon 56.8 mg/L, N-NH_4^+ 29 mg/L, N-NO_3^- <0.22 mg/L) originated from a wastewater lagoon at Murviel-les-Montpellier (Hérault, France, 43.605034 N, 3.757292 E) and was stored at 4°C until the experiment.

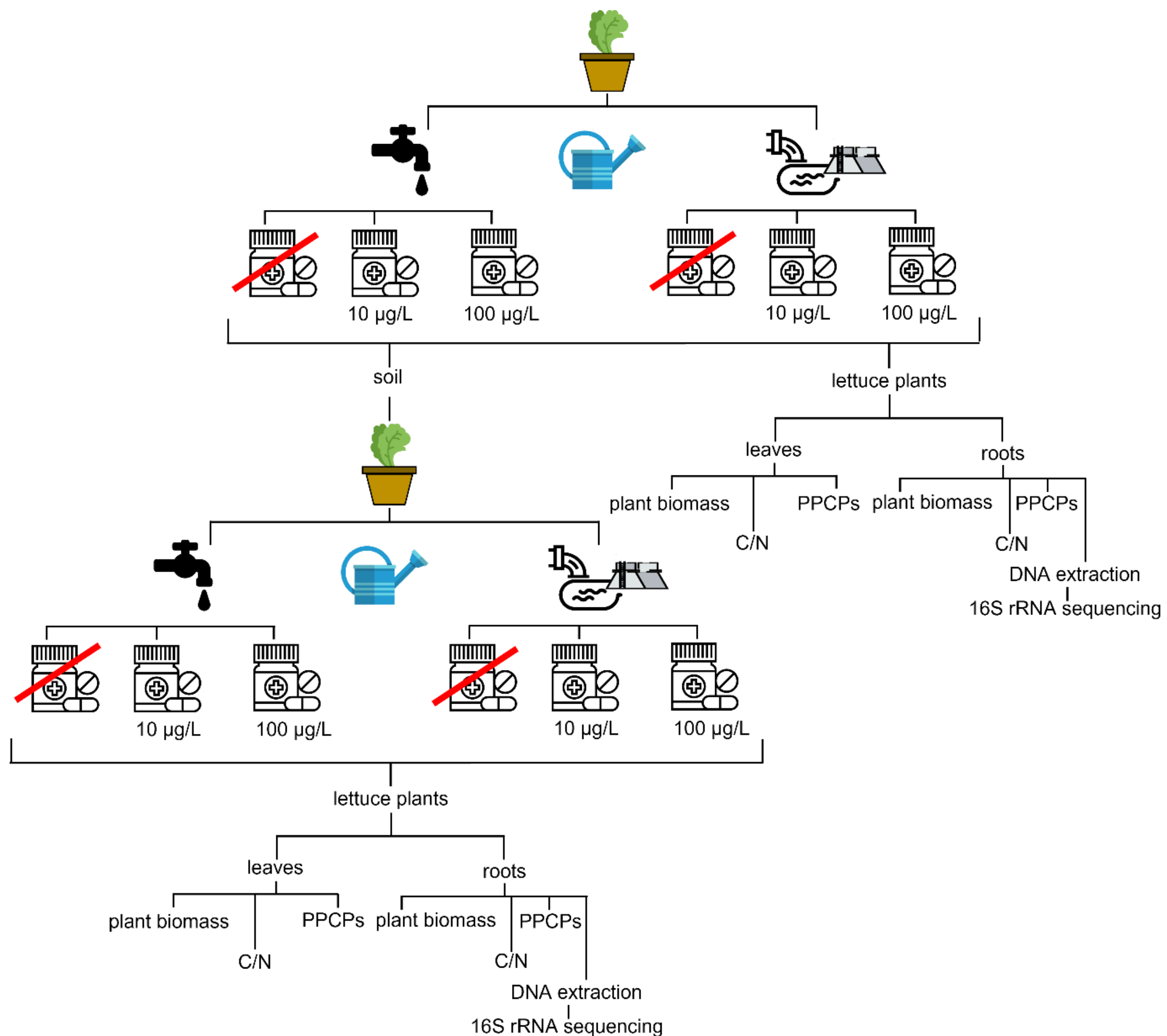

Figure 2: Flow chart of the experimental setup of the greenhouse experiment studying the effect of a complex mixture of PPCPs in water and treated wastewater on lettuce root-associated bacteria. Plants were irrigated with either tap water or treated wastewater, spiked or not with a mixture of 14 PPCPs at a concentration of 10 µg/L or 100 µg/L. After the harvest of lettuce plants for several measurements, soil was used for a consecutive cultivation campaign under the same conditions.

During both campaigns pots containing lettuce plants were irrigated daily (ca. 30-80 mL per day) with six different solutions and with tap water to keep the water holding capacity at 70% for 7 weeks per campaign. After the first campaign the soil was stored at 4°C for two weeks and afterwards used for a consecutive second campaign. The 6 different irrigation solutions

were (1) tap water, (2) tap water spiked with a mixture of 14 xenobiotics at a concentration of 10 µg/L, (3) tap water spiked with a mixture of 14 xenobiotics at 100 µg/L, (4) wastewater, (5) wastewater spiked with a mixture of 14 xenobiotics at 10 µg/L, and (6) wastewater spiked with a mixture of 14 xenobiotics at 100 µg/L. The mixture of PPCPs was prepared by combining 14 individual solutions of the following compounds at the above-mentioned concentrations: acesulfame, benzotriazole, carbamazepine, ciprofloxacin, citalopram, clarithromycin, climbazole, diclofenac, hydrochlorothiazide, irbesartan, metoprolol, sucralose, sulfamethoxazole and valsartan. Each pot (five replicates per treatment) was irrigated with the same volume of the respective irrigation solution with or without PPCPs (total irrigation volume of 3 L (1st campaign) and 2.7 L (2nd campaign)). Additionally, to compensate the nutrient depletion 4 × 60 mL of modified Hoagland ¼ solution (Hoagland & Arnon, 1938) was added during the second campaign. At the end of both campaigns, soil samples were collected and stored at -20°C for chemical analysis. Lettuce leaves and roots were separated and the fresh total plant biomass was measured. Afterwards, lettuce samples were freeze dried for further analysis.

2.2 Chemical analysis

2.2.1 Quantification of hydrogen peroxide

The H_2O_2 concentration in lettuce roots and leaves was quantified according to the method published by Shin et al. (2005) using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen, Carlsbad, CA). Briefly, 60 mg of liquid nitrogen frozen and ground plant material was mixed with 400 µl 20 mM potassium-phosphate buffer (pH 6.5). After centrifugation (20 min, 12,000 × g, 4°C), supernatants were incubated with 100 µM Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and 0.2 U ml^{-1} horseradish peroxidase under dark conditions at room temperature for 30 min before quantifying with a fluorescence/absorbance microplate reader (TECAN Spark®, Tecan Group Ltd., Switzerland) at excitation/emission at 530/590 nm using a H_2O_2 calibration curve (0 – 10 µM).

2.2.2 Total nitrogen and carbon measurements in lettuce

To assess the total nitrogen and carbon content in lettuce roots and leaves, lyophilized samples were ground to a fine powder and afterwards quantified by a FLASH 2000 CHN Analyzer (Thermo Fisher Scientific).

2.2.3 LC-MS/MS analysis of lamotrigine from liquid media samples

Proteins from liquid media samples were precipitated by mixing with 5-sulfosalicylic acid (10:1 v/v, 1.9 M in water) prior to injection. After a centrifugation step at 20,000 × g for 10 min at 4°C, 10 µl of the supernatants were injected in triplicates by an auto sampler (Dionex UltiMate 3000TRS, Thermo Scientific) into an UHPLC (Dionex UltiMate 3000RS, Thermo Scientific) coupled to a triple quadrupole mass spectrometer (HESI-MS/MS, TSQ Quantum Access Max, Thermo Scientific). The chromatographic separation was performed on an Accucore PFP column (100 mm x 2.1 mm, 2.6 µm particle size, Thermo Scientific) at a flow rate of 0.450 mL/min. The elution took 10 min (for details see Table 1) and was carried out using ACN + 0.1% formic acid and Mili-Q water + 0.1% formic acid as solvents.

Table 1: LC gradient for the elution of lamotrigine extracted from liquid media samples.

Time (min)	**Mobile phase composition/vol. %**		**Flow rate, (mL/min)**
	Mili-Q water + 0.1% formic acid	ACN + 0.1% formic acid	
0.0	95	5	0.450
2.0	95	5	
8.0	0	100	
9.0	0	100	
9.1	95	5	
10.0	95	5	

The mass spectrometer was operated in positive HESI mode with capillary voltage of 4000 V, nitrogen dumping gas temperature of 350°C, sheath gas pressure 50 psi, auxiliary gas pressure 5 psi, capillary temperature 380°C, skimmer offset of 6, collision energy of 28 eV and tube lenses of 97 V. Samples were analyzed in scheduled multiple-reaction-monitoring (SMRM) mode following the precursor ion [M+H]+ 256.01 m/z and the product ions 186.8 and 211.0 m/z and quantified against a calibration curve (r^2=0.987). Chromatographic procedures were selective for the quantification of lamotrigine as the retention time and mass spectrum was similar between standards and fortified matrices (RSD<20%). The limit of detection (LOD) of the method was 0.24 µg/L and the limit of quantification (LOQ) 0.71 µg/L.

2.2.4 Extraction of PPCPs and major metabolites from lettuce and soil

For the extraction of PPCPs and their major metabolites from lettuce leaves according to Montemurro et al. (2020), 1 g of homogenized freeze-dried plant material was rehydrated with 9 mL of HPLC water. After vortexing for 2 minutes at 2500 rpm using a BenchMixer XLQ QuEChERS Vortexer (Benchmark Scientific, Sayreville NJ, US), samples were incubated for 1 hour with 50 µL of internal standard (IS) mix to achieve the final concentration 10 ng/mL. Samples were vortexed (2500 rpm, 2.5 min) a second time and incubated for another 30 min. 10 mL of acetonitrile and 50 µL of concentrated formic acid were added to the tubes before vortexing a third time. Afterwards, the Original QuEChERS extraction kit (Bekolut, Hauptstuhl, Germany) was directly added into the tubes and hand shaken for 30 seconds before samples were centrifuged for 10 min at 4000 rpm and 4ºC. The supernatants were transferred into glass tubes and frozen overnight (at -20°C) to promote the precipitation of fatty acids and lipids. After 12 h, 6 ml of the organic phase were added to PSA tubes (150mg PSA, 150mg C18, 900mg $MgSO_4$) before vortexing and centrifugation (5 min, 4000 rpm, 4°C). Finally, 1 mL of the supernatant was evaporated until total dryness under nitrogen stream at room temperature and reconstituted with 1 mL of water/MeOH (90/10, v/v) for injection. For the equivalent extraction from lettuce roots and soil a similar modified QuEChERS method established by Manasfi et al. (2022) was used. In comparison to the extraction of PPCPs from lettuce leaves, 1 g of freeze-dried root material was hydrated with 9 mL of EDTA-McIlvaine buffer (pH 4) for 1 hour and the IS mix was then added afterwards. For soil, 10 g of air-dried soil sample was incubated with 3 mL of acetone and the IS mix overnight at room temperature before 8 mL of EDTA-McIlvaine buffer was added, respectively. The extraction was then performed by adding 10 mL acetonitrile without formic acid during the further procedure. Moreover, no freezing or clean-up step was performed for root and soil extracts.

2.2.5 LC-MS analysis of the PPCPs and major metabolites from lettuce and soil

Lettuce leaves, roots and soil extracts were analyzed on a SCIEX X500R QTOF hybrid system with Turbo V™ source and Electrospray Ionization (ESI) operating in positive and negative mode (Sciex, Redwood City, CA, U.S.). A reverse phase Hibar® HR Purospher® STAR RP-C18 column (100 mm x 2.1 mm i.d., 2 µm particle size, Merck, Darmstadt, Germany) and a flow rate of 0.5 mL/min was applied for the chromatographic separation. For a linear gradient elution, the mobile phases (A) water: either 5 mM ammonium acetate + 0.1% formic acid (positive ion mode) or 2 mM ammonium fluoride (negative ion mode) and (B) ACN were used to apply the following gradient program: 0–0.1 min 5% Buffer B, 0.1-6 min 5-40% B, 6-10 min 40-98% B, 10-10.9 min 98% B, 10.9-11.1 min 98-5% B, 11.1-12 min 5% B. The injection volume was 10 µL. High resolution data were acquired in positive electrospray ionization in

MRM^{HR} acquisition using fragment scanning mode with ion spray voltage of 5500 V (-4500 V for negative), atomizing gas pressure 55 psi, auxiliary gas pressure 55 psi, air curtain gas pressure 30 psi, source temperature TEM 550°C, collision energy of 10 V/-10 V, declustering potential of 80 V/-80 V and collision gas of 7. Additional details of the methodology are reported in Montemurro et al. (2020).

2.3 Enzyme activity analysis

2.3.1 Extraction of cytosolic enzymes from plants

To study plant cytosolic enzyme activities, soluble proteins were first extracted according to Schröder et al. (2005). Briefly, a maximum of 3.5 g of liquid nitrogen frozen and freshly ground plant material was stirred on ice for 30 min with 10 mL per gram extraction buffer (0,1 M Tris/HCl pH 7,8, 5 mM EDTA (Ethylenediaminetetraacetic acid), 1% PVP K90 (Polyvinylpyrrolidone), 1% Nonidet und 5 mM DTE (Dithioerythritol)). Extracts were then centrifugated at 4°C and 20,000 × g for 30 min and filtrated through a layer of Miracloth filtration material (pore size: 22-25 µm, Calbiochem, Merck, Darmstadt) to remove residual plant material. For the following precipitation of proteins, fine grounded solid ammonium sulphate ($(NH_4)_2SO_4$) was added slowly to the ice-cooled extract which was stirring for 30 min to reach a salt saturation of 40% in the first step. After another centrifugation at 4°C and 20,000 × g for 30 min the supernatant was collected and in a second step the salt saturation was increased up to 80% using the same procedure. The extract was then centrifuged again (30 min, 20,000 × g, 4°C) and the pellet was kept and re-suspended in 2 mL of 25 mM Tris/HCl buffer (pH 7.8). Lastly, to avoid any influence on the enzyme activities by high salt concentrations, the extract was desalted by passing through PD-10 gel filtration columns (GE Healthcare, Freiburg) and eluted in 2.5 mL 25 mM Tris/HCl buffer (pH 7.8). Aliquoted samples were afterwards stored at -80°C until they were used for protein content determination or enzyme activity measurements.

2.3.2 Protein content determination

Protein contents were determined according to Bradford (1976). Therefore, 10 µL of the enzyme extract was added to 200 µL 1:10 diluted Coomassie Brilliant Blue G250 dye in a 96-well plate and incubated for 10 min under dark conditions at room temperature. After the incubation, the OD was measured at 595 nm in a 96-well spectrophotometer (Spectra MAX 190, Molecular Devices, Germany) and the protein content was quantified against a BSA calibration curve.

2.3.3 Glutathione-*S* transferase and Peroxidase assay

Glutathione-*S* transferase (GST, EC 2.5.1.18) activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate and reduced glutathione (GSH) as a co-substrate (Habig et al., 1974). To start the measurements, 190 µL of the reaction mixture containing 0,1 M Tris/HCl (pH 6,4), 1 mM CDNB and 1 mM GSH were mixed with 10 µL enzyme extract in 96-well plates. The absorption was measured over time at room temperature at 340nm (ε = 9.6 mM^{-1} cm^{-1}) in a 96-well spectrophotometer (Spectra MAX 190, Molecular Devices, Germany) to follow the formation of the substrate-GSH conjugate.

Peroxidase (POX, EC 1.11.1.7) activity was determined by the oxidation of guaiacol (2-methoxyphenol) to tetra-guaiacol in the presence of H_2O_2 at a wavelength of 420 nm (ε= 26.6 mM^{-1} cm^{-1}) according to Diekmann et al. (2004). To perform the peroxidase assay, 10 µL enzyme extract was added to 190 µL reaction mixture (50 mM Tris/HCl (pH 6,0), 68 µM guaiacol and 18 µM H_2O_2) in 96-well plates and the absorption was measured for 300 sec using a 96-well spectrophotometer (Spectra MAX 190, Molecular Devices, Germany).

2.4 Analysis of plant stress gene expression

2.4.1 Primer design

For the primer design of genes involved in oxidative stress reactions and the detoxification of xenobiotics, target genes were selected based on the comparison with functional genes from *A. thaliana* using ‘The Arabidopsis Information Resource’ (www.arabidopsis.org, Berardini et al. 2015). The complete sequences of the target genes were acquired from the *Lactuca sativa* whole genome sequencing project at NCBI (www.ncbi.nlm.nih.gov/bioproject/PRJNA68025). After the Primer3Plus software (Untergasser et al., 2007) was used to design all primer pairs (Table 2) for the corresponding qPCRs, primer pairs were validated according to the Applied Biosystems Real-time PCR handbook guidelines (Thermo Fisher Scientific). To determine the relative gene expression of the selected genes in lettuce the housekeeping gene coding for the glyceraldehyde-3-dehydrogenase (*gapdh*) was selected as an endogenous control.

Table 2: List of plant genes selected for expression analysis with their corresponding loci and primer sequences in *A. thaliana*. Adapted from (Bigott et al., 2021).

Name of gene*	Primer sequences (5‘ -3‘)
cat1 **(AT1G20630)**	5’ – GGTCCAAGGCGATGTCTTTG -3’ 5’ – ATGAACAGCTGGCGTTTTGT – 3’
per50 **(AT4G37520)**	5’ – CTGTCAACACATGGGCTTCC – 3’ 5’ – TCCCACTTCGACCCGTTTTA – 3’
gst-f8 **(AT2G47730)**	5’ – GCCCAAATACTTGCTCTCCG – 3’ 5’ – TTGGGATGACTACCGACGAG – 3’
gst-u5 **(AT2G29450)**	5’ – AGCATTGGACTTTTGTTTGGGA – 3’ 5’ – TGAAGCTATTGGGATTTTGGGG – 3’
gst-f6 **(AT1G02930)**	5’ – TTGGGATGACTACCGACGAG – 3’ 5’ – RGCCCAAATACTTGCTCTCCG -3’
gapdh	5’ – AGGTAGCGATCAACGGATTC – 3’ 5’ – AGGTGGGATGCTTGTTTGAC – 3’

* Locus tag in *Arabidopsis thaliana* in parenthesis.

2.4.2 RNA isolation and reverse transcription

To isolate the RNA from liquid nitrogen frozen, pulverized plant roots and leaves the RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) was used. Afterwards, the RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s protocol.

2.4.3 Quantitative real-time PCR of plant stress genes^{y}

The qPCR analyses were performed according to Chowdhury et al. (2019). Therefore, the Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, U.S.A.), 0.4 µM of each primer (Eurofins MVG Operon, Ebersberg, Germany) and cDNA dilutions (1 µl, 1:4) as PCR templates were used. PCR reactions were heated in a peqSTAR 96Q thermal cycler (PEQLAB Biotechnologie) to 95°C for 3 min and afterwards for 40 cycles with steps of 95°C for 30 s, 60°C for 30 s, and 60°C for 30 s. Melting curve analysis and gel electrophoresis were carried out to confirm the generation of specific PCR products before the relative quantification by the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). After the ΔCt values were calculated relative to the

endogenous control, the data obtained at each time point was normalized to the initial time point 0. The standard error of the mean of the three biological replicates (*n*=3) was calculated.

[γ] The isolation and reverse transcription of RNA and the quantitative real-time PCRs (qPCRs) of plant stress genes were prepared with the help of Dr. Soumitra Chowdhury (Institute of Network Biology, Helmholtz Zentrum München).

2.5 Determination of the total bacterial abundance

2.5.1 DNA extraction and quantification

The bacterial DNA from liquid nitrogen frozen, pulverized lettuce roots was extracted using the DNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany). To quantify the concentration of DNA the Quant-iT™Pico Green® dsDNA assay Kit (Invitrogen, Carlsbad, CA, USA) and a Tecan Spark® microplate spectrofluorometric reader (Tecan, Männedorf, Switzerland) were used.

2.5.2 Quantitative real-time PCR of the 16S rRNA gene

A qPCR assay of the 16S rRNA gene using the 335Fc (5’-CADACTCCTACGGGAGGC-3’) as a forward primer and 769Rc (5’-ATCCTGTTTGMTMCCCVCRC-3’) as a reverse primer (Dorn-In et al., 2015) was performed on an ABI 7300 Real-Time PCR System (Thermo Fisher Scientific Inc.) to quantify the total bacterial communities associated with lettuce roots. Therefore, a PCR reaction mixture containing 12.5 µl Power SYBR® Green PCR Master Mix (Life Technologies Ltd, United Kingdom), 0.5 µL of each primer (10 pmol/µL), 0.5 µL of 3% BSA, and 2 µl template DNA (diluted 1:8) was prepared in a final volume of 25 µl. A 10 min denaturation at 95°C was followed by 40 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. A melting curve analysis confirmed the absence of primer dimers. For the absolute quantification of the qPCR products, an external linear standard curve (R^2=0.99) was used. The sequence of the positive standard of the target gene (Table 3) originated from *Pseudomonas anguilliseptica* strain YSH-14 (obtained from IDT Technologies, San Diego, CA). Based on the linear standard curve and according to the formula Eff = [10(−1/slope)−1] (Töwe et al., 2010) the efficiency of the qPCR was at 65.51%.

Table 3: Source sequence of the qPCR linear standard. Adopted from Bigott et al. (2022).

Gene	Sequence	Originating organism
16S rRNA	5'-CAGACTCCTACGGGAGGCAGCAGTGGGG AATATTGGACAATGGGCGAAAGCCTGATCCA GCCATGCCGCGTGTGTGAAGAAGGTCTTCG GATTGTAAAGCACTTTAAGTTGGGAGGAAGG GTAGTAACCTAATACGTTGCTACTTTGACGTT ACCGACAGAATAAGCACCGGCTAACTTCGTG CCAGCAGCCGCGGTAATACGAAGGGTGCAA GCGTTAATCGGAATTACTGGGCGTAAAGCGC GCGTAGGTGGTTCAGTAAGTTGGAAGTGAAA TCCCCGGGCTCAACCTGGGAACTGCTTTCAA AACTGCTGAGCTAGAGTACGGTAGAGGGTG GTGGAATTTCCTGTGTAGCGGTGAAATGCGT AGATATAGGAAGGAACACCAGTGGCGAAGG CGACCACCTGGACTGATACTGACACTGAGGT GCGAAAGCGTGGGGAGCAAACAGGATTAGA TA-3'	*Pseudomonas anguilliseptica* strain YSH-14

2.6 Statistical analysis of the chemical-, enzyme- and gene expression analyses

Statistical analyses were performed with the software R version 3.6.1.. For the quantification of hydrogen peroxide and the determination of the GST and peroxidase activity a two-way analysis of variance (ANOVA) with Bonferroni post-test was used to determine significant differences between control and treated plant groups. To test for differences in the gene expression between the different treatments of plant stress genes and the 16S rRNA gene as well as for the nitrogen and carbon measurements, an one-way ANOVA with post-hoc Tukey's test was performed. Furthermore, the PPCP residues analyzed by LC-MS in lettuce and soil were compared by one-way ANOVAs with corresponding post-hoc Lincon testing to determine significant differences (p–value ≤ 0.05) between the treated samples or time points.

2.7 Sequencing of the bacterial 16S rRNA gene

2.7.1 Library preparation and Illumina sequencing

The library preparation for next generation sequencing (NGS) of the 16S rRNA gene was performed on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). For the sequence specific PCR, the same primer sequences mentioned in "2.5.2 Quantitative real-time PCR of the 16S rRNA gene" with added Illumina adaptors (5'-CADACTCCTACGGG AGGC-3') on the forward primer and (5'-ATCCTGTTTGMTMCCCVCRC-3') on the reverse primer were used (for the complete sequence see Table 4). Negative controls using empty extraction tubes and buffers were prepared in parallel to the samples to examine that no contaminations were introduced by the DNA extraction procedure or any consecutive step.

Table 4: Sequence of the primer pairs used for next generation sequencing of the 16S rRNA gene. Adapted from Bigott et al. (2022).

Name	Sequence	Length ca.	Length without adapters
S-D-Bact-0335-a-S-17 (338f)	5'-TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCADACTCCTACGGGAGGC-3'	~498bp	431 bp
S-D-Bact-0769-a-A-19 (789r)	5'-GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGATCCTGTTTGMTMCCCVCRC-3'		

PCR reactions contained: 12.5 µl 2 × NEBNext High Fidelity Master Mix (New England BioLabs Ltd., United Kingdom), 0.5 µL of each primer (10 pmol/µL), 2.5 µL of 3% BSA, and 5 ng of template DNA filled to a final volume of 25 µl. During the PCR, samples were initially heated to 98°C for 5 min, followed by 28 cycles with three steps at 98°C for 10 s, 60°C for 30 s and 72°C for 30 s, and an additional final elongation step at 72°C for 5 min. PCR reactions were afterwards purified using MagSi-NGS (0.8 × sample volume; Magtivio B.V., Geleen, Netherlands) before the absence of primer-dimers and the concentration of DNA was determined on a Fragment Analyzer Automated CE System (Advanced Analytical Technologies Inc., United States). For performing the Illumina indexing PCR, the Nextera XT Index kit v2 (Illumina Inc.) was used. In short, 10 ng of amplicon DNA, 12.5 µL 2 × NEB Next High Fidelity Master Mix, and 2.5 µl of each indexing-primer were added to a final volume of 25 µl. Conditions for the indexing PCR included an initial denaturation step at 98°C for 5 min, followed by eight cycles of denaturation (98°C; 10 s), annealing (55°C; 30 s) and elongation (72°C; 30 s), followed by a final elongation step at 72°C for 5 min. After the indexed amplicons

were purified and quantified as described above, libraries were diluted to 4 nM and paired-end sequenced on a MiSeq instrument using the MiSeq Reagent Kit v3 (600 cycles) (Illumina Inc.).

2.7.2 Bioinformatic analysis of the sequencing data

The adapter sequences of the sequenced and demultiplexed reads were trimmed from both ends using AdapterRemoval v2.1.0 (Lindgreen, 2012). Afterwards, further processing was performed with QIIME 2 (v2018.8.01; Caporaso et al., 2010) according to Michas et al. (2020). Briefly, after merging of paired reads and removing of chimeras with the *qiime dada2 denoise-paired command* (DADA2 R package v1.3.4; Callahan et al., 2016) N-terminal trimming was set to 15 bp, and the C-terminal trimming of the forward and reverse reads was adjusted to 260 bp and 220 bp based on the quality scores of the data set.
Amplicon sequence variants (ASVs) were inferred and taxonomically assigned against reference sequences (*P*-confidence ≥ 0.9) using the *feature-classifier classify-sklearn* command. Before this assignment of the 16S rRNA amplicons it was necessary to extract the reference sequences from the SILVA database (release 132; Quast et al., 2013) and to train the Qiime2 classifier using the *feature-classifier extract-reads* and *fit-classifier-naive-bayes* commands first.

2.7.3 Statistical and data analysis of the sequencing data

For the data analysis and visualization the program R (version 3.6.2) was used. ASV output tables and taxonomic data from QIIME 2 were imported into R using the qiime2R package (Bisanz, 2018). ASVs present in the blank extraction non-template controls and the samples were considered as contaminants and removed from the dataset together with the ASVs that could not be assigned to any taxonomic level. Afterwards the datasets were rarefied to the minimum number of reads per sample using the *rrarefy* command of the vegan package (Oksanen et al., 2020) to compensate their unequal sequencing depths. A sufficient sequencing coverage was indicated by rarefaction curves reaching a plateau calculated with the *rarecurve* command. The normality of the NGS data was calculated using Shapiro Wilk's test ($p>0.05$) and the homogeneity of variance was checked using Levene's test ($p>0.05$). A two-way ANOVA followed by Tukey's test (using time and treatment as factors) and pairwise Student's *t-test* were used to determine differences for parametric distributions. Differences among the relative abundance of genera were therefore tested with the mvabund package (Wang et al., 2012) and the changes among the diversity indices with the WRS2 package (Mair & Wilcox, 2020). The sample ordination by non-metric multidimensional scaling (NMDS) on the Bray-Curtis dissimilarity and the stacked barplots were generated with the phyloseq package (McMurdie and Holmes, 2013). Subsequently, the Adonis function from the vegan

package (Oksanen et al., 2020) was used on the Bray-Curtis dissimilarity to perform a PERMANOVA. Differences of all data were considered as significant when the p-value was ≤ 0.05. Data was displayed graphically with phyloseq and ggplot2 (Wickham, 2009).

2.8 Data analysis of the scientific literature on plant uptake of pharmaceuticals

A bibliographic online search was performed with the search engine Google Scholar to collect the scientific literature on plant uptake and translocation of PPCPs of the last 7 years (2013 - 2020). Therefore, all articles containing the keywords “plant uptake + pharmaceutical group” or “plant uptake + compound name” were scrutinized for parameters such as the concentration of applied PPCP in the study, the time of exposure or the type of experiment (hydroponic, pot or plate experiment). Only studies with clear units and defined plant species were considered for the consecutive data analysis. Studies performed in the field or in lysimeters were excluded from this data analysis because of their higher complexity and additional influencing factors which result in a reduced comparability with studies performed under more controlled conditions. When numerical data was missing in the studies and only barplots were provided the approximate values were determined by using the ImageJ software (version 1.52a) and the “set scale” and “analyse” commands. Chemical parameters such as the molar mass (g/mol), the water solubility (mg/L) and the logarithmic octanol-water partition coefficient (log K_{OW}) were extracted from the PubChem and/or DrugBank website, pH dependent properties like the acid dissociation constant (p*K*a) and the logarithmic distribution coefficient (log D_{OW}) from the SPARC (SPARC Performs Automated Reasoning in Chemistry) system. After the collection of data, bioconcentration factors (BCF; ratio of the PPCP in the plant vs. in the surrounding environment) and translocation factors (TF; relative translocation from roots to shoots, stems and/or leaves) were calculated by the following equations:

$$\mathrm{BCF} = \frac{\mathrm{concentration_{root}\ (ng/kg)}}{\mathrm{concentration_{soil}\ (ng/kg)}} \quad \mathrm{or}$$

$$\mathrm{BCF} = \frac{\mathrm{concentration_{root}\ (ng/kg)}}{\mathrm{concentration_{nutrient\ media}\ (ng/L)}}$$

$$\mathrm{TF} = \frac{\mathrm{concentration_{shoot}\ (ng/kg)}}{\mathrm{concentration_{root}\ (ng/kg)}}$$

In this regard, the concentration in soil or nutrient media was calculated by the difference between the spiked concentration in the beginning and the concentration found at the particular time point of sampling to not include the remaining concentration of the PPCPs in the media/soil. For the evaluation of the results, TFs > 1 demonstrate a translocation of the PPCPs from roots to shoots whereas TFs < 1 indicate rather an accumulation in the roots.

3. Manuscript Overview

List of publications included in the present thesis

Publication I - Research Article (first author, published)

Bigott, Y., Chowdhury, S. P., Pérez, S., Montemurro, N., Manasfi, R., & Schröder, P. (2021). Effect of the pharmaceuticals diclofenac and lamotrigine on stress responses and stress gene expression in lettuce (*Lactuca sativa*) at environmentally relevant concentrations. Journal of Hazardous Materials, 403, 123881. https://doi.org/10.1016/j.jhazmat.2020.123881

Publication II - Research Article (co-first author, published)

Bigott, Y.*, Gallego, S.*, Montemurro, N., Breuil, M.-C., Pérez, S., Michas, A., Martin-Laurent, F., & Schröder, P. (2022). Fate and impact of wastewater-borne micropollutants in lettuce and the root-associated bacteria. Science of The Total Environment, 831, 154674. https://doi.org/10.1016/j.scitotenv.2022.154674

Publication III - Peer-reviewed Book Chapter incl. Data Analysis (first author, published)

Bigott, Y., Khalaf, D. M., Schröder, P., Schröder, P. M., & Cruzeiro, C. (2020). Uptake and translocation of pharmaceuticals in plants: principles and data analysis. In S. Pérez Solsona, N. Montemurro, S. Chiron, & D. Barceló (Eds.), Interaction and Fate of Pharmaceuticals in Soil-Crop Systems. The Handbook of Environmental Chemistry (Vol. 103, pp. 103-140). Springer, Cham. https://doi.org/10.1007/698_2020_622

Contribution to additional publications

- Gallego, S., **Bigott, Y.**, Mounier, A., Spor, A., Schröder, P., & Martin-Laurent, F. (2022). Impact of repeated irrigation of lettuce cultures with municipal wastewater on the diversity and composition of root-associated arbuscular mycorrhizal fungi. Biology and Fertility of Soils, 58(5), 607-611. https://doi.org/10.1007/s00374-022-01641-0

Publication I

Effect of the pharmaceuticals diclofenac and lamotrigine on stress responses and stress gene expression in lettuce (*Lactuca sativa*) at environmentally relevant concentrations

Yvonne Bigott, Soumitra Paul Chowdhury, Sandra Pérez, Nicola Montemurro, Rayana Manasfi and Peter Schröder

Brief description:

Pharmaceuticals and personal care products can be taken up and trigger stress symptoms in crop plants. However, many studies focused on the application of high concentrations of PPCPs. We investigated the uptake and translocation of diclofenac and lamotrigine as well as their effects on plant stress responses and on the stress gene expression in lettuce at environmentally relevant concentrations. Therefore, lettuce plants were grown in hydroponic systems under controlled conditions in a phytochamber with 16/8 h light/dark cycle and treated with either diclofenac (20 µg/L) or lamotrigine (60 µg/L). Root and leaf samples were collected separately at the time points 0, 6, 12, 24, 30, 36 and 48 h after the application of the substances. Diclofenac and its metabolite 4'–hydroxydiclofenac were only detected in lettuce roots. After 6 h, a transient reduction of the expression of stress related genes was observed in roots of diclofenac treated plants. Furthermore, with decreasing concentrations of the parent compound the influence on the stress gene expression was reduced. In contrast, low concentrations of lamotrigine were not only determined in lettuce roots but also in leaves which putatively triggered a systemic response starting with an oxidative burst in both tissues. Additionally, we observed a phase shift in the diurnal expression pattern of genes involved in plant stress reactions (*PER50*, *CAT1* and *GST–F6*) in roots and a significantly different expression of stress genes compared to the control plants at the time points T24, T36 and T48 in leaves and roots after the treatment with lamotrigine. Thus, we showed that although the uptake rates were relatively low, pharmaceuticals such as lamotrigine can potentially act as signals or *zeitgebers*, affecting the circadian expression of stress related genes in lettuce.

Contributions:

- Formed the hypothesis and designed the experiment together with P. Schröder
- Performed experiment, measured hydrogen peroxide and enzyme activities, performed LC-MS/MS analysis of spiked nutrient media and prepared lettuce samples for LC/HRMS QTOF measurements
- Interpretation of gene expression and enzyme data together with S. P. Chowdhury and P. Schröder
- Interpretation of analytical results together with N. Montemurro, S. Pérez and P. Schröder
- Wrote the manuscript
- Contribution to the comments given in the review process together with all authors

Publication II

Fate and impact of wastewater-borne micropollutants in lettuce and the root-associated bacteria

Yvonne Bigott, Sara Gallego, Nicola Montemurro, Marie-Christine Breuil, Sandra Pérez, Antonios Michas, Fabrice Martin-Laurent and Peter Schröder

Brief description:

The factors which are influencing the uptake and translocation of pharmaceuticals and personal care products (PPCP) in plants and the effects of these emerging contaminants on the plant-associated bacteria are not completely understood. We determined the concentration of 14 different PPCPs and their main metabolites in lettuce roots and leaves after the irrigation with tap water or treated wastewater spiked with and without the compounds. The experiment was performed in pots under a realistic and worst-case scenario (10 and 100 µg/L) and afterwards repeated for a consecutive cultivation campaign to test for the accumulation of substances. Moreover, the effects of the PPCPs and the type of irrigation water on the root-associated bacterial communities was investigated to gain insight into the impact of these contaminants on the plant microbiome. In this regard, almost all spiked PPCPs (thirteen out of fourteen) were present in the edible part of lettuce and the uptake of PPCPs by lettuce was higher when plants were irrigated with spiked treated wastewater compared to spiked tap water. The absolute bacterial abundance was comparable in all treatments and during both cultivation campaigns but the irrigation with treated wastewater had a significant effect on the alpha diversity indices at the end of the second cultivation campaign. Furthermore, we detected a change of the bacterial community composition and structure at the end of both campaigns. Five and fourteen bacterial taxonomic families were affected by the type of irrigation water (water vs. wastewater), the concentration of PPCPs or both at the end of the first and second cultivation campaign, respectively. On genus level, the relative abundance of the taxonomic clade *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was increased whereas the abundance of *Haliangium* was significantly decreased in response to the exposure of the PPCPs at a concentration of 100 µg/L at the end of the second campaign.

Contributions:

- Formed the hypothesis together with S. Gallego, F. Martin-Laurent and P. Schröder
- Preparation of the sequencing library and bioinformatical analysis of the next generation sequencing data and qPCR performance
- Data analysis together with S. Gallego
- Interpretation of the bacterial community and analytical results together with S. Gallego, F. Martin-Laurent and P. Schröder
- Wrote major parts of the manuscript in discussion with S. Gallego
- Contribution to the comments given in the review process together with all authors

Publication III

Uptake and translocation of pharmaceuticals in plants: principles and data analysis

Yvonne Bigott, David Mamdouh Khalaf, Peter Schröder, Peter M. Schröder and Catarina Cruzeiro

Brief description:

Pharmaceuticals and personal care products can be taken up and translocated in plants. In this regard, several parameters are influencing these processes such as the physico-chemical properties of the compounds, the plant physiology and different environmental factors. Besides providing the theoretical background on the main principles of the uptake and translocation of PPCPs in plants we furthermore performed an analysis of the current literature to compare the uptake and translocation rates of various pharmaceutical groups in different plant species. Therefore, we performed a bibliographic online search to collect the scientific literature of the last 7 years (2013 - 2020) on the uptake and translocation of PPCPs in plants to calculate the bioconcentration and translocation factors and compare them between different pharmaceutical groups and plants. As an outcome of this comparison, we were able to gain several insights into cross-study results and, among other things, could confirm an important role of the specific plant species for the uptake and translocation of PPCPs. Moreover, we revealed a great importance of performing uptake and translocation studies not only at high but also at environmentally relevant concentrations of the PPCPs as for some substances a higher uptake and translocation was detected when lower concentrations were applied. Finally, we concluded that basic guidelines for the standardization of uptake and translocation studies including appropriate meta data which should be additionally collected, would provide a useful tool to make the data more comparable and to reduce misinterpretation of results.

Contributions:

- Conception of the book chapter together with D. M. Khalaf, C. Cruzeiro and P. Schröder
- Collection of data and data analysis for the experimental section together with D. M. Khalaf and C. Cruzeiro
- Extensive literature research
- Wrote major parts of the book chapter
- Data visualization together with C. Cruzeiro
- Contribution to the comments given in the review process together with all authors

4. General Discussion

The research performed in the present thesis is embedded within the framework of the AWARE project, which received support and funding from the Joint Programming Initiative on "Water challenges for a changing world" (Water-JPI) of the European Research Area Network (ERA-NET). In this context, different approaches were used to study the uptake and translocation of PPCPs in crops as well as to investigate the influence of these compounds at environmentally relevant concentrations on the stress response in lettuce and on the plant-associated bacterial community composition and diversity (Figure 3).

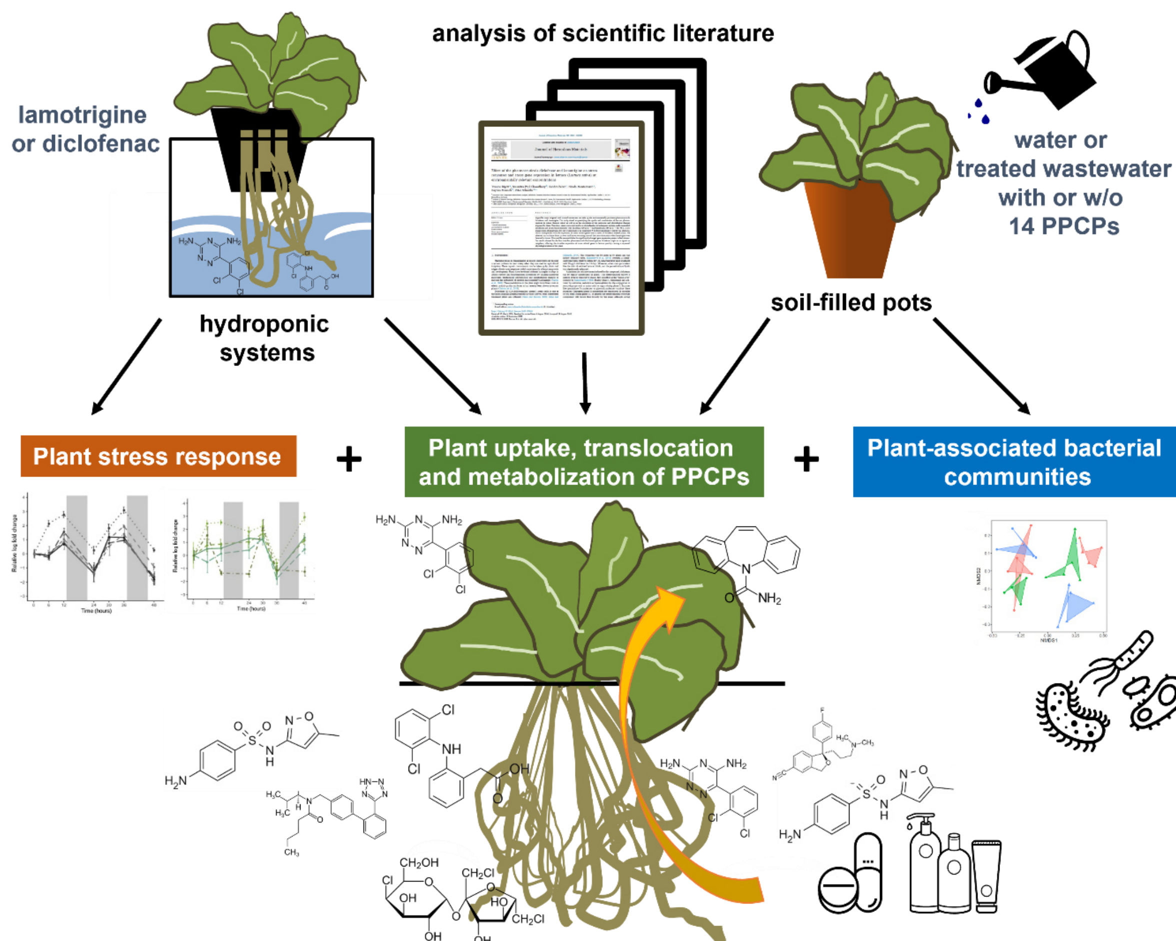

Figure 3: Scheme to illustrate the approach of the present thesis: experiments (with plants in hydroponic systems and in soil-filled-pots) were accompanied by analysis of scientific literature to study the uptake, translocation and metabolization of pharmaceuticals and personal care products (PPCPs) in crops as well as to investigate the influence of PPCPs at environmentally relevant concentrations on lettuce stress responses and on the plant-associated bacterial community composition and diversity.

Considering the discussion on the reuse of treated wastewater for agricultural practices, it was demonstrated on the one hand that crops such as lettuce (*Lactuca sativa*) can take up various pharmaceuticals and personal care products (PPCPs) which were also translocated to the edible part of the plant. On the other hand, however, the concentrations found in the plant tissues after the exposure to the currently relevant environmental levels of PPCPs were relatively low. Nevertheless, already at these detected concentrations a significantly changed gene expression as well as a putative effect on the circadian expression of stress related genes in lettuce was observed. Consequently, such molecular and physiological changes in response to PPCPs might become competitive advantages or disadvantages to plants and even impact quality traits. Furthermore, after the irrigation with treated wastewater but also in response to PPCPs in a worst-case scenario an influence on the plant-associated bacterial community composition and diversity was identified. Plant-associated bacterial communities are highly relevant for the health and the optimal growth of plants. Hence, changes in their composition and diversity accompanied by detectable xenobiotic residues might result in unknown consequences for the crops and their use as sources for human food and fodder for cattle.

4.1 Ability of crops to take up and transform PPCPs

Freshwater resources in many parts of the world are progressively threatened because of the consequences of climate change and the urban development. The demand for freshwater in agriculture is enormous as the irrigation of crops accounts for 70% of the global freshwater withdrawals (FAO, 2017). In this regard, treated wastewater can be an alternative water source for the irrigation of crops especially in arid and semi-arid regions worldwide to reduce the pressure on our water reserves. Nevertheless, this water source can contain residual contaminants such as PPCPs in concentrations between ng/L to µg/L (Bartelt-Hunt et al., 2009; Roberts & Thomas, 2006; Vanderford & Snyder, 2006). These PPCPs can be taken up, translocated and metabolized by plants, and even worse, by crops (Malchi et al., 2014; Goldstein et al., 2014; Boxall et al., 2006). Most probably due to their specific physico-chemical properties different uptake and translocation rates are expected for every single compound under the same condition in the same plant and plant part as stated in Hypothesis 1. Moreover, increased uptake rates in lettuce were assumed when PPCPs are spiked to treated wastewater compared to spiked tap water as the uptake of PPCPs follows the uptake of other substances in the treated wastewater which was postulated in Hypothesis 2. Already while comparing two pharmaceuticals which were applied individually to a hydroponic system, different uptake and translocation behaviors in the same lettuce cultivars were identified (Publication I). Diclofenac was taken up into roots very fast at relatively high concentrations without reaching the edible plant parts whereas lamotrigine was present in lower rates in the roots but was also

translocated in low concentrations to lettuce leaves. These specific uptake and translocation behaviors could be explained by the different physico-chemical properties of the substances as all other conditions and parameters were identical. In this context, it is also important to mention that metabolization processes might lead to the formation of phase II metabolites of the parent compounds which can be stored in plant vacuoles instead of being translocated to other plant organs or they might not be followed by targeted analysis.

The trend for specific uptake and translocation behaviors of different PPCPs under otherwise identical conditions was also observed in a greenhouse experiment where lettuce plants were grown in pots filled with soil (Publication II) as well as after a data analysis of 53 published ISI scientific articles and one technical report (Publication III). For some pharmaceuticals a clear trend could be observed: e.g. for diclofenac, which was detected in roots but not or only at minimum concentrations in leaves or above ground tissues of plants grown in the hydroponic experiment (Publication I), in soil-filled pots in the greenhouse experiment (Publication II), as well as for the data collected from hydroponic studies (Publication III). Similar to diclofenac also for carbamazepine a specific trend could be observed. Carbamazepine is not only frequently detected in the environment but also one of the most studied PPCPs in plant uptake studies. As detected in the greenhouse experiment (Publication II) and confirmed by the hydroponic and soil studies collected (Publication III) for carbamazepine a translocation to the leaves rather than an accumulation in the roots is frequently reported. This uptake and translocation behavior might be explained by the intermediate hydrophobicity and uncharged ionization status of the molecule as also assumed by Miller et al. (2016). In general, the hydrophobicity and ionization status of the PPCPs seem to be main parameters influencing their uptake and translocation. As the ionization status depends on environmental conditions such as the pH, it is crucial to collect those parameters during the experimental phase of the studies to reduce misinterpretations.

As several studies reported the uptake of PPCPs but have differences in the experimental design and analytical methods, the development of a fundamental knowledge of the accumulation of PPCPs in plants is impeded as studies are hardly comparable (Miller et al., 2016). Therefore, reliable predictions for the uptake and translocation behavior for many substances are not easy to be made by comparing different uptake studies (Publication III) and the underlying influencing parameters still need to be elucidated. Furthermore, the uptake and translocation behavior can also be different for several PPCPs, when the compounds are applied to soils or as a mixture. Both, soil experiments and the application of mixtures are on the one hand more realistic environmental scenarios but on the other hand they represent an additional layer of complexity influencing the availability of PPCPs for plants. The characterization of the soil and the collection of several soil parameters (soil type, pH, organic

matter (%), sand (%), silt (%), clay (%), moisture at 1/3 bar (%), Cation exchange capacity (meq/100 g)) could therefore help for a coherent interpretation and conclusive classification of the results.

In addition, when PPCPs are applied as a mixture with other PPCPs or even with different contaminants the possibilities of combinations are innumerable. Significant interactions between different PPCPs in soil and beets (*Beta vulgaris* L.) were identified as being almost exclusively synergistic (Papaioannou et al., 2020). Synergistic effects and higher uptake rates were also observed for lamotrigine in cucumber plants (*Cucumis sativus*) when carbamazepine was present under hydroponic conditions. However, the uptake of carbamazepine was not affected in presence of lamotrigine (Goldstein et al., 2018). Besides the interaction with other PPCPs, the presence of heavy metals, metalloids or micro- and macroelements was also reported to partly influence the uptake and translocation of PPCPs (Papaioannou et al., 2019; Papaioannou et al., 2020). Those synergistic effects triggered by substances in the treated wastewater could also be an explanation for the significantly higher concentration of PPCPs in lettuce when plants were irrigated with PPCP-spiked treated wastewater compared to spiked tap water, as detected in Publication II. Furthermore, transporter proteins such as OCTs or less specific nitrate and peptide transporters could be another reason for the increased uptake of PPCPs in lettuce roots irrigated with spiked treated wastewater. The knowledge about plant transporters specifically involved in the uptake of PPCPs is scarce, except for the observed involvement of OCTs in the uptake of metformin and tramadol (Cui et al., 2015; Khalaf et al., 2022). However, as shown for the distribution of a pesticide in crops, an amino acid transporter-like protein (*OsATL15*) plays an important role for the uptake and translocation of the insecticide thiamethoxam in rice (Xiao et al., 2022). As previously shown, such transporter- or transporter-like proteins also exist facilitating the uptake and translocation of PPCPs in plants (Eggen & Lillo, 2016).

Interestingly, the application of higher concentrations of PPCPs in soil or media does not necessarily lead to higher uptake rates in plants. This was observed for different plants and PPCPs while comparing 53 published ISI scientific articles and one technical report (Publication III). Recently, the relationship between application rates and residue concentrations in crops was tested for another group of organic compounds, namely for pesticides (including their metabolites) by using three models and the data of more than 5000 individual trials. As a result of this study, the assumption of a direct proportionality between the application rates and the concentration of residues in food commodities was not confirmed to be statistically significant (Gloe et al., 2023). However, compared to pesticide research and especially registration, relevant metabolites of PPCPs are often unknown although the metabolization of PPCPs can be rapid and parent compounds are therefore not detectable

anymore by targeted analysis. This rapid metabolization was observed for diclofenac during the hydroponic experiment (Publication I). Already after 6 hours the phase I metabolite 4'-hydroxydiclofenac was identified and after 24 hours the concentration of the metabolite was higher than that of the parent compound. Similar results were also obtained during the greenhouse experiment (Publication II). Several metabolites of the tested PPCPs were not only detected in lettuce, but also quantified at higher concentrations than the parent compounds. Interestingly, the metabolite valsartan acid was present at concentrations up to 39 ng/g dry weight in lettuce roots at the end of the second cultivation campaign but the parent compound valsartan could not even be detected in the roots of the plant. Like valsartan acid, 4'-hydroxydiclofenac was also measured at higher concentrations than the parent compound in lettuce roots which highlights the importance of identifying and quantifying the relevant metabolites of the PPCPs to not underestimate the uptake and translocation of organic contaminants in plants. Unfortunately, this important identification of metabolites is expensive and challenging, since metabolites can be specific for plant species or even for different organs of the same plant. This is probably one of the reasons why studies for the identification of the metabolism and/or degradation products of pesticides in crops should be performed for each type of crop group for which the use is intended (i.e. cereals, fruits, leafy crops, pulses and oilseeds, and root vegetables) if they are in accordance with the OECD guideline for the testing of chemicals 501 (OECD, 2007). Therefore, radiolabeled active ingredients (often several ones with different radiolabel positions depending on the structure of the molecule) are used for the quantification of the total radioactive residue (TRR) in a specific crop part and for further identification or characterization of the components depending on their concentration, percentage or toxicity. This identification of the components includes the exact structural determination of metabolites and degradation products by various spectroscopic methods (mass spectrometry (MS) or nuclear magnetic resonance (NMR)). In this regard, Dodgen and colleagues (2013) cultivated lettuce (*Lactuca sativa*) and collards (*Brassica oleracea*) for 21 days in hydroponic systems containing nutrient solution spiked with ^{14}C-labelled diclofenac sodium, naproxen, bisphenol A and 4-nonylphenol at concentrations previously found in reclaimed water. As a result, the group observed that the vast majority of ^{14}C was detected in the non-extractable residues and only a minor amount was solvent-extractable which is showing the importance of experiments using radiolabeled compounds to provide detailed insights into the distribution of the substances. Nevertheless, this experimental approach is expensive and often needs special precautions, which might be a reason why plant uptake and translocation studies using radiolabeled PPCPs are very limited and the majority of PPCP metabolites are often unknown.

The concentration of PPCPs in plants irrigated with real treated wastewater or with spiked water at environmentally relevant concentrations were in a range between ng/g to low µg/g dry weight and therefore relatively low (Wu et al., 2014; Riemenschneider et al., 2016; Manasfi et al., 2021; Christou et al., 2019). Similar concentrations were also observed during the hydroponic- and greenhouse experiments in Publication I and II. In this context, several authors concluded that the tested environmentally relevant concentrations of PPCPs would not have any negative effect on human health or only a de minimis risk when crops are consumed at realistic levels (Marsoni et al., 2014; Manasfi et al., 2021). However, when using the threshold of toxicological concern (TTC) approach, Malchi et al. (2014) calculated that for children the TTC for lamotrigine could be reached after the daily consumption of half a carrot (~60 g). Thus, the authors concluded that certain PPCPs, exceeding the TTC values, should be classified as contaminants of emerging concern.

The highest concentrations in lettuce roots as well as in leaves of the fourteen spiked PPCPs in the greenhouse experiment were detected for sucralose with measured concentrations of up to 2.14 µg/g dry weight in the below ground tissue (Publication II). As published by the World Health Organization in May 2023, sucralose as a non-sugar sweetener might have potential undesirable effects from long-term usage like an increased risk of type-2 diabetes, cardiovascular diseases, and all-cause mortality in adults and should not be consumed for weight control as it was not shown to be beneficial for reducing body fat (WHO, 2023). As an important toxicological endpoint for the chronic risk assessment, the Acceptable Daily Intake (ADI) is the estimated residual amount of substance in food and beverages without measurable risk for human health when consumed daily over a lifetime. Even though the highest concentrations out of the tested PPCPs were detected for sucralose in the greenhouse experiment (Publication II), it would be impossible to exceed the current ADI for sucralose with 15 mg/kg/body weight/day (European Commission, 2021) by eating lettuce irrigated with 100 µg/L spiked treated wastewater. Similarly, Carter and colleagues (2014) showed that the ADI was not exceeded for any of the five analyzed pharmaceuticals in ryegrass and radish when plants were grown in soil-filled pots containing the tested substances.

To summarize, the risk for human health in terms of consuming PPCP-contaminated crops seems relatively low at the current concentrations found in the environment. Nevertheless, it might be still too early to exclude any negative effects on human health especially as PPCPs are introduced to the agroecosystems as complex mixtures with different PPCPs or other organic contaminants and also show high detection frequencies in the crops. Thirteen out of fourteen applied PPCPs were present in the edible part of lettuce plants in the greenhouse experiment at the end of the second cultivation campaign (Publication II). Likewise, Wu et al. (2014) also observed relatively high detection frequencies of the analyzed PPCPs of 64% and

91% in all of the eight selected vegetables after the irrigation with treated wastewater or PPCP-fortified water under real field conditions, respectively. Furthermore, various other PPCPs were shown to be translocated to the edible part of the plants, such as paracetamol in maize (Hammad et al., 2018) and sulfamethoxazole and trimethoprim in tomato fruits (Christou et al., 2019).

4.2 Influence of PPCPs on plant stress responses

PPCPs as bioactive compounds are designed to effect target molecules or structures in humans or animals and can thus also potentially influence the physiology and biochemistry of other living beings, e.g. plants (Malchi et al., 2022; Fu et al., 2019). One of the first responses of plants to PPCP stress is the production of reactive oxygen species (ROS). On the one hand, ROS possess a function as secondary messengers triggering downstream stress responses in plants such as an altered expression of stress genes and on the other hand an over-accumulation of ROS can cause oxidative damage to plant biomolecules and even result in cell death. To prevent these adverse effects, antioxidant defense mechanisms including ROS detoxifying enzymes like superoxide dismutases, catalases or peroxidases and scavenging molecules such as glutathione and ascorbate are crucial for the protection of the plant (Mittler, 2002). These PPCP-induced plant stress responses might differ in their type and intensity between the individual PPCPs possibly because the substances are translocated and metabolized differently as stated in Hypothesis 3. Additionally, stress responses are expected to be only locally detected in the corresponding plant part where the PPCP is present.

After its uptake, the concentration of diclofenac in lettuce roots was shown to decrease over time while the phase I metabolite 4'-hydroxydiclofenac was detected already after 6 hours and its concentration further increased during the experiment (Publication I). Therefore, a rapid metabolization of diclofenac in lettuce was suggested. However, no diclofenac or 4'-hydroxydiclofenac was detected in leaves of the treated lettuce plants at any time point. It is noteworthy that phase II reactions can frequently lead to a formation of metabolites that are stored in the vacuole, so that long-range transport to other plant organs might be impossible. Corresponding to the decreasing presence of diclofenac in lettuce roots, the influence on the expression of stress genes was decreasing over time. At the highest detected concentration of diclofenac in lettuce roots 6 hours after the exposure, a significant transient reduction of the expression of all tested genes was observed. With decreasing concentrations of diclofenac after 12 hours, the expression of less genes (*CAT1*, *PER50*, *GST-F6* and *GST-F8*) was significantly reduced and the influence on the stress gene expression further decreased with decreasing concentrations of diclofenac. Furthermore, in leaves where no diclofenac or its metabolite 4'-hydroxydiclofenac was detected, the influence on the expression of stress genes

was generally low. In comparison to diclofenac, the concentration of lamotrigine in lettuce roots stayed constant after an initial uptake phase without any decrease over time. Moreover, low but increasing concentrations of lamotrigine were detected in lettuce leaves. In general, a phase shift in the diurnal expression of the tested stress genes was observed in lamotrigine treated lettuce after the pharmaceutical was translocated to the leaves. The high and low peaks of the gene expression, which is following a specific diurnal pattern for several genes, were shifted and lamotrigine therefore might act as a *zeitgeber* affecting the circadian rhythm of the selected stress genes after a putative systemic response when lamotrigine was detected in lettuce leaves. The concentration of lamotrigine in lettuce roots, in contrast to diclofenac, did not decrease over time and also the observed effects on the expression of stress genes did not decrease. Therefore, it can be summarized that the different pharmaceuticals trigger different stress responses in lettuce in presence of the substances. In this regard, Leitão and colleagues (2021) detected different patterns of stress protein species and diverse metabolic responses in lettuce plants varying between the three applied individual pharmaceuticals (metformin, acetaminophen and carbamazepine) at 1 mg/L for 8 days. Among others, the abundance of specific stress related protein species like catalases, superoxide dismutases and peroxidases was increased in roots when plants were treated with carbamazepine, whereas the abundance of ascorbate peroxidases were higher when exposed to metformin. In contrast, acetaminophen caused the biggest differences in the abundance of cell respiration protein species in pharmaceutical-treated lettuce suggesting a potential dysregulation of respiratory pathways. Moreover, pharmaceutical-specific responses of the antioxidant and redox homeostasis were also proposed to be a possible explanation for the differences observed in stress physiology markers and detoxification responses in alfalfa between diclofenac, sulfamethoxazole, trimethoprim and 17a-ethinylestradiol treatments (Christou et al., 2016).

The concentration of lamotrigine in lettuce roots as well as the effects on the expression of various stress genes did not decrease over time. In comparison, diclofenac was metabolized, leading to a reduction of the parent compound and to a decreased effect on the stress gene expression. However, for lamotrigine the changes in the stress gene expression were initiated after a putative systemic response starting with an oxidative burst occurring in both tissues at the same time point when lamotrigine was also detected in lettuce leaves (Publication I). Also, Christou and colleagues (2016) revealed that after regular irrigation and therefore constant supply with four pharmaceuticals (diclofenac, sulfamethoxazole, trimethoprim and 17a-ethinylestradiol) at a realistic environmental concentration of 10 µg/L, several stress genes i.a. different GSTs like *GST17* were still significantly affected after 50 days in alfalfa. Potentially, this constantly altered gene expression can have positive or negative effects for the plant. On

the one hand, these chronic stress responses could cause a higher susceptibility of the plant because of their increased fitness costs. On the other hand, as several studies already observed potential hormetic responses triggered by different antibiotics in several crop plants (*Cucumis sativus*, *Lactuca sativa*, *Phaseolus vulgaris* and *Raphanus sativus*, *Zea mays*) and in the weed *Lythrum salicaria* L. (Migliore et al., 2003; Migliore et al., 2010a; Migliore et al., 2000; Migliore et al., 2010b) low concentrations of the tested pharmaceuticals could also provoke hormetic effects in lettuce. These responses are dose-dependent relationships implying positive effects such as an increased growth for the organism after the exposure to low doses of a toxin or pollutant and negative consequences at higher concentrations (Minden et al., 2017).

Despite the change in the expression of stress genes and the disturbance of their diurnal rhythm, no effects on the stress enzyme activity after the treatment with diclofenac nor lamotrigine were observed except for a reduced guaiacol-peroxidase activity in lamotrigine treated lettuce roots. The concentrations used in the experiment were at an environmentally relevant level (Publication I). Several studies observing a significant effect on various stress enzyme activities were performed at PPCP concentrations markedly higher than those found in the environment (Bartha et al., 2014; Sun et al., 2019; Pierattini et al., 2018). However, Christou et al. (2016) also detected a changed activity of catalases and superoxide dismutases after the exposure to some of the spiked pharmaceuticals (diclofenac, sulfamethoxazole, trimethoprim and 17a-ethinylestradiol) at 10 µg/L after 50 days in alfalfa and Kummerová and colleagues (2016) reported an increased glutathione reductase activity already at 0.1 µg/L of diclofenac and paracetamol after 10 days in *Lemna minor*. For the relatively short exposure time of 48 hours of the experiment published in Publication I, concentrations were probably too low to trigger effects on the stress enzyme activity, except for the guaiacol-peroxidase activity in lettuce roots exposed to 60 µg/L lamotrigine. Thus, it can be concluded, even if the concentration of PPCPs is probably too low to induce some stress symptoms such as an altered stress enzyme activity, the expression of stress related genes can be influenced by PPCPs and some pharmaceuticals potentially act as *zeitgebers* affecting the circadian expression of stress genes in lettuce.

4.3 Influence of PPCPs on plant-associated bacterial communities

Plant-associated bacteria play an important role for their host plant. Besides the improvement of the nutrient availability for the plant, the promotion of plant growth and the ability to increase the tolerance of the plant against diverse biotic and abiotic stresses, plant-associated bacteria were also capable to degrade PPCPs (Aeron et al., 2020; Compant et al., 2005; Kumar et al., 2020; Vargas et al., 2017; Sauvêtre & Schröder, 2015; Sauvêtre et al., 2020a). Moreover, isolated endophytic bacteria from *Phragmites australis* were observed to activate specific degradation pathways in hairy root cultures of horseradish for the metabolization of carbamazepine (Sauvêtre et al., 2018).

Nevertheless, PPCPs were also shown to change bacterial community structures and influence the microbial diversity (Man et al., 2020; Syranidou et al., 2018; Zhang et al., 2016; Zhao et al., 2015; Zheng et al., 2021). In this regard, the bacterial alpha diversity in lettuce roots, shoots and soil was decreased after the exposure to a mixture of 8 antibiotics (carbadox, lincomycin, monensin sodium, oxytetracycline, sulfadiazine, sulfamethoxazole, trimethoprim, and tylosin) and 3 other pharmaceuticals (acetaminophen, caffeine, and carbamazepine) as observed by Shen et al. (2019). Previously, a reduced microbial diversity had been shown to have negative impacts on the denitrification activity in soil (Philippot et al., 2013) and on the terrestrial ecosystem multifunctionality e.g. by influencing soil fertility, and food and fiber production (Delgado-Baquerizo et al., 2016).

When lettuce was exposed to a mixture of PPCPs a decreased plant-associated bacterial diversity and an altered community composition was expected as stated in Hypothesis 4. Furthermore, especially bacterial groups which were shown to be resistant to PPCPs or other abiotic stressors were expected to be enriched. As shown in Publication II, no reduction of the alpha diversity was observed for the PPCP treatments. Instead, a significantly increased Chao1 (indicator of species richness) for samples irrigated with 10 µg/L PPCP-spiked tap water at the end of the second cultivation campaign was detected. Thus, the tested environmentally relevant concentrations of PPCPs showed no negative impact on alpha diversity indices and furthermore even an increased diversity was identified at the lower applied concentration of spiked PPCPs. Ciprofloxacin was one of the fourteen spiked PPCPs in the experiment published in Publication II. Li et al. (2020) also detected an enhanced bacterial alpha diversity in a planted constructed wetland system after the exposure to a mixture of different fluoroquinolone antibiotics including ciprofloxacin, norfloxacin and ofloxacin. Hence the group postulated that besides their antibacterial mode of action the antibiotics might be assimilated as a carbon source by some bacteria, stimulating their growth and abundance at low concentrations of the compounds.

In contrast to the PPCP application, negative effects on the alpha diversity (Chao1 and Shannon) of lettuce root-associated bacterial communities were visible after the irrigation with treated wastewater at the end of the second cultivation campaign as shown in Publication II. Therefore, a possible explanation could be a decrease of those bacteria in the community which were susceptible for at least one substance originating from treated wastewater such as the high ammonia level that accumulated during the two cultivation campaigns or due to a potential depletion of soil nutrients because of repeated lettuce cultivation. Moreover, an influence of treated wastewater on the beta diversity at the end of both campaigns was also investigated. In contrast, PPCPs affected the beta diversity only at the end of the second cultivation campaign.

Similar results were also obtained for the composition of the plant-associated bacterial communities, where more taxonomic families were significantly affected by the irrigation with treated wastewater compared to the PPCP application (5 and 9 families influenced by treated wastewater compared to 0 and 4 by the PPCP application at the end of the first and second cultivation campaign, respectively). These results highlight the more pronounced influence of the irrigation with treated wastewater on the diversity and community composition of plant-associated bacteria compared to the effects of the PPCP treatment at both concentrations (10 µg/L and 100 µg/L). However, a shift of bacterial genera was observed for both, the treatment with treated wastewater and PPCPs. Two genera were decreased (*Caulobacter* und *Rhizobacter*) and two other genera increased (*Cellvibrio* and *Hydrogenophaga*) when lettuce was irrigated with treated wastewater. Comparably, one genus/clade was negatively (*Haliangium*) and one positively (*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*) affected by the PPCP treatment at the higher concentration of 100 µg/L at the end of the second cultivation campaign. For this observed shift of bacterial taxa two major possibilities can be considered: (i) the plant might select specific microbes which are beneficial for the optimal plant growth and health as proposed previously (Hartman & Tringe, 2019; Sauvêtre et al., 2020b), or (ii) the respective bacteria are more susceptible or resistant against the stressors. For example, the genus *Haliangium* which was found to be decreased in the experiment in Publication II had previously been detected to be sensitive to veterinary antibiotics in plant–soil systems (Uddin et al., 2019) and the clade *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* showed tolerance against the plasticizer DEHP before (Bai et al., 2020). Thus, some bacteria can be competed by other bacteria, which are less susceptible to the changed environmental conditions. This disturbance can increase the likelihood of antibiotic-resistant bacteria (ARB), or human- or plant pathogens to occupy the ecological niche, which could have adverse effects on human health, especially after the consumption of raw consumed food commodities like lettuce. In this regard, Cui and Liang

(2019) showed that the abundance of the human pathogen *Legionella sp.* was significantly enriched in the lettuce rhizosphere after the irrigation with raw and treated wastewater and additionally of *Aeromonas hydrophila*, *Arcobacter sp.* and *Escherichia coli* in the lettuce phyllosphere after plants were irrigated with raw wastewater. Furthermore, after the irrigation with undiluted reclaimed wastewater the abundance of the plant pathogen *Pseudomonas syringae* and also of the human pathogens *A. hydrophila* and *E. coli* was significantly higher in pepper endophytes (Cui et al., 2020).

However, it might be possible to reduce the risk of contaminants and pathogens for consumers by choosing the type of irrigation technique. For crops whose above ground parts are eaten, subsurface drip irrigation can introduce several advantages. When the system was used for watering tomato and cucumber plants none of the human viruses which were seeded to the secondary wastewater irrigation water (i.a. hepatitis A, enteric adenovirus 40, poliovirus type 1) were detected on any of the above ground plant surfaces (Alum et al., 2011). Furthermore, pharmaceutical residues were substantially lower in greenhouse grown lettuce shoots when crops were watered by soil surface- compared to overhead irrigation, which was also observed for the substances monensin sodium, trimethoprim and tylosin after washing the plants (Bhalsod et al., 2018). In contrast, for reducing the risk of a contamination of root crops with human pathogens subsurface drip irrigation seems not appropriate as pathogens added to the irrigation water were detectable on all tested potato samples after using this irrigation system (Forslund et al., 2011).

5. Conclusion and Recommendations

An increasing number of regions in the world are enduring high water stress. Treated wastewater can therefore be an alternative water source for the irrigation of crops to reduce the pressure on water resources. However, this alternative water source might still contain various contaminants such as pharmaceuticals and personal care products (PPCPs) which can accumulate in soils or even be taken up in plants.

As observed in the context of the present thesis, lettuce can take up and translocate thirteen out of fourteen selected PPCPs to the edible part of the plant showing the high potential for the contamination of crops. Even though the detected concentrations were relatively low, the frequency of occurrence of PPCPs in crops should not be underestimated due to the limited knowledge about stress responses in plants and on the plant-associated bacterial communities after the exposure to PPCPs at environmentally relevant concentrations. In this thesis, an altered stress gene expression was observed in lettuce at environmentally relevant concentrations of two pharmaceuticals with differing responses between the selected compounds. Lamotrigine was shown to potentially act as a signal or *zeitgeber* influencing the diurnal expression pattern of stress related genes in lettuce which could possibly cause a repressed physiological status of the plant. In contrast, diclofenac triggered a transient reduction of the expression of several stress genes in lettuce roots when the compound was present. This effect was reduced with decreasing concentrations of diclofenac, but as the irrigation with treated wastewater under real-life conditions results in a constant supply with many different contaminants, the plants are chronically exposed to several xenobiotics, possibly leading to further unknown consequences.

For the plants' health and its' optimal performance plant-associated bacteria play a crucial role. The results of the present thesis revealed that the impact of treated wastewater on the diversity and composition of lettuce root-associated bacterial communities was more pronounced than the changes triggered by PPCPs at environmentally relevant concentrations. Negative effects on the alpha diversity of plant-associated bacterial communities, when plants in soil-filled pots were irrigated with treated wastewater at the end of a second cultivation campaign, as well as an impact on the beta diversity at the end of both campaigns were observed. Furthermore, the bacterial community composition changed after the irrigation with treated wastewater but also in response to PPCPs. The decreased microbial diversity and disruptions of the community composition can lead to reduced ecosystem services as well as to negative consequences for the nutrient availability or a higher susceptibility of the plant against different biotic and abiotic stressors which can ultimately lead to a reduced agricultural productivity. On genus level, treated wastewater significantly affected the abundance of four taxa (two decreased, two

increased), whereas two other taxa were significantly affected by the treatment with PPCPs in the worst-case scenario. Therefore, the decreased genus *Haliangium* might be more susceptible against the pollution with PPCPs or against a specific PPCP, the increased clade *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* in contrast more tolerant against the contaminants.

Treated wastewater was not only shown to affect the bacterial diversity and community composition but also to influence the uptake of PPCPs in lettuce. The concentration of most of the PPCPs was higher in lettuce roots irrigated with spiked treated wastewater compared to spiked tap water, showing that besides the relevance of the plant and the physico-chemical parameters of the compounds for the uptake and translocation of PPCPs in crops, also substances and/or factors in the treated wastewater are important. As the composition of the complex mixtures found in treated wastewater is dynamic and can differ daily, the extent of the uptake and distribution of PPCP residues in crops and therefore the risk of a potential intake by the consumer gets more difficult to assess.

Above all, the assurance of safety for consumers and the environment is the key factor in the discussion about the reuse of treated wastewater for the irrigation of crops. Therefore, it is crucial to understand the uptake and translocation of residual contaminants such as PPCPs by crops and to elucidate the consequences for plants and associated organisms. The present thesis which is embedded in the AWARE project within the framework of the EU Water-JPI initiative aimed at closing knowledge gaps on the fate of PPCPs in crops, as well as on their effects on plant stress responses and on the plant-associated bacterial communities to secure a save and sustainable use of biogenic resources in accordance with the precautionary principle. As demonstrated in this thesis, raw consumed leafy green vegetables like lettuce irrigated with spiked treated wastewater or tap water containing PPCPs at currently relevant environmental levels can take up various compounds and translocate the majority of them to the edible part of the plant. Even though the detected concentrations were relatively low in the crop tissues, PPCPs still triggered significant changes in plant stress responses and affected the gene expression of stress related genes in lettuce treated with PPCPs at environmentally relevant concentrations. Furthermore, PPCPs in the worst-case scenario at 100 µg/L were also identified to cause a shift in the bacterial community composition, and treated wastewater was even shown to have negative effects on the alpha diversity and influenced the beta diversity of the lettuce root-associated bacterial communities.

The results of the present thesis have given valuable insights on the uptake and translocation of PPCPs as residual contaminants of treated wastewater in lettuce and demonstrated an influence on the gene expression of stress related genes as well as on the plant-associated

bacterial community composition and diversity in the worst-case scenario even though the detected concentrations in the plants' tissues were relatively low. However, as the long-term consequences of these observed changes for the crops' physiological status and yield are still unknown, environmental and economic consequences of residual PPCPs in treated wastewater used for the irrigation of crops cannot be excluded at the current level of knowledge.

In any case, as stated in the Water-JPI program, the availability of water in sufficient quantities and adequate quality is indeed a public issue of high priority and addresses a pan-European and global environmental challenge. Hence it would be advantageous to reduce the PPCP loads in the treated wastewater by a responsible consumption and disposal to lower their concentrations from the beginning on or by advanced treatment techniques of WWTPs to filter these but also other contaminants so that less of the substances are entering into surface- and ground waters, agricultural soils, and crops used as food and feed commodities.

IV. Bibliography

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VI. Appendix

Publications I – III

Appendix: Publication I – Manuscript

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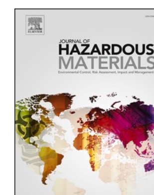

Effect of the pharmaceuticals diclofenac and lamotrigine on stress responses and stress gene expression in lettuce (*Lactuca sativa*) at environmentally relevant concentrations

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Diurnal rhythm

ABSTRACT

Vegetable crops irrigated with treated wastewater can take up the environmentally persistent pharmaceuticals diclofenac and lamotrigine. This study aimed at quantifying the uptake and translocation of the two pharmaceuticals in lettuce (*Lactuca sativa*) as well as on the elucidation of the molecular and physiological changes triggered by them. Therefore, plants were cultivated in a phytochamber in hydroponic systems under controlled conditions and treated independently with diclofenac (20 μg L^{-1}) and lamotrigine (60 μg L^{-1}) for 48 h. A low translocation of lamotrigine but not of diclofenac or its metabolite 4'-hydroxydiclofenac to leaves was observed, which corresponded with the expression of stress related genes only in roots of diclofenac treated plants. We observed an oxidative burst in roots and leaves occurring around the same time point when lamotrigine was detected in leaves. This could be responsible for the significantly changed gene expression pattern in both tissues. Our results showed for the first time that pharmaceuticals like lamotrigine or diclofenac might act as signals or zeitgebers, affecting the circadian expression of stress related genes in lettuce possibly causing a repressed physiological status of the plant.

1. Introduction

Pharmaceuticals as contaminants in treated wastewater can become a serious problem for food safety when they are used for agricultural irrigation. These organic contaminants can be taken up by plants and trigger abiotic stress responses which can eventually affect plant growth and development. Plants have developed different strategies to adapt to abiotic stresses and environmental fluctuations by utilizing numerous molecular, biochemical, physiological and morphological changes to increase the probability of survival and competitive advantages (Pareek et al., 2009). These modulations in the plant might have fitness costs or effects on fruit quality attributes as has recently been shown in tomato plants (Christou et al., 2019).

Diclofenac ([2-(2,6-dichloroanilino) phenyl] acetic acid), is one of the most abundant pharmaceuticals in water derived from wastewater treatment plants and effluents (Pérez and Barceló, 2008; Vieno and Sillanpää, 2014). This compound can be taken up by plants and can induce oxidative stress. Kummerová et al. (2016) detected a significantly increased relative content of H_2O_2 in *Lemna minor* upon treatment with 10 μg/L diclofenac for 10 days. Moreover, other stress parameters like the ratio of oxidized/reduced thiols, and the peroxidation of lipids was significantly enhanced.

Apart from the oxidative stress induced by this compound, diclofenac can be rapidly metabolized in plants. This metabolization follows a pattern of three consecutive phases, first described as the "Green Liver" concept by Sandermann (1992). During phase I, compounds are activated by oxidation, reduction or hydroxylation for the conjugation to reactive groups such as amino acids or sugars during phase II. Enzymes like glutathione *S*-transferases or glycosyltransferases catalyze these reactions. Conjugated phase II metabolites are sequestered in vacuoles or cell walls during phase III. In general the metabolization of foreign compounds will reduce their toxicity for the plant, although during

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phase I activation ROS may be produced, that need to be controlled by scavenging enzymes. Huber et al. (2012) observed phase I and phase II metabolization products of diclofenac in *Hordeum vulgare* (barley) and in the hairy root cell culture of *Armoracia rusticana* (horseradish). The activated hydroxylated metabolite 4′OH-diclofenac as well as the subsequently conjugated glucopyranoside were detected already after three hours of exposure.

Similar to diclofenac, the anti-epileptic drug lamotrigine is highly persistent in the environment and could be detected in crops (Paz et al., 2016) even if the concentration found in plant tissue is low and the specific translocation mechanism still unknown. Therefore, Goldstein et al. (2018) hypothesized an adsorption of lamotrigine to the roots or a trapping in root vacuoles with only limited transport to the shoots. Information about lamotrigine-triggered stress responses in plants is lacking, but could provide useful hints for the translocation and perception of this pharmaceutical.

Genes involved in abiotic stress responses are often expressed in diurnal rhythms. Mutations in key circadian clock genes caused a greater sensitivity to salt, osmotic, and heat stress in *Arabidopsis thaliana*, which demonstrates the importance of the diurnal rhythms in the modulation of multiple stress responses (Kant et al., 2008). Many cold- and drought-responsive stress genes are rhythmically expressed in *A. thaliana* (Covington et al., 2008; Wilkins et al., 2010). Furthermore, Lai et al. (2012) demonstrated a circadian-regulation of reactive oxygen species (ROS) response. ROS act as secondary messengers involved in stress-response signaling but they are also cellular indicators of stress. High levels of ROS cause oxidative damage such as membrane lipid peroxidation, protein oxidation, DNA and RNA damage and can lead to induced cell death. Consequently, scavenging of ROS in cells is essential and catalyzed by enzymes including peroxidases and catalases (Mittler, 2002). The expression of a peroxidase (*NtPXC8.1*), a cytochrome P450 (*NtCYP71D21*) and different other genes involved in the metabolism of xenobiotic compounds and clock genes were significantly affected in *Nicotiana tabacum* hairy root culture under phenol treatment (Alderete et al., 2018). However, the putative influence of residual pharmaceuticals in wastewater on the expression of circadian controlled genes coding for stress enzymes in plants has not been investigated so far.

In this exploratory research, we aimed to elucidate the influence of environmentally relevant concentrations of diclofenac and lamotrigine on the physiology and biochemistry of edible plants under controlled conditions. Hydroponic systems therefore offer several advantages like the usage of nutrient solution, which can be modified easily and homogeneously to test toxic effects of elements and different contaminants under controlled and known conditions. However, the results may vary in magnitude compared to plants grown in soil experiments (Nguyen et al., 2016). Lettuce, the species used in our experiment is frequently grown in hydroponic systems in commercial production, as growth and yield are independent of soil type and quality of the cultivated area (Maucieri et al., 2019). Therefore, the usage of hydroponic systems for lettuce experiments represent a realistic growing scenario for the food producing industry. A multidisciplinary approach was used, (1) to quantify the concentrations of the two pharmaceuticals and their key metabolites in lettuce roots and leaves to investigate their uptake and translocation. These results were related (2) to the analysis of the oxidative stress level in the plant and, (3) to the investigation of the expression of genes involved in abiotic stress response and the metabolization of xenobiotics such as peroxidase (*PER50*), catalase (*CAT1*), and glutathione *S*-transferases (*GST-F6*, *GST-F8*, *GST-U5*).

2. Materials and methods

2.1. Experimental design

Lettuce (*Lactuca sativa* L. var. capitata cv. 'Tizian', Syngenta, Bad Salzuflen, Germany) was grown for 21 days after germination in hydroponic systems in a phytochamber with 16/8 h light/dark cycle at 20/15 °C, and an average humidity of 50 %. Each pot contained one plant and was filled with clean perlite to avoid possible adsorptions of the pharmaceuticals to the substrate. Modified 0.5 × Johnson`s solution pH 5.4 containing 20 μM $FeSO_4 \times 7 H_2O$ was used as nutrient media. The experiment was performed in triplicates. For the treatments the nutrient media was renewed and either lamotrigine (60 μg L^{-1}), diclofenac (20 μg L^{-1}) or pure ethanol (control) was added to it. Plant leaves and roots were harvested separately at time points 0, 6, 12, 24, 30, 36 and 48 h post treatment, snap frozen in liquid nitrogen and stored at – 80 °C until processing. Frozen material was ground in liquid nitrogen with mortar and pestle to a fine powder for either RNA, enzyme or H_2O_2 extraction. For the analytical procedure, plant cultivation and treatments were repeated and samples of time points 0, 6, 12, 24 and 48 h were lyophilized for further processing.

2.2. Extraction and analysis of diclofenac & lamotrigine and metabolites

Extraction of pharmaceuticals from lettuce root and leaf samples was carried out using the Original QuEChERS extraction kit (Bekolut, Hauptstuhl, Germany) followed by LC/QTOF-MS analysis according to (Nicola Montemurro et al. in prep.). Briefly, 1 *g* of homogenized freeze-dried lettuce leaves was placed in 50-mL Falcon tubes and 9 mL of HPLC water were added. Then, the tubes were vortexed for 2 min at 2500 rpm using a BenchMixer XLQ QuEChERS Vortexer (Benchmark Scientific, Sayreville NJ, US). After 1 h from the complete hydration, 50 μL of internal standard (IS) mix were added to achieve the final concentration 10 ng mL^{-1}, vortexed (2500 rpm, 2.5 min) and allowed to rest for another 30 min. Then 10 mL of acetonitrile and 50 μL of concentrated formic acid were added and the tubes were vortexed again. After that, the Original QuEChERS extraction kit was added directly into the tubes and instantly hand shaken for 30 s. All tubes were vortexed again and centrifuged (4000 rpm, 10 min, 4 ºC). The supernatant was transferred into a glass tube and left overnight at −20 °C, to promote the precipitation of co-extractives like waxes and sugars contained in lettuce leaves. After 12 h, 6 mL of the organic phase were transferred into PSA tube (150 mg PSA, 150 mg C18, 900 mg $MgSO_4$), vortexed for 2 min, and centrifuged at 4000 rpm for 5 min, 4 °C. One mL of the supernatant was transferred to a 2-mL vial and evaporated until total dryness under a nitrogen stream and then reconstituted with 1 mL of water/MeOH (90:10) solution before it was injected for LC–MS/MS analysis. For the roots, a similar modified QuEChERS procedure was used which consists of a single extraction step according to the following protocol (Manasfi et al., in preparation). Briefly, 1 *g* of homogenized freeze-dried root tissue was transferred in a 50-mL falcon tube and hydrated with 8 mL of EDTA-McIlvaine buffer (pH = 4), vortexed, and allowed to rest for 30 min. After adding 50 μL of IS mix, the tubes were vortexed (2500 rpm, 2.5 min) and allowed to rest for another 30 min. Then, 10 mL of acetonitrile were added to the samples and they were vortexed for 2 min at 2500 rpm. Finally, the Original QuEChERS extraction kit was transferred into the falcon tubes, hand shaken and vortexed another time and finally, the tubes were centrifuged (4000 rpm, 10 min, 4 ºC) as for lettuce. No freezing or cleanup step took place in this case. Just 1 mL of the supernatant was transferred to a 2-mL vial, evaporated to dryness under a nitrogen stream, reconstituted with 1 mL of water/MeOH (90:10) solution and injected for LC/QTOF-MS/MS analysis. Details about chemicals, EDTA-McIlvaine buffer preparation, LC/QTOF-MS/MS conditions are reported in (see Supplementary Methods (SM)). An one-way ANOVA with corresponding post-hoc Lincon testing was performed to determine significant differences between time points within the diclofenac or lamotrigine treated samples ($n = 3$). Significant differences were indicated with different letters (p–value ≤ 0.05).

Liquid media samples were collected for each exposure time point, mixed 1:2 with 200 mM 5-sulfosalicylic acid and centrifuged at 16,100 x g for 10 min at 4 °C for protein precipitation. Afterwards supernatants were injected for LC–MS/MS analysis. Further details are described in SM.

2.3. Quantitative-PCR analysis of gene expression

Target genes involved in oxidative stress reactions and the detoxification of xenobiotics were selected based on the comparison with functional genes from *A. thaliana* using 'The Arabidopsis Information Resource' (www.arabidopsis.org, Berardini et al., 2015). Complete sequences of those genes were acquired from the *Lactuca sativa* whole genome sequencing project at NCBI (www.ncbi.nlm.nih.gov/bioproject/PRJNA68025). All primer pairs for qPCR (Table S4) were designed by Primer3Plus software (Untergasser et al., 2007) and validated (Applied Biosystems Real-time PCR handbook guidelines, Thermo Fisher Scientific). Afterwards primer/gene-specificities were checked by PCR on cDNAs. The housekeeping gene, coding for the glyceraldehyde-3-dehydrogenase (*GAPDH*), was used as an endogenous control for the qPCR analyses.

The RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) was used to extract RNA from 100 mg pulverized lettuce leaves and roots. After quantification of RNA by NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), cDNA was synthesized from 2 µg of RNA with the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA). The following qPCR of the three biological replicates was performed as described previously (Chowdhury et al., 2019) in three technical replicates. Specific PCR products were confirmed by melting curve analysis and gel electrophoresis before the relative quantification by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). ΔCt values were calculated relative to the endogenous control and subsequently the data of each time point was normalized to the initial time point 0. The standard error of the mean was calculated from the average of the triplicates.

To compare which genes were differentially expressed in the diclofenac and lamotrigine treatments compared to controls, one-way ANOVA with post-hoc Tukey's HSD tests were performed based on ΔCt data.

2.4. Quantification of H_2O_2

H_2O_2 production in roots and leaves was measured according to Shin et al. (2005) using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen, Carlsbad, CA). Ground frozen plant tissue was mixed with 20 mM potassium-phosphate buffer pH 6.5 and centrifuged. Supernatants were incubated with 100 µM Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and 0.2 U ml^{-1} horseradish peroxidase at room temperature for 30 min in the dark before quantifying with a fluorescence/absorbance microplate reader (TECAN Spark®, Tecan Group Ltd., Switzerland) at excitation/emission at 530/590 nm against a H_2O_2 standard curve (0–10 µM).

2.5. Protein extraction and enzyme activity analysis

Soluble protein was extracted according to Schröder et al. (2005), and protein content was quantified (Bradford, 1976) before assaying enzyme activities in a 96-well spectrophotometer (Spectra MAX 190, Molecular Devices, Germany). GST activity was determined at 340 nm (ε =9.6 mM^{-1} cm^{-1}) using the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as a co-substrate (Habig et al., 1974). Peroxidase (POX, EC 1.11.1.7) activity was evaluated by the oxidation of guajacol to tetraguajacol in the presence of H_2O_2 at an extinction of 420 nm (ε =26.6 mM^{-1} cm^{-1}, Diekmann et al., 2004).

2.6. Statistics

Statistical analyses were performed with the software R version 3.6.1. If not indicated differently, a two-way analysis of variance (ANOVA) with Bonferroni post-test was applied to determine significant differences between control plants and treated groups (n = 3). Significance levels were determined as "*" for 0.01 ≤ p–value ≤ 0.05, "**" for 0.001 ≤ p–value ≤ 0.01, and "***" for p–value ≤ 0.001.

3. Results and discussion

3.1. Uptake and translocation of pharmaceuticals in lettuce

The highest concentration of diclofenac was detected in root tissue 6 h after treatment (6.02 µg g^{-1} DW) and a significant reduction of this concentration occurred during the experiment. Simultaneously the analysis of diclofenac treated root samples revealed the formation of the metabolite 4'-hydroxydiclofenac at the same time point and onwards (Fig. 1). Corroborating our results, hydroxylated metabolites had been already detected after 3 h of exposure in a hairy root cell culture of *Armoracia rusticana* (horseradish) (Huber et al., 2012). We observed a rapid metabolization of diclofenac and a higher concentration of the phase I metabolite than the initial compound after 24 h, similar to results published for *Typha latifolia* by Bartha et al. (2014).

However, we were not able to detect diclofenac and the phase I metabolite 4'-hydroxydiclofenac in leaves of the treated lettuce plants at any time point. Similar to our observation, in *Typha latifolia* exposed to high concentrations of diclofenac (1 mg L^{-1}) under hydroponic conditions, barely small amounts of the pharmaceutical (4% of amount in roots after 24 h) were quantified in shoots (Bartha et al., 2014). Additionally, it has been reported that only when plants were treated with diclofenac for a prolonged period, this compound was translocated to tomato fruits (Christou et al., 2017) or to the leaves of *Scirpus validus* (Zhang et al., 2012) in higher rates.

Unlike diclofenac, the concentration of lamotrigine in lettuce roots increased during the first 6 h but stayed constant at a similar concentration (2.14 ± 0.22 µg g^{-1DW}) afterwards until the end of the experiment (Fig. 2). Moreover, a translocation of lamotrigine to the leaves in low but increasing concentrations was detected. It has been proposed that lamotrigine may be restricted from passing through plant cell walls or membranes because of its ionic character and therefore might rather accumulate in roots than in shoots (Chuang et al., 2019). At the initial pH of the liquid media at pH 5.4, ~ 50 % of lamotrigine (pKa 5.7) is charged to form a cation. Charged lamotrigine putatively remains in the apoplastic space and is adsorbed to the root surface, whereas uncharged lamotrigine might be transported by passive diffusion into root cells (pH 7–7.4) or to the leaves. After entering root vacuoles (pH 4–5.5) the molecule is again charged and cannot pass the tonoplast (Nason et al.,

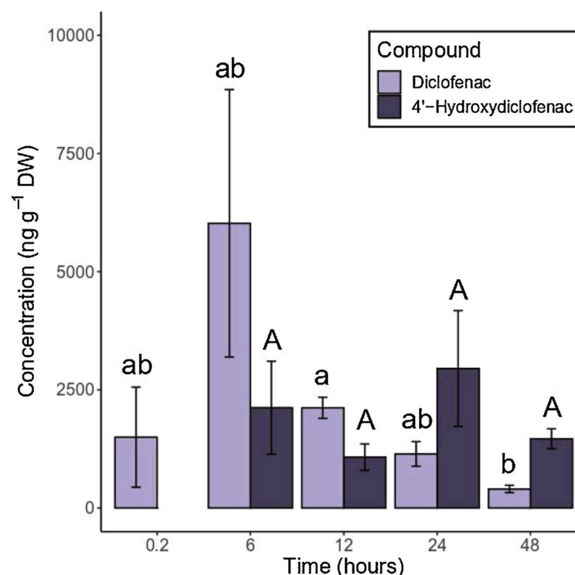

Fig. 1. Concentration of diclofenac and its metabolite 4'-hydroxydiclofenac (ng g^{-1}) in lettuce roots of diclofenac treated groups. Data are mean concentrations (ng g^{-1}dry weight, DW) ± standard error (n = 3). Different letters indicate statistical significance among different time points after exposure to diclofenac (one-way ANOVA, p–value ≤ 0.05).

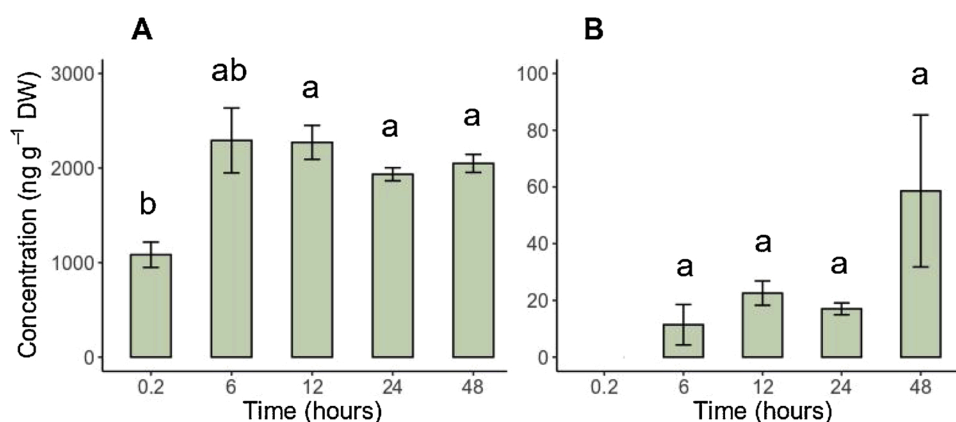

Fig. 2. Concentration of lamotrigine (ng g^{-1}) in lettuce tissue ((A) roots, (B) leaves) of lamotrigine treated groups. Data are mean concentrations (ng g^{-1}dry weight, DW) ± standard error ($n = 3$). Different letters indicate statistical significance among different time points after exposure to lamotrigine (one-way ANOVA, p–value ≤ 0.05).

2018). Consequently, the highest accumulation of lamotrigine was detected in roots and only low concentrations were translocated to leaves (Fig. 2).

In general, our findings highlight a putative passive transport of lamotrigine to leaves, occuring in low concentrations and at slow rates. This reduced mobility might be caused by the cationic charge of the molecule depending on the pH. The high hydrophobicity of diclofenac is hypothesized to be the main reason for the lacking translocation of this compound to aboveground tissues. As already reported in previous studies, the octanol-water partitioning coefficient (log *Kow*) plays a crucial role to predict the uptake of xenobiotics by plants (Briggs et al., 1982). Therefore, highly hydrophobic substances (like diclofenac; log *Kow* = 4.51) have a large potential for bioconcentration in roots but a low possibility for translocation to shoots and leaves. Moreover, when diclofenac had entered plant tissue, the molecule underwent rapid metabolization, as was observed by a decrease of the parent compound and a simultaneous increase of the phase I metabolite (Fig. 1). Such a decrease was not verified for lamotrigine in the present study, but there is also no information about possible metabolism in plants available in literature.

None of the pharmaceuticals was present in control plants growing in liquid media only. Moreover, for the tested concentrations and exposure time of lamotrigine or diclofenac neither visual signs of toxicity nor changes in growth were observed in lettuce (Fig. S1). However, exposure was only for 48 h and at low concentrations (diclofenac: 20 μg L^{-1}; lamotrigine: 60 μg L^{-1}).

Concentrations of lamotrigine were also analyzed in liquid media of treated plants and plant-free control groups. During the 48h-experiment, we detected a relatively stable concentration of lamotrigine in the plant-free control groups (Fig. S2), showing there was negligible loss of the pharmaceutical by sorption to perlite or non-plant related photo- or biodegradation. In the presence of plants, the initial concentration of 58.32 ± 6.74 μg L^{-1} of lamotrigine in nutrient media was reduced to 45.48 ± 2.96 μg L^{-1} within 48 h (Fig. S3).

3.2. H_2O_2 production

H_2O_2 is an important signaling molecule in plant cells that can cause damage to various cell structures in high concentrations. On the one hand, H_2O_2 in high concentrations mediates oxidative stress, which causes damage to cellular components such as proteins, DNA or lipids (Møller et al., 2007). On the other hand, H_2O_2 also acts as a secondary messenger for further downstream signaling, leading to plant responses and to diverse functions of growth and development (Choudhury et al. 2013). After specific perception, H_2O_2 as one of the primary reactive oxygen species (ROS) in plants, is formed as an initial reaction in almost all plant compartments during different enzyme reactions e.g. by plasma membrane bound NADPH oxidases. The apoplastic ROS accumulation can activate ion channels leading to an influx of calcium (Ca^{2+}) into the cytoplasm, which can then vice versa enhance the induction of the apoplastic ROS production during abiotic stress conditions (Lamers et al., 2020). Consequently, these common ROS-calcium signaling pathways enable cell-to-cell communication and thereby long-distance transmission besides the signaling on the single-cell level (Steinhorst and Kudla, 2019; Mittler, 2002). The information presented in the Ca^{2+} signatures can be decoded by diverse Ca^{2+} sensors (e.g. calcium dependent protein kinases (CDPKs), Calmodulins (CaM) or Calmodulin-like proteins (CMLs)) into phosphorylation events, changes in protein–protein interactions or regulation of gene expression by binding at Ca2+/calmodulin-binding transcription factors (Hashimoto and Kudla, 2011). The concentration of H_2O_2 in lamotrigine-treated roots and leaves was significantly elevated (p–values ≤ 0.001) after 12 h compared to control plants (Fig. 3). For the other time points no difference was detected, indicating that lamotrigine is not triggering cellular ROS production but rather a transient oxidative burst, as has been shown for *Salvia officinalis* leaves after they were exposed to ozone for 5 h (Marchica et al., 2019). Interestingly, this transient oxidative burst was detected in roots and leaves at the same time point, when we were also able detect lamotrigine for the first time in the lettuce leaves. We postulate that this oxidative burst appeared due to systemic signaling activities from leaves to roots triggered by the presence of lamotrigine or its metabolites in the leaves. Whether lamotrigine or its degradation products have a direct influence on a leaf specific cell structure remains to be elucidated. Since there are no plant related metabolites of lamotrigine published to date, we were not able to test this hypothesis.

In contrast, upon diclofenac treatment we observed a trend of a reduced H_2O_2 concentration in roots but not in leaves during the experiment (Fig. 3), indicating that the pattern was only detected in the tissue where we were able to quantify the compound.

3.3. Gene expression analysis

Our earlier work showed that the two genes *GST-F6* and *GST-U5* were induced in roots in *Brassica* upon Paracetamol treatment (Bartha, 2012). However, the influence of residual pharmaceuticals in water on the circadian rhythm/control of stress signaling genes in plants has not been investigated so far. We determined the expression of these two genes as well as of an additional GST (*GST- F8*) and two other genes involved in the detoxification of ROS (*PER50 and CAT1*) in lettuce after the exposure to diclofenac or lamotrigine over a time period of 48 h. The expression of all tested genes in the control plants, without exposure to

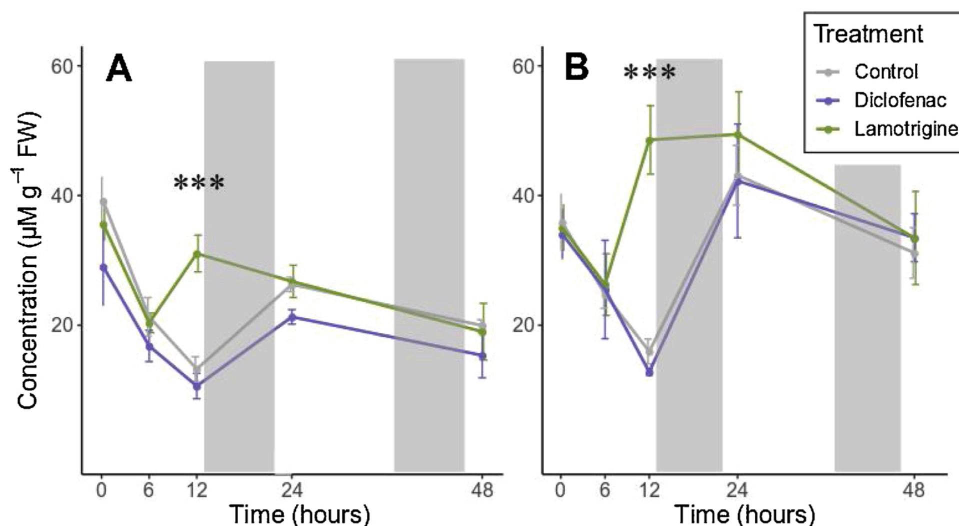

Fig. 3. Line diagram showing change in the concentration of hydrogen peroxide (µM g^{-1}) in lettuce tissue ((A) roots, (B) leaves) in control plants and diclofenac or lamotrigine treated groups as measured over a time period of 48 h. Data are mean H_2O_2 concentrations (g^{-1}fresh weight, FW) ± standard error (n = 3). Significant differences between samples of treated groups and control plants are indicated according to ANOVA as "***" for p–value ≤ 0.001. Grey bars: subjective night.

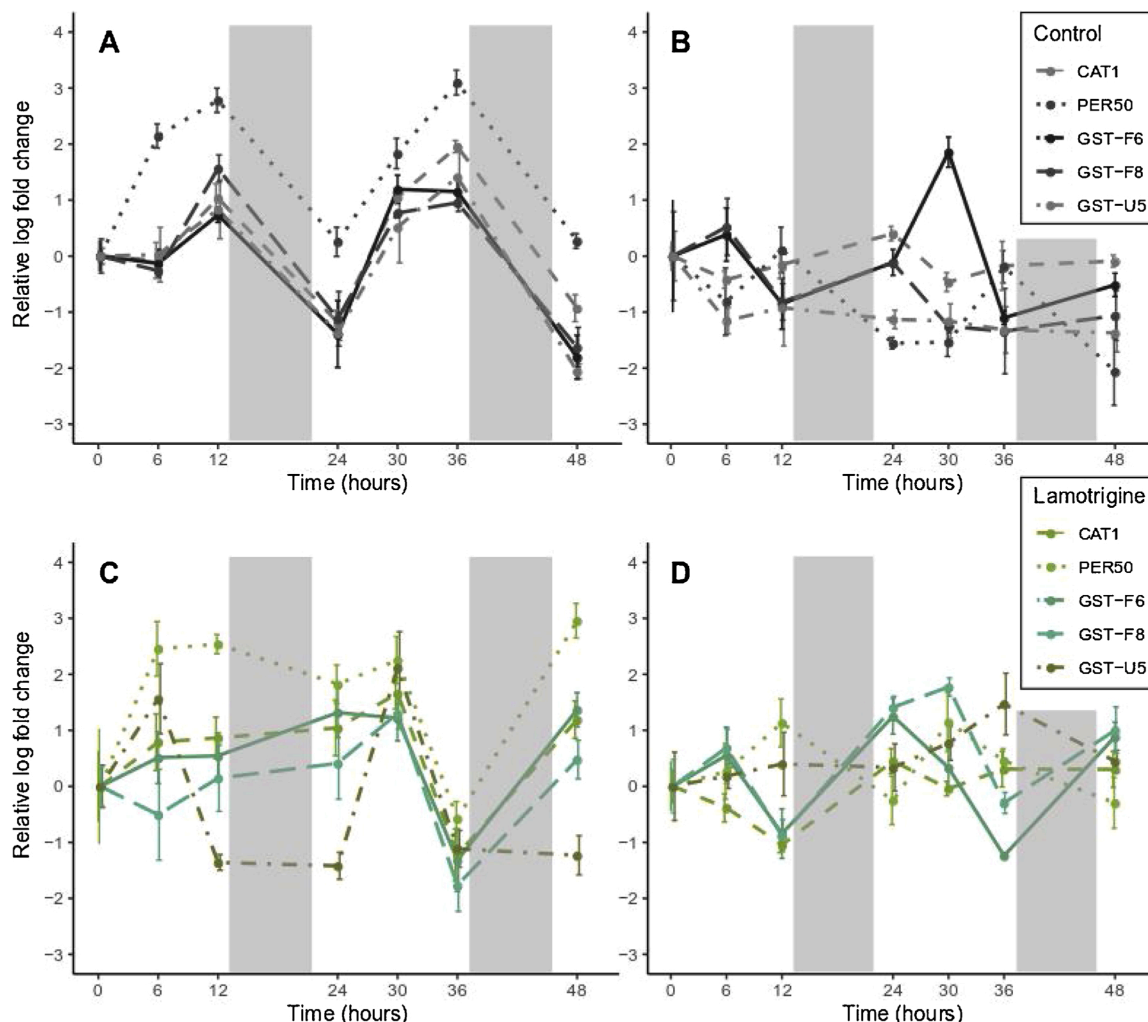

Fig. 4. Line diagram showing changes in relative gene expression (log fold change) over the measured time period as compared to time 0, of three glutathione S-transferases (*GST F6*, *GST-F8* and *GST-U5*), one catalase (*CAT1*) and one peroxidase (*PER50*) gene in (A + B) control and (C + D) lamotrigine treated lettuce in different plant tissues ((A + C) roots and (B + D) leaves). Error bars indicate 95 % confidence interval. Significant differences were observed in the expression pattern of all genes when compared to control plants at different time points revealed by Tukey`s HSD pairwise testing (Supplementary Table S 1). Grey bars: subjective night.

any pharmaceuticals, followed a diurnal pattern over the duration of the experiment (Fig. 4; A and B). In lettuce roots, all five tested genes showed maximal expression in the last hour before subjective dusk (T12 and T36), whereas in the leaves the peaks of the expression were observed at different time points for different genes. We detected the highest expression of the genes coding for the two GSTs belonging to the plant specific phi class (*GST-F6* and *GST-F8*) during the first 8 h after subjective dawn (T6 and T30), the one coding for the peroxidase (*PER50*) in the last hour before subjective dusk (T12 and T36) (Fig. 4; B and D). The diurnal cycles of gene expression in shoots and roots of plants are not usually in-sync. This had been demonstrated in a previous study comparing the circadian clock in roots and shoots in *Arabidopsis*. The rhythmic behavior of the gene expression markedly differed between the tissues. Furthermore, a photosynthesis-related signal from the shoots was identified, affecting the setting of the clock in the roots (James et al., 2008). However, the rhythmic diurnal expression of these genes in lettuce has not been described so far, which makes this an interesting observation.

As an exception to the obvious diurnal expression pattern, the gene coding for the tau-class GST (*GST-U5*) was expressed at constant levels in lettuce leaves in control plants. A constitutive expression of the gene *GST-U5* in leaves had been reported previously, suggesting its housekeeping functions (Wagner et al., 2002) although it was also found to be induced by auxin in roots by another study (van der Kop et al., 1996). Interestingly, the expression of *GST-U5* was significantly increased over all analyzed time points in lamotrigine treated lettuce leaves compared to control plants, indicating a lamotrigine-triggered effect on *GST-U5* (Fig. 4; D).

All other tested genes (*PER50*, *CAT*, *GST-F6* and *GST-F8*) measured in lamotrigine treated plant roots, which were previously shown to be induced by H_2O_2 (Chen et al., 1996; Guan et al., 2000; Wagner et al., 2002) had a similar expression pattern, differing from the control plants (Fig. 4; C). In general, we observed a phase shift in the diurnal expression of the genes. There was a trend for an earlier increased expression after 6 h and an enhanced expression over time for *PER50*, *CAT1* and *GST-F6* in roots. The expression high and low peaks in the circadian rhythm were shifted for most of the genes and their expression at T24, T36 and T48 was significantly different to that in the control plants in roots and leaves (Fig. 4 A–D; Table S5). Shortly before this significant change in gene expression, we detected a significant increase of the H_2O_2 concentration in both tissues at T12 in lamotrigine treated plants, highlighting the role of H_2O_2 in intracellular communication and its connection to subsequent downstream signaling like changes in gene expression (Choudhury et al., 2017).

It has been proven that amongst several other signals, ROS, metabolism and nutrients can act as zeitgebers (external or internal signals acting as time cues) which can affect the functioning of circadian clock of the plants. They can affect a shift in the phase, period or the amplitude of the circadian clock (Lai et al., 2012). The circadian clock has been shown to influence several biological processes in plants, within a complex network of pathways which has been studied in detail for *Arabidopsis* (Harmer et al., 2000; Lai et al., 2012). However, since such information is lacking for lettuce, we may only postulate that lamotrigine or its metabolites could either directly or indirectly act as a stimulus (zeitgeber) or cause a disruption of the circadian clock in lettuce plants.

A significant transient reduction of the expression of all genes was observed at T6 in roots of diclofenac treated plants (Fig. 5). Moreover, the expression of *CAT1*, *PER50*, *GST-F6* and *GST-F8* was also significantly reduced at T12. With decreasing concentrations of diclofenac we detected a reduced influence on stress gene expression compared to control plants in lettuce roots. In leaves, where we were not able to detect diclofenac or its metabolite 4'-hydroxydiclofenac, the influence on the expression of stress genes was generally low (Fig. 6). Nevertheless, a reduced expression of stress genes might lead to a decreased defense status against biotic and abiotic stressors and therefore to a higher susceptibility of the plant when the compound was present.

3.4. Stress enzyme activity

Since reactive oxygen species in high concentrations produced during the activation of xenobiotics can cause oxidative stress to the plant, it is crucial to strictly regulate intracellular H_2O_2 concentrations because of its additional role in cell signaling. Peroxidases (POX) are important enzymes involved in the antioxidant network and catalyze the conversion of H_2O_2 to water (Mittler, 2002). We observed a significantly reduced POX activity in roots exposed to lamotrigine during the whole experiment (Fig. 7).

In *Typha latifolia*, POX activity was inhibited during the first 14 days of the exposure and began to increase only after 21 days of exposure to carbamazepine (Dordio et al., 2011). This change was detected also in leaves, since carbamazepine is taken up by the plants' roots and translocated to the aerial parts of the plants. However, since the translocation of lamotrigine to lettuce leaves is relatively low; hence we measured no change of POX activity in the leaves compared to control plants. Plant peroxidases were reported to oxidize diclofenac to activate the molecule for further conjugation (Huber et al., 2016). When *Typha latifolia* was incubated with 1 mg L^{-1} of diclofenac, enzyme activities were significantly increased after 24 h (Bartha et al., 2014). In the present case, exposing plants to a much lower concentration (20 μg L^{-1}) for up to 48 h, we were not able to detect differences in POX activities in roots or leaves (Fig. 7).

The activity of enzymes involved in the conjugation of activated xenobiotics to glutathione during detoxification processes was comparable between diclofenac (20 μg L^{-1}) treated and control plants in lettuce, as also shown for a concentration of 10 μg L^{-1} in *Lemna minor* (Kummerová et al., 2016). Only higher diclofenac concentrations (100 μg L^{-1}) caused significantly increased *Lemna* GST activities. Moreover, no change of GST activities was caused by the exposure to lamotrigine, as this compound might not be a substrate for these enzymes.

The present observations showed that the alterations of the antioxidant enzyme POX might be explained as a reaction to the uptake of lamotrigine by lettuce roots and the low translocation to the leaves. In contrast, the concentration of diclofenac in the tissue seemed too low to induce a change of enzyme activities.

4. Conclusions

Our results indicate that low concentrations of diclofenac and lamotrigine do not trigger measurable inductions of stress enzyme activities in lettuce, but a significant change in the expression of several stress related genes. The alterations of gene expression in case of diclofenac were predominantly pronounced in the roots where the pharmaceutical was localized whereas lamotrigine triggered a putative systemic response after the pharmaceutical was translocated to the leaves. We show for the first time that pharmaceuticals like lamotrigine and diclofenac can possibly act as signals or zeitgebers, which affect the circadian expression of the selected genes in lettuce plants.

Irrigation of vegetable crops using treated wastewater is a common growing practice in modern agriculture. The presence of various pharmaceuticals in the wastewater and their constant uptake by crops may influence the expression of plant stress genes in different ways. Especially circadian dysfunction of the stress gene expression could lead to chronic reactions and cause a repressed physiological status resulting in a reduced resistance to biotic stresses, an inferior tolerance to other abiotic stresses or in general to reduced growth and yields.

Author contribution

YB designed and performed the experiments, evaluated the data, styled the figures and wrote the draft manuscript.

SPC performed the Q-PCR and aided in gene expression interpretation and figure design.

SP supervised the MS-measurements.

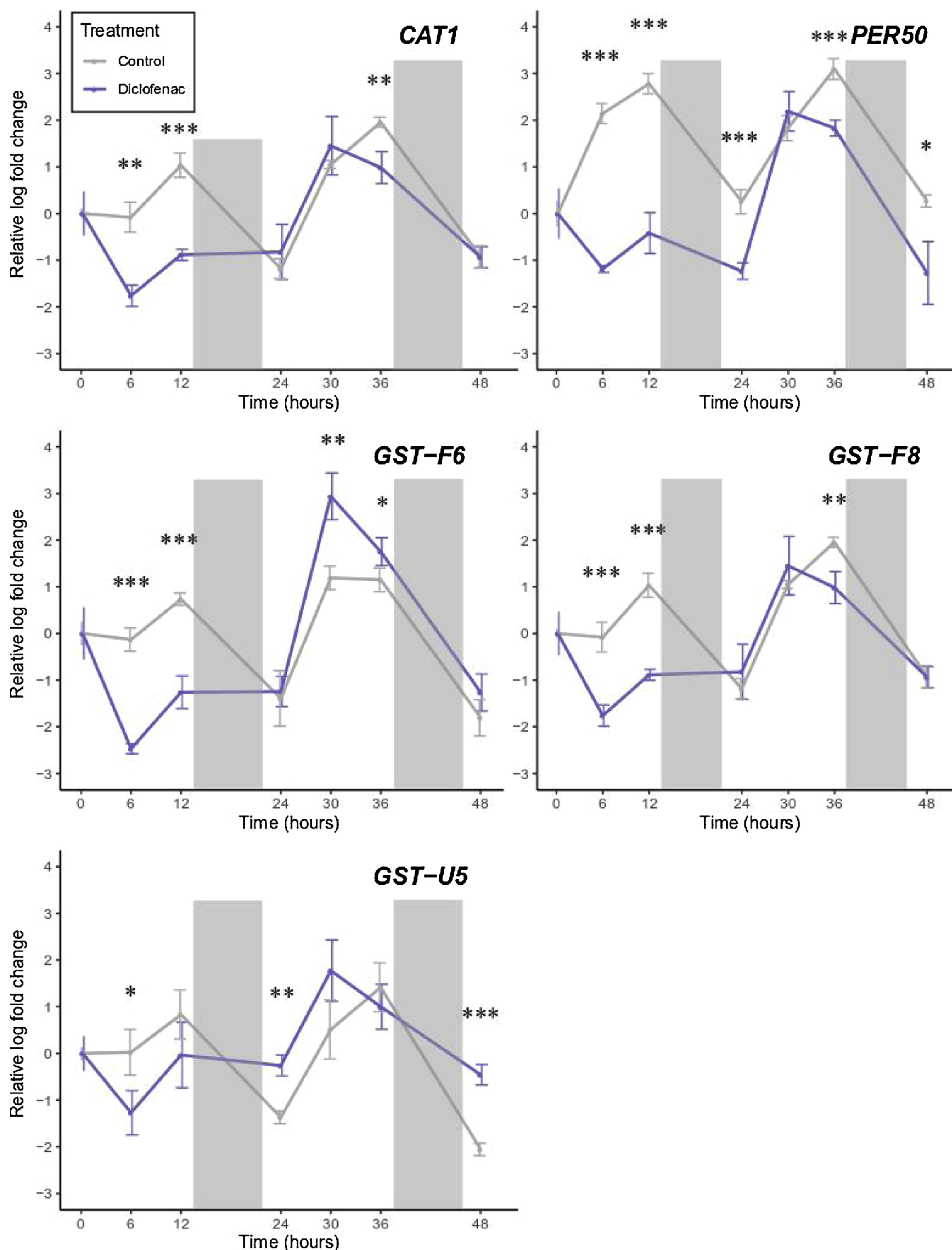

Fig. 5. Line diagram showing changes in relative gene expression (log fold change) in lettuce roots over the measured time period as compared to time 0, of three glutathione *S*-transferases (*GST F6*, *GST-F8* and *GST-U5*), one catalase (*CAT1*) and one peroxidase (*PER50*) gene in controls and diclofenac treated plants. Error bars indicate 95 % confidence interval. Significant differences between treated and control plants are indicated according to Tukey`s HSD pairwise testing as "*" for 0.01 ≤ p–value ≤ 0.05, "**" for 0.001 ≤ p–value ≤ 0.01, and "***" for p–value ≤ 0.001. Grey bars: subjective night.

NM performed the MS-Analyses and interpreted the data.

RM extracted the plants and evaluated the data.

PS acquired funding, composed hypotheses and experiments jointly with YB, discussed gene expression experiments and finalized the manuscript.

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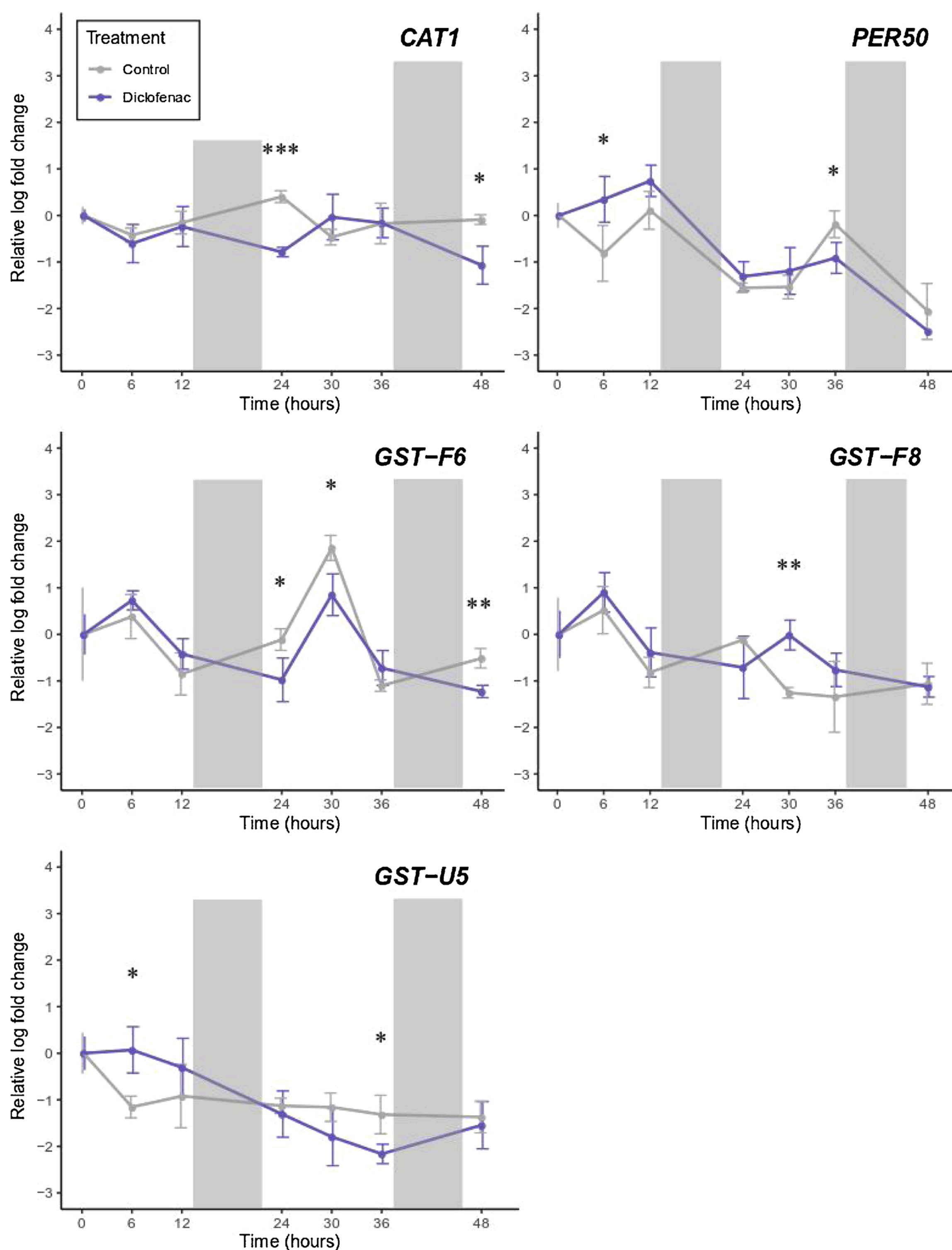

Fig. 6. Line diagram showing changes in relative gene expression (log fold change) in lettuce leaves over the measured time period as compared to time 0, of three glutathione *S*-transferases (*GST F6*, *GST-F8* and *GST-U5*), one catalase (*CAT1*) and one peroxidase (*PER50*) gene in controls and diclofenac treated plants. Error bars indicate 95 % confidence interval. Significant differences between treated and control plants are indicated according to Tukey`s HSD pairwise testing as "*" for 0.01 $\leq$ p–value $\leq$ 0.05, "**" for 0.001 $\leq$ p–value $\leq$ 0.01, and "***" for p–value $\leq$ 0.001. Grey bars: subjective night.

AWARE (Assessing the fate of pesticides and water-borne contaminants in agricultural crops and their environmental risks) project, and the Federal Office for Agriculture and Food (Bundesamt für Landwirtschaft und Ernährung; BLE. grant number: 2816ERA04W). SPC was supported by the German Federal Ministry of Education and Research (BMBF) project DiControl (031B0514E). SP and NM thank the Spanish Ministry of Science and Innovation (Project CEX2018−000794-S). The authors also acknowledge SCIEX for providing the loan instrument LC/HRMS QTOF X500R and Bekolut GmbH & Co. KG for the contribution with QuEChERS kits extraction.

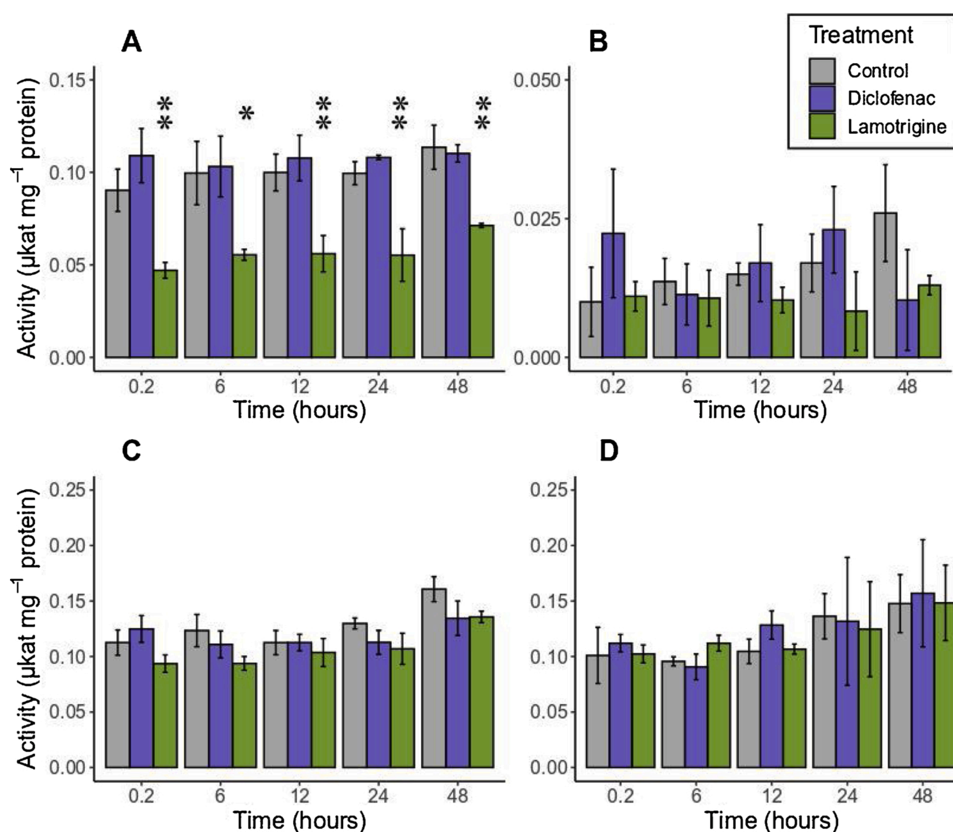

Fig. 7. Stress enzyme activities of (A + B) guajacol-peroxidase and (C + D) glutathione *S*-transferase in different plant tissues ((A + C) roots and (B + D) leaves) in control plants and treated groups. Data are mean activities ± standard error ($n = 3$). Significant differences between treated groups and control plants are indicated according to ANOVA as "*" for $0.01 \leq$ p–value ≤ 0.05 and "**" for $0.001 \leq$ p–value ≤ 0.01.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

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Appendix: Publication I – Supplementary Material

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Supplementary Material

Effect of the pharmaceuticals diclofenac and lamotrigine on stress responses and stress gene expression in lettuce (Lactuca sativa) at environmentally relevant concentrations

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Figure S1: Lettuce biomass fresh weight (g) of (A) roots and (B) leaves of control plants and diclofenac or lamotrigine treated groups over the time of the experiment of 48h. Data are mean concentrations ± standard error (*n* = 3).

Figure S2: Relative concentration (Ci/C0) of lamotrigine in the plant-free control groups over the time of the experiment of 48h. Ci is the measured concentration at a specific time point, C0 the concentration at T0.

Figure S3: Concentration of lamotrigine (µg L^{-1}) in nutrient media over the time of the experiment of 48h. Data are mean concentrations ± standard error (*n* = 3).

Supplementary Methods

1. Chemicals

Lamotrigine (3,5-Diamino-6-(2,3-dichlorphenyl)-1,2,4-triazin, pharmaceutical secondary standard) and Diclofenac sodium salt (2-[(2,6-Dichlorophenyl)amino]benzeneacetic acid sodium salt) were purchased from Sigma-Aldrich (Germany). High purity reference standards (4'-hydroxydiclofenac, diclofenac, and lamotrigine) purchased from Sigma Aldrich (St. Luis, MO, U.S). Isotopically labelled compounds (IS) (diclofenac-$^{13}C_6$ and lamotrigine-$^{13}C_3$) were high purity (mostly 90%) and were obtained from Sigma-Aldrich (St. Luis, MO, USA) and Toronto Research Chemicals (Toronto, ON, Canada), respectively.

LC-MS grade acetonitrile (ACN) (≥99.9%), methanol (MeOH) (≥99.9%), HPLC water, and formic acid (98%) were purchased from Merck (Darmstadt, Germany). The Original (OR) QuEChERS extraction salts kit (4g $MgSO_4$ + 1g NaCl) was obtained from BEKOlut GmbH & Co. KG (Hauptstuhl, Germany). Working solutions mixture (2 µg mL^{-1}) and internal standard (IS) working solution (2 µg mL^{-1}), for analysis and calibration purposes, were prepared by diluting adequate volumes of the individual stock solutions (1000 mg L^{-1}) with MeOH. All the solutions were stored at -20 °C. For preparation of the EDTA-McIlvaine buffer (pH=4) for roots extraction, Di-Sodium hydrogen phosphate dehydrate ($Na_2HPO_4 \cdot 2H_2O$) Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) and ethylendiaminetetraacetic acid anhydrous (EDTA) (≥99%) were supplied by Sigma-Aldrich (St. Luis, MO, USA). The EDTA buffer was prepared by dissolving 1.5 g of disodium hydrogen phosphate dehydrate, 1.3 g of citric acid monohydrate, and 0.372 g EDTA in 100 mL HPLC water.

70 **2. LC/QTOF-MS/MS analysis of lettuce samples**

71 **2.1. Method Performance**

72 Qualitative and quantitative analysis were performed using SCIEX OS™ Software
73 version 1.6 (Sciex, Redwood City, CA, U.S.). The two highest resolution ions were used
74 for positive confirmation and identification through HR-QToF-MS analysis: the most
75 abundant product ion for the quantification and the precursor ion for the confirmation
76 (SANTE/11813) (Commission 2018). Calibration curves were constructed using linear
77 weighted least-squares regression (1/x as weighting factor) by plotting the ratio of the
78 analyte signal to that of its corresponding IS and presenting coefficients of determination
79 (R^2) above 0.99. Linearity of the method was evaluated using calibration curves ranging
80 between 0.5 and 2000 ng g^{-1} DW in lettuce tissues, with a minimum of eight calibration
81 points. Sensitivity, Limits of Detection (LODs) and Limits of Quantification (LOQs)
82 were estimated from the matrix-matched calibration curves using linear regression
83 analysis and a signal-to-noise ratio of 3.3 and 10, respectively (Table S1).

84

85 **Table S1:** Linearity, LODs and LOQs of 4´-hydroxydiclofenac, diclofenac and

86 lamotrigine extracted from lettuce roots and leaves.

	Linearity (ng g^{-1})	R^2	LOD (ng g^{-1})	LOQ (ng g^{-1})	Internal standard
LEAVES					
4'-Hydroxydiclofenac	2.5-2000	0.9935	0.09	0.26	Diclofenac-$^{13}C_6$
Diclofenac	5-2000	0.9917	0.05	0.17	Diclofenac-$^{13}C_6$
Lamotrigine	1-2000	0.9930	0.05	0.14	Lamotrigine-$^{13}C_3$
ROOTS					
4'-Hydroxydiclofenac	5-2000	0.9888	0.04	0.13	Diclofenac-$^{13}C_6$
Diclofenac	1-2000	0.9933	0.03	0.10	Diclofenac-$^{13}C_6$
Lamotrigine	5-2000	0.9957	0.02	0.05	Lamotrigine-$^{13}C_3$

87

88

89 **2.2. Analysis of lettuce samples**

90 Lettuce leaves and roots samples were analysed using a SCIEX X500R QTOF hybrid

91 system (Sciex, Redwood City, CA, U.S.). Chromatographic separation was performed on

92 a reverse phase Hibar® HR Purospher® STAR RP-C18 column (100 mm x 2.1 mm i.d.,

93 2 µm particle size, Merck, Darmstadt, Germany), thermostated at 40 °C in the column

94 oven. A 12 min fast elution was carried out using of ACN and water (5 mM ammonium

95 acetate + 0.1% formic acid) as mobile phases, at a flow rate of 0.5 mL/min (Table S2).

96

97

98 **Table S2:** LC gradient for the elution of the target compounds extracted from lettuce roots and

99 leaves.

Time (min)	**Mobile phase composition/vol. %**		**Flow rate, (μL min^{-1})**
	Water*	ACN	
0.0	95	5	0.5
0.1	95	5	0.5
6.0	60	40	0.5
10.0	2	98	0.5
10.9	2	98	0.5
11.1	95	5	0.5
12.0	95	5	0.5

100 *(5 mM ammonium acetate + 0.1% formic acid) for the positive electrospray ionization. ACN:

101 acetonitrile.

102

103 The injection volume was 10 µL, and the auto-sampler temperature was maintained at

104 8 °C. High resolution data were acquired in positive electrospray ionization in MRM^{HR}

105 acquisition using fragment scanning mode. Data acquisition method and source

106 conditions are listed in Table S3. Exhaustive details of the methodology are reported

107 elsewhere (Montemurro et al., in preparation; Manasfi et al., in preparation).

108

109 **Table S3:** Ion source parameters and MRM^{HR} acquisition parameters used for analysing

110 lettuce sample.

Ion source Voltage:	5500V	**Source Temperature TEM:**	550°C
Atomizing gas GS1:	55 psi	**TOF-MS**	100 to 950 *m/z*, 0.12s acc. time
Auxiliary gas GS2:	55 psi	**Collision energy**	10 V
Air curtain gas:	30 psi	**Collision gas:**	7

Analyte	**4'-Hydroxydiclofenac**	**Diclofenac**	**Lamotrigine**
Chemical Formula	$C_{14}H_{11}C_{l2}NO_3$	$C_{14}H_{11}C_{l2}NO_2$	$C_9H_7C_{l2}N_5$
Adduct/Charge	[M+H]+	[M+H]+	[M+H]+
Precursor Mass (m/z)	312.0188	296.0239	256.0151
Fragment Mass (m/z)	230.0277	214.0424	210.9719
Declustering potential (V)	65	55	145
Collision energy (V)	45	40	35
Retention Time (min)	7.54	8.49	4.02
IS Name	Diclofenac-$^{13}C_6$	Diclofenac-$^{13}C_6$	Lamotrigine-$^{13}C_3$

111

112 **3. LC-MS/MS analysis of liquid media samples**

113 **3.1. Quality assurance procedures**

114 The performance of the methods was checked daily, using method blanks (solvent
115 controls), fortified samples spiked with internal standard using, new calibration curves
116 weekly. The limits of detection (LODs) and quantification (LOQs) for each
117 pharmaceutical were defined as LOD = 3.3(α /S) and LOQ= 10(α /S); here, α is the
118 standard deviation slope and S is the average slope of the calibration curves. Precision
119 and accuracy were evaluated following the criteria established by following the ICH
120 (2005).

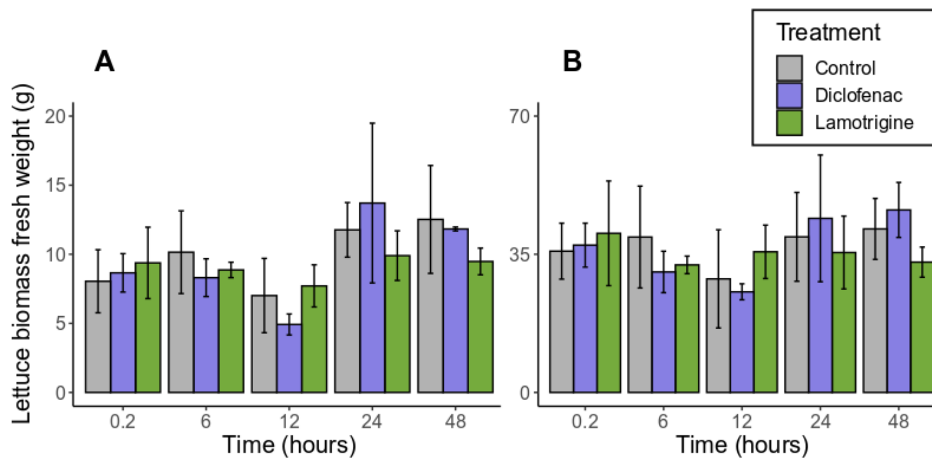
121

122

3.2. Analysis of liquid media samples

The protein precipitated liquid media samples were injected (10 µL) in triplicates by an auto sampler (Dionex UltiMate 3000TRS, Gemering, Germany) into an UHPLC (Dionex UltiMate 3000RS, Gemering, Germany) coupled to a triple quadrupole mass spectrometer (HESI-MS/MS, TSQ Quantum Access Max, San Jose, USA) from Thermo Scientific. An Accucore PFP column (100 mm x 2.1mm, 2.6 µm particle size, Thermo Scientific) with an Accucore PFP pre-column (10 x 2.1mm, 2.6 µm particle size, Thermo Scientific) at a flow rate of 0.450 mL min^{-1} was applied for chromatographic separation. For a linear gradient elution, the mobile phases 0.1% formic acid in Mili-Q water (A) and 0.1% formic acid in acetonitrile (B) were used to apply the following gradient program: 0–2 min 5% Buffer B, 2-8 min 5-100% B, 8-9 min 100% B, 9-9.1 min 100-5% B, 9.1-10 min 5% B. The mass spectrometer was operated in positive HESI mode with capillary voltage of 4000 V; nitrogen dumping gas temperature of 350 °C; sheath gas pressure 50 psi, auxiliary gas pressure 5 psi, capillary temperature 380 °C, skimmer offset of 6, collision energy of 28 eV and tube lenses of 97 V. Analysis of samples was in scheduled multiple-reaction-monitoring (SMRM) mode following the precursor ion [M+H]+ 256.01 m/z and the product ions 186.8 and 211.0 m/z. Afterwards, samples were quantified against a calibration curve with five nominal concentrations from 7.5 to 120 µg L^{-1}, using Lamotrigine-^{13}C as internal standard (20 µg L^{-1}).

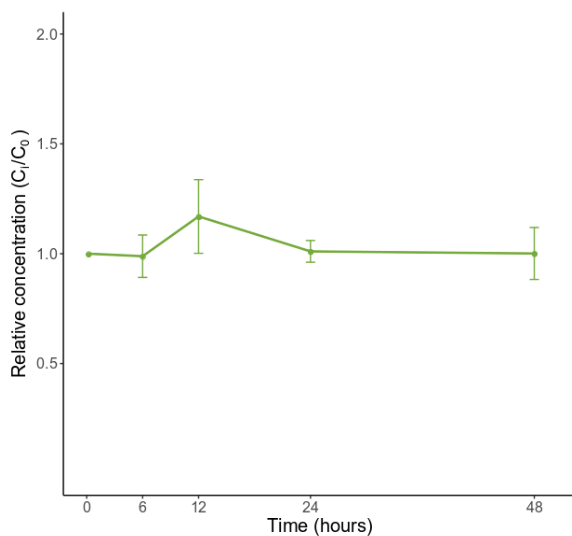
Retention times and mass spectra were similar between standards and fortified matrices (RSD<20%), thus proving that the chromatographic procedures were selective for the quantification of all pesticides. The calibration curves proved to have good fits, with r^2 ranging from 0.0987. LOD and LOQ was 0.24 µg L^{-1} and 0.71 µg L^{-1}, respectively.



147

148 **Figure S1:** Lettuce biomass fresh weight (g) of (A) roots and (B) leaves of control plants
149 and diclofenac or lamotrigine treated groups over the time of the experiment of 48h. Data
150 are mean concentrations ± standard error (n = 3).

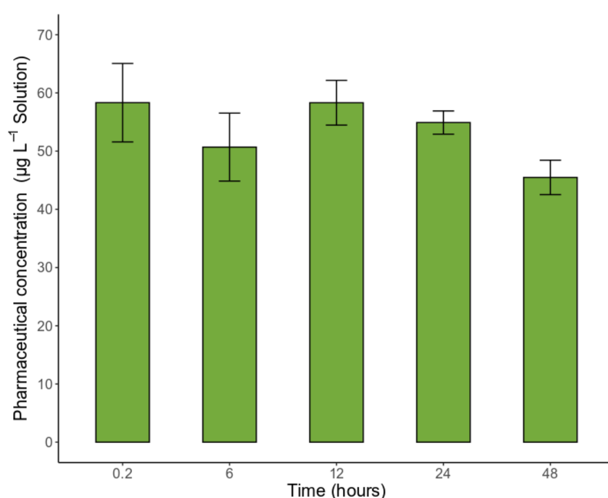
151



152

153 **Figure S2:** Relative concentration (Ci/C0) of lamotrigine in the plant-free control groups
154 over the time of the experiment of 48h. Ci is the measured concentration at a specific time
155 point, C0 the concentration at T0.

156



157

158 **Figure S3:** Concentration of lamotrigine (µg L^{-1}) in nutrient media over the time of the

159 experiment of 48h. Data are mean concentrations ± standard error (*n* = 3).

160

161 **Table S4:** List of plant genes selected for expression analysis with their corresponding

162 loci, functions in *A. thaliana* and primer sequences.

Name of gene (Locus tag in *Arabidopsis thaliana*)	**Documented functions in *Arabidopsis thaliana***	**Primer sequences (5‘ -3‘) All primers were designed in this study and have an annealing temperature of 55°C**
***CAT1* (AT1G20630)**	Catalase, induced by hydrogen peroxide, abscisic acid (ABA), drought, and salt stress.	5’ – GGTCCAAGGCGATGTCTTTG -3’ 5’ – ATGAACAGCTGGCGTTTTGT – 3’
***PER50* (AT4G37520)**	Peroxidase; Response to environmental stresses such as wounding, pathogen attack and oxidative stress.	5’ – CTGTCAACACATGGGCTTCC – 3’ 5’ – TCCCACTTCGACCCGTTTTA – 3’
***GST-F8* (AT2G47730)**	Glutathione S-transferase expressed in response to auxin, SA and hydrogen peroxide.	5’ – GCCCAAATACTTGCTCTCCG – 3’ 5’ – TTGGGATGACTACCGACGAG – 3’
***GST-U5* (AT2G29450)**	Tau Family, involved in glutathione metabolic processes, response to oxidative stress, toxin catabolic processes. Upregulated by Paracetamol Treatment in *A. thaliana*	5’- AGCATTGGACTTTTGTTTGGGA – 3’ 5’ - TGAAGCTATTGGGATTTTGGGG – 3’
***GST-F6* (AT1G02930)**	Phi class, involved in defense response to bacteria, glutathione metabolic processes, oxidative and water stress, toxin catabolic processes. Upregulated by Paracetamol Treatment in *A. thaliana*	5’ – TTGGGATGACTACCGACGAG – 3’ 5’ – RGCCCAAATACTTGCTCTCCG -3’
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase, used as internal standard /housekeeping gene	5’ – AGGTAGCGATCAACGGATTC – 3’ 5’ – AGGTGGGATGCTTGTTTGAC – 3’

163

164 **Table S5:** P values obtained from Tukey's HSD pairwise comparison of stress gene
165 expression in lamotrigine treated lettuce tissue ((A) roots, (B) leaves) compared to control
166 plants at different time points. Different significance graduation is indicated by different
167 colors as "light green" for 0.01 ≤ p–value ≤ 0.05, "light red" for 0.001 ≤ p–value ≤ 0.01,
168 and "dark red" for p–value ≤ 0.001.

	A					**B**				
Time [h]	***CAT1***	***PER50***	***GST-F8***	***GST-U5***	***GST-F6***	***CAT1***	***PER50***	***GST-F8***	***GST-U5***	***GST-F6***
T6	0.0468	0.3169	0.5860	0.0191	0.0742	0.8021	0.0536	0.6069	0.0012	0.6546
T12	0.5066	0.1589	0.0123	0.0014	0.4384	0.0034	0.0271	0.8001	0.0426	0.9845
T24	0.0013	0.0020	0.0202	0.7355	0.0020	0.7560	0.0045	0.0001	0.0031	0.0026
T30	0.0149	0.1739	0.0209	0.0257	0.9241	0.0175	0.0009	0.0000	0.0009	0.0023
T36	0.0002	0.0001	0.0004	0.0013	0.0014	0.1540	0.0246	0.0594	0.0014	0.1030
T48	0.0005	0.0001	0.0011	0.0125	0.0002	0.0835	0.0100	0.0026	0.0008	0.0012

169

170 **References**

171 ICH Harmonised Tripartite Guideline. *International conference on harmonization,*
172 *Geneva, Switzerland 2005,* 11.

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174 method validation procedures for pesticide residues and analysis in food and feed
175 (SANTE 11813/2017).

176

Appendix: Publication II – Manuscript

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Fate and impact of wastewater-borne micropollutants in lettuce and the root-associated bacteria

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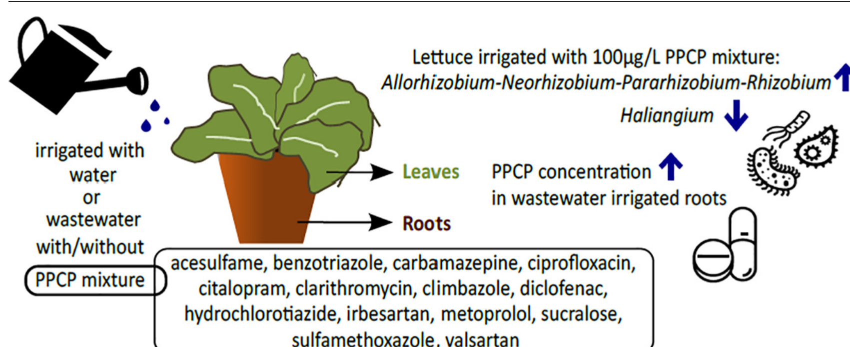
HIGHLIGHTS

- Thirteen out of fourteen spiked PPCPs were detected in the edible part of lettuce.
- Higher PPCP uptake in spiked wastewater- than water irrigated lettuce roots
- Wastewater had an impact on the diversity and composition of root-associated bacteria.
- *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* + *Haliangium* were affected by PPCP.
- *Caulobacter*, *Cellvibrio*, *Hydrogenophaga* and *Rhizobacter* were affected by wastewater.

GRAPHICAL ABSTRACT



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ABSTRACT

The reuse of water for agricultural practices becomes progressively more important due to increasing demands for a transition to a circular economy. Treated wastewater can be an alternative option of blue water used for the irrigation of crops but its risks need to be evaluated. This study assesses the uptake and metabolization of pharmaceuticals and personal care products (PPCPs) derived from treated wastewater into lettuce as well as the impact on root-associated bacteria under a realistic and worst-case scenario. Lettuce was grown in a controlled greenhouse and irrigated with water or treated wastewater spiked with and without a mixture of fourteen different PPCPs at 10 µg/L or 100 µg/L. After harvesting the plants, the same soil was reused for a consecutive cultivation campaign to test for the accumulation of PPCPs. Twelve out of fourteen spiked PPCPs were detected in lettuce roots, and thirteen in leaves. In roots, highest concentrations were measured for sucralose, sulfamethoxazole and citalopram, while sucralose, acesulfame and carbamazepine were the highest in leaves. Higher PPCP concentrations were found in lettuce roots irrigated with spiked treated wastewater than in those irrigated with spiked water. The absolute bacterial abundance remained stable over both cultivation campaigns and was not affected by any of the treatments (type of irrigation water (water vs. wastewater) nor concentration of PPCPs). However, the irrigation of lettuce with treated wastewater had a significant effect on the microbial α-diversity indices at the end of the second cultivation campaign, and modified the structure and community composition of root-associated bacteria at the end of both campaigns. Five and fourteen bacterial families were shown to be responsible for the observed changes at the end of the first and second cultivation campaign,

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respectively. Relative abundance of *Haliangium* and the clade *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was significantly affected in response to PCPPs exposure. *Caulobacter*, *Cellvibrio*, *Hydrogenophaga* and *Rhizobacter* were significantly affected in microcosms irrigated with wastewater.

1. Introduction

The key concept of circular economy is to prevent resource scarcity in the context of an increasing demand of resources, the advancing climate change and the loss of resources worldwide. In this regard, circular economy aims at minimizing the total consumption of natural resources by maximizing their reuse (Breure et al., 2018). Nevertheless, the reuse of these resources needs to be safe and their human and environmental risks must be assessed.

The production of food and other biomass is highly dependent on healthy soil and water. Treated wastewater can be a cheap and readily available option to cover blue water demands for irrigation of crops in arid and semi-arid regions. A large body of literature has demonstrated the advantages of the irrigation of crops with treated wastewater (Cirelli et al., 2012; Singh et al., 2012; Vergine et al., 2017). For instance, nutrients introduced by wastewater can reduce the application of agrochemical fertilizers and improve plant growth (Montemurro et al., 2017a; Urbano et al., 2017). Despite numerous benefits of wastewater reuse in agriculture, wastewater-borne microbiological and chemical contaminants can alter soil physicochemical and microbiological properties (Becerra-Castro et al., 2015) with serious consequences on soil health and functioning (Cycon et al., 2016; Wagg et al., 2014).

Among the plethora of contaminants present in treated wastewater, pharmaceuticals and personal care products (PPCPs) are of special concern due to the human health and environmental risks they can pose. To date, there is no uniform regulation available for the risk assessment of complex mixtures of PPCPs (Godoy and Kummrow, 2017) although these micropollutants have been shown to accumulate in soils or plants (Carter et al., 2014; Manasfi et al., 2020), influence plant performance (Bártíková et al., 2016; Bigott et al., 2021a; Chen et al., 2017) and ultimately enter the human food chain in concentrations above the threshold of toxicological concern (Malchi et al., 2014). The most frequently occurring pharmaceuticals in soils are analgesics and anti-inflammatories (especially, nonsteroid anti-inflammatory drugs (NSAID)), antibiotics, cardiovascular pharmaceuticals (β-blockers/diuretics), psychostimulants, hormones, and antiepileptic drugs (Ferrer and Thurman, 2013; Loos et al., 2013; Li, 2014). In this regard, the antiepileptic drug carbamazepine is one of the most abundant PPCPs in wastewater effluents (Zhang et al., 2008) and was furthermore detected together with its metabolites in human urine after the consumption of fresh produce which was irrigated with treated wastewater (Paltiel et al., 2016). Non-nutritive artificial sweeteners are another class of PPCPs commonly used in toothpaste or pharmaceutical formulations. These recalcitrant substances like acesulfame or sucralose were already detected in surface water, tap water, groundwater, seawater and in the atmosphere (Praveena et al., 2019).

The importance of plant-associated bacteria has been recognized (Matilla and Krell, 2018) to notably promote plant growth due to the bacterial production of plant growth regulators (auxins, gibberellins or cytokinins), to improve the nutrient availability for the plant (N fixation, P solubilization or production of siderophores) or to increase the tolerance of plants against various biotic and abiotic stresses (Compant et al., 2005; Goswami and Deka, 2020; Reinhold-Hurek and Hurek, 2011). The plant microbiome can also play a key role in the degradation of PPCPs (Li et al., 2016a, b; Nguyen et al., 2019; Sauvêtre and Schröder, 2015; Syranidou et al., 2018) in addition to the detoxification and metabolization mechanisms in plants (Dordio et al., 2011; Edwards et al., 2011; Huber et al., 2009; Martínez-Piernas et al., 2019; Riemenschneider et al., 2017; Wu et al., 2016). Endophytes isolated from *Phragmites australis* were shown to contribute to the degradation of the anticonvulsant carbamazepine by activating specific metabolic pathways in horseradish hairy root culture (Sauvêtre et al., 2018). Furthermore, bacteria (mainly *Streptomyces*) isolated from roots and rhizomes of *Miscanthus* × *giganteus* plants exhibited removal capacity for sulfamethoxazole and diclofenac (Sauvêtre et al., 2020b). While the exposure to PPCPs can affect the composition and diversity of plant-associated microorganisms, it has been proposed that plants can also impose a selective control on plant-associated microbes favoring the enrichment of specific beneficial bacterial traits within and nearby the plant organs (Hartman and Tringe, 2019; Reinhold-Hurek and Hurek, 2011; Sauvêtre et al., 2020a).

The irrigation of lettuce with a mixture of 8 antibiotics (carbadox, lincomycin, monensin sodium, oxytetracycline, sulfadiazine, sulfamethoxazole, trimethoprim, and tylosin) and 3 other pharmaceuticals (acetaminophen, caffeine, and carbamazepine) was reported to decrease the bacterial alpha diversity in root, shoot and soil samples (Shen et al., 2019). Especially wastewater-borne sulfonamides were shown to reduce the microbial diversity in constructed wetlands planted with either *Cyperus alternifolius*, *Cyperus papyrus* or *Juncus effuse* and to significantly increase putative sulfonamide-degrading methylotrophs (Man et al., 2020). Additionally, the irrigation of lettuce with a mixture of trimethoprim, ofloxacin and sulfamethoxazole antibiotics negatively affected the relative abundance of Rhizobiales in roots (Cerqueira et al., 2020). The effects of treated wastewater on the structure and diversity of plant-associated microbial communities in tomato and lettuce were also studied by Zolti et al. (2019) who reported that 13% of the variation of the rhizoplane bacterial community composition was explained by the type of irrigation water (water vs. wastewater). However, to our best knowledge, until now there are no studies available assessing both the plant accumulation and the impact of complex mixtures of wastewater borne PPCPs on root-associated communities.

This study aimed to investigate the impact of wastewater irrigation on root-associated bacteria and assess the accumulation of 14 PPCPs (acesulfame, benzotriazole, carbamazepine, ciprofloxacin, citalopram, clarithromycin, climbazole, diclofenac, hydrochlorothiazide, irbesartan, metoprolol, sucralose, sulfamethoxazole and valsartan) on lettuce roots and leaves. Lettuce was grown under controlled greenhouse conditions in pots filled with arable soil and irrigated with water or treated wastewater, with or without the addition of a complex mixture of fourteen PPCPs (at 10 μg/L and 100 μg/L each) to study realistic agronomical conditions and the worst-case scenario, respectively. After seven weeks, plants were harvested and the soil was reused for a second cultivation campaign of lettuce. A multiple approach was used to quantify the fourteen targeted PPCPs and their major metabolites in soil and plants by liquid chromatography-high resolution mass spectrometry (LC-HRMS), to assess plant performance and to link those results to the total bacterial community diversity and composition determined by MiSeq sequencing of 16S rRNA amplicons generated from endophytes DNA.

2. Material and methods

2.1. Experimental design

The greenhouse experimental design consisted of 3 L pots containing 2 kg and 1.1 kg dry weight (d.w.) of sieved (4 mm) soil (for the first and the second campaign respectively) per treatment (type of irrigation water (water vs. wastewater) or concentration of PPCPs; five replicates per treatment). Soil pots were pre-incubated for four weeks at 50% of the soil water holding capacity (71.91%). Four-week old lettuce plantlets (*Lactuca sativa* var. Tizian) were transferred into the pots (one lettuce per pot). Lettuce plants were daily irrigated with the same volume of irrigation solution (ca. 30–80 mL per day) and water to adjust the water holding capacity to

70%. The irrigation solutions were: water (W), water spiked with a mixture of 14 compounds at 10 μg/L each (W10), water spiked with a mixture of 14 compounds at 100 μg/L each (W100), wastewater (WW), wastewater spiked with a mixture of 14 compounds at 10 μg/L each (WW10) and wastewater spiked with a mixture of 14 compounds at 100 μg/L each (WW100) (see SM1.1 Soil and wastewater characteristics in Supplementary Materials). The mixture of the compounds was selected based on their frequency of detection and concentration in treated wastewater in addition to including a broad spectrum of PPCPs with various physico-chemical properties (Montemurro et al., 2020). The majority of compounds chosen for the mixture were pharmaceuticals (carbamazepine (antiepileptic drug); ciprofloxacin, clarithromycin and sulfamethoxazole (antibiotics); citalopram (antidepressant); diclofenac (NSAID)); hydrochlorothiazide (diuretics); irbesartan, metoprolol and valsartan (cardiovascular pharmaceuticals)), exceptions were climbazole (antifungal, used as active substance in antidandruff shampoos), benzotriazole (restrainer or anti-fogging agent) as well as acesulfame and sucralose (artificial sweetener). For providing the mixture of compounds, individual solutions of the 14 PPCPs were prepared by dissolving the substances in methanol, ethanol, acetonitrile or water at 10 or 100 μg/L final concentration each. Different concentrations were used to mimic a realistic and the worst-case scenario. The same quantity of water-solvent mixture (with or without the mixture of compounds) was added to all the irrigation solutions (0.2% vol:vol). More details about soil and wastewater characteristics, reference standards and the purity of the solvents are reported in the Supplementary Materials (see SM1.1 Soil and wastewater characteristics and SM1.2 Chemicals), the most relevant physicochemical properties of each compound have been published in Montemurro et al., 2021. Two successive lettuce campaigns planted on the same soil were performed and 3 L and 2.7 L of irrigation solution (water or wastewater with/without PPCPs) were added per pot for the first and second campaign in total. For the second campaign, to overcome nutrient deficiency symptoms, plants were watered four times (once per week) with 60 mL of modified Hoagland ¼ solution (Hoagland and Arnon, 1938). The experiment was carried out in a greenhouse under controlled conditions at 20 °C (±5 °C) with a 16/8 h light/dark period. Soil pots were daily randomized. At the end of each campaign, soil samples and lettuce plants were collected. Soil samples were stored at −20 °C for chemical analysis. Lettuce plants (separated in leaves and roots) were thoroughly washed first with distilled water and then with ethanol to remove soil particles and microorganisms from the root surface but to not destroy DNA of interest (Lundberg et al., 2012). Fresh total plant biomass was weighed. Lettuce leaves and roots were subsequently freeze dried for chemical and DNA based analyses.

2.2. Soil and lettuce chemical analysis

Total nitrogen and carbon in lettuce roots and leaves were estimated from freeze-dried samples ground to a fine powder using a FLASH 2000 CHN Analyzer (Thermo Fisher Scientific).

Analysis of the fourteen compounds and their main metabolites and transformation products in soil and lettuce roots and leaves was performed using a QuEChERS method coupled to LC-HR/MS analysis from soil and lettuce samples irrigated with 100 μg/L spiked water or 100 μg/L spiked wastewater. Information about the extraction protocol of the compounds and their main metabolites, and the chemicals used for extraction and for soil and lettuce chemical analysis can be found in the Supplementary Materials (see SM1.2 Chemicals–SM1.4 Lettuce chemical extraction) and in Montemurro et al. (2020) for the extraction of pharmaceuticals from lettuce leaves and in Manasfi et al. (2022, in preparation) for the extraction from lettuce roots and soil.

After this step, a targeted analysis of the extracts was performed using an integrated SCIEX X500R QTOF system (Sciex, Redwood city, CA, U.S.) with Turbo V™ source and Electrospray Ionization (ESI) operating in positive and negative mode. Full details on chromatography and mass spectrometry parameters are reported elsewhere (Montemurro et al., 2020).

2.3. Quantitative-PCR analysis of 16S rRNA gene expression

DNA was extracted from roots using the DNeasy Plant Mini kit (Qiagen) extraction kit. The concentration of DNA was quantified using the Quant-iT™Pico Green® ds DNA assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and measured on a Tecan Spark® microplate spectrofluorometric reader (Tecan, Männedorf, Switzerland). Total bacterial communities were quantified by a qPCR assay of the 16S rRNA gene using the 335Fc (5′-CADACTCCTACGGGAGGC-3′) as a forward primer and 769Rc (5′-ATCCTGTTTGMTMCCCVCRC-3′) as a reverse primer (Dorn-In et al., 2015). The qPCR assay was carried out on an ABI 7300 Real-Time PCR System (Thermo Fisher Scientific Inc.) with a PCR reaction mixture containing 12.5 μL Power SYBR® Green PCR Master Mix (Life Technologies Ltd., United Kingdom), 0.5 μL of each primer (10 pmol/μL), 0.5 μL of 3% BSA, and 2 μL template DNA (diluted 1:8) added in a final volume of 25 μL. PCR reaction was initiated after 10 min denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s and elongation with data acquisition at 72 °C for 30 s. The specificity of the amplified products was confirmed by a melting curve analysis. For quantification, serial dilutions of an external linear standard (source sequence stated in Table S1; obtained from IDT Technologies, San Diego, CA) were used to produce a linear standard curve with an R^2 above 0.99. Additionally, the efficiency of the qPCR was calculated based on the linear standard curve according to the formula $Eff = [10^{(-1/slope)} - 1]$ (Töwe et al., 2010) and was at 65.51%.

2.4. Library preparation and Illumina sequencing

A library preparation for next generation sequencing on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) was performed with the same DNA used for the qPCR analysis. To ensure that no contamination was introduced by the DNA extraction procedure negative controls were introduced using empty extraction tubes and buffers. The sequence specific polymerase chain reaction (PCR) of the 16S rRNA region was performed using the same primer sequences mentioned above with added Illumina adaptors. PCR reactions contained 12.5 μL 2 × NEBNext High Fidelity Master Mix (New England BioLabs Ltd., United Kingdom), 0.5 μL of each primer (10 pmol/μL), 2.5 μL of 3% BSA and 5 ng of template DNA added in a final volume of 25 μL. PCR conditions included an initial denaturation step at 98 °C for 5 min, followed by 28 cycles of denaturation (98 °C; 10 s), annealing (60 °C; 30 s) and elongation (72 °C; 30 s), and afterwards a final elongation step at 72 °C for 5 min. PCR reactions were purified using MagSi-NGS (0.8 × sample volume; Magtivio B.V., Geleen, Netherlands). The absence of primer-dimers was confirmed and the concentration of DNA measured on a Fragment Analyzer Automated CE System (Advanced Analytical Technologies Inc., United States). The Illumina indexing PCR was performed using the Nextera XT Index kit v2 (Illumina Inc.). Therefore, the PCR reaction contained 10 ng of amplicon DNA, 12.5 μL 2 × NEB Next High Fidelity Master Mix, and 2.5 μL of each indexing-primer filled to a final volume of 25 μL. PCR conditions for the indexing PCR were as following: 98 °C for 5 min, eight cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s, followed by a final elongation at 72 °C for 5 min. After a purification and quantification of the indexed amplicons as described above, the libraries were diluted to 4 nM and paired-end sequenced on a MiSeq instrument using the MiSeq Reagent Kit v3 (600 cycles) (Illumina Inc.).

2.5. Sequencing data and statistical analysis

Demultiplexed, sequenced reads were processed with QIIME 2 (v2018.8.01; Caporaso et al., 2010). Paired reads were merged and chimeras removed with the DADA2 plugin (DADA2 R package v1.3.4; (Callahan et al., 2016). Therefore, the N-terminal trimming was adjusted to 15 bp, and the C-terminal trimming of the forward and reverse reads was set to 260 bp and 220 bp based on the quality scores. Amplicon sequence variants (ASVs) were inferred and taxonomically assigned (*P*-confidence ≥ 0.9) as described in Michas et al. (2020). The amplicon sequence

dataset is available in the Sequence Read Archive (SRA) under the accession numbers PRJNA717020: SAMN18475103–SAMN18475166. Details on statistical analyses as well as on the sample ordination by non-metric multidimensional scaling (NMDS) and the PERMANOVA are described in the Supplementary Materials (see SM1.5 Statistical analyses of sequencing data).

3. Results

3.1. Plant performance and accumulation of C and N in roots and leaves

Regardless of the treatment, the fresh weight of lettuce plants collected at the end of the second campaign was significantly lower than that from the first campaign ($p < 0.01$). In both cultivation campaigns, irrigation with treated wastewater (spiked or not) increased plant growth, with significantly higher biomass recorded in lettuce collected at the end of the second cultivation campaign ($p < 0.0001$) (Fig. 1).

In roots, for both campaigns, the total C:N ratio was lower in wastewater-irrigated samples than in water-irrigated samples, although not significant ($p > 0.09$) (Fig. 2A). This trend was due to the significant accumulation of nitrogen during the first campaign ($p = 0.03$) and to the significant decrease in the carbon content in the second campaign ($p = 0.005$) (Table S3).

In the leaves, the C:N ratio remained stable along both cultivation campaigns, and no significant differences were observed in the total nitrogen and carbon content between water and wastewater-irrigated samples ($p = 0.204$) (Fig. 2B).

3.2. Chemical analysis of PPCPs and metabolites

Fourteen spiked chemicals and four major metabolites were analyzed in soil, and in lettuce roots and leaves collected at the end of the second campaign. The eighteen compounds were detected in all analyzed matrices except for 4′-hydroxydiclofenac in soil, ciprofloxacin and valsartan in roots, and diclofenac, 4′-hydroxydiclofenac and 4-nitro-sulfamethoxazole in leaves (Fig. 3, Tables S4, S5 and S6).

The highest concentrations in soils were detected for clarithromycin (218.7 ± 49.0 to 357.0 ± 33.1 ng/g), hydrochlorothiazide (26.6 ± 4.7 to 32.8 ± 2.4 ng/g) and citalopram (5.3 ± 1.1 to 12.5 ± 1.3 ng/g) (see Gallego et al., 2021a and Table S4). In lettuce roots highest concentrations were measured for sucralose (135.3 ± 140.4 to 1335.6 ± 773.0 ng/g), sulfamethoxazole (16.1 ± 13.3 to 628.27 ± 260.0 ng/g) and citalopram (9.8 ± 4.7 to 201.1 ± 109.3 ng/g) (Fig. 3 and Table S5). In leaves, the compounds quantified with the highest concentrations were sucralose (299.3 ± 128.5 to 636.6 ± 196.9), acesulfame (115.0 ± 32.0 to 428.0 ± 184.6) and carbamazepine (83.0 ± 6.9 to 164.2 ± 15.7) (Fig. 3 and Table S6). In contrast, lowest concentrations were observed in soil samples for acesulfame, sulfamethoxazole, metoprolol, diclofenac, and valsartan (from 0.3 ± 0.2 ng/g to below limit of quantification (BLOQ)), in root samples for benzotriazole, diclofenac and carbamazepine epoxide (from 10.2 ± 11.2 ng/g to BLOQ) and in leaves for sulfamethoxazole, valsartan and irbesartan (from 0.8 ± 1.4 ng/g to 0.1 ± 0.2 ng/g).

To compare the accumulation of a given compound between samples collected from soil microcosms irrigated with water or wastewater, the differences [expressed in %] between the concentration of PPCPs in 100 μg/L spiked water and 100 μg/L spiked wastewater were calculated (Fig. S2). The concentration of PPCPs was generally higher in soil irrigated with spiked wastewater than in those irrigated with spiked water but for most of the compounds this trend was not statistically significant ($p > 0.14$). Only the climbazole concentration was significantly increased in soil irrigated with wastewater as compared to those irrigated with water in both campaigns ($p < 0.007$) (accounting up to 322.8 ± 180.3% for the first campaign) while carbamazepine and irbesartan concentrations significantly increased in soil irrigated with wastewater only in the second campaign ($p = 0.001$ and $p < 0.001$, respectively). In roots, the concentration of PPCPs in samples collected from cultures irrigated with spiked wastewater was generally higher or remained at the same concentration as compared to those of cultures irrigated with water. The highest differences were quantified for metoprolol at the end of the first campaign (1333 ± 1674.9%) and citalopram at the end of the second campaign (1036.7 ± 552.4%). Citalopram and irbesartan significantly increased in roots of lettuce plants irrigated with spiked wastewater as compared to those irrigated with spiked water for both campaigns ($p < 0.05$), while acesulfame only increased in the roots collected at the end of the first campaign ($p = 0.02$). Furthermore, the concentration of carbamazepine, climbazole, hydrochlorothiazide, sucralose and sulfamethoxazole increased significantly only in the roots irrigated with spiked wastewater collected at the end of the second campaign ($p < 0.05$). The differences in the concentration of the three metabolites (carbamazepine epoxide, 4′OH-diclofenac and valsartan acid) measured in the roots were significantly higher in roots collected at

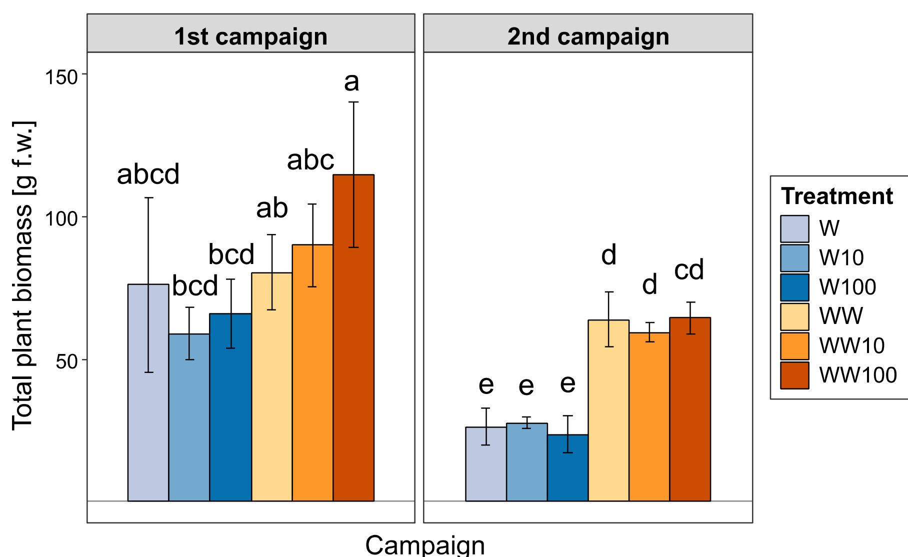

Fig. 1. Total plant biomass [g f.w.] of lettuce plants treated with water (W), 10 μg/L or 100 μg/L spiked water (W10 and W100), wastewater (WW), 10 μg/L or 100 μg/L spiked wastewater (WW10 or WW100) and collected at the end of the first and second cultivation campaign (n = 5). Different letters indicate significant differences ($p < 0.05$) calculated by ANOVA and Tukey's post hoc testing. Standard deviations are indicated by error bars.

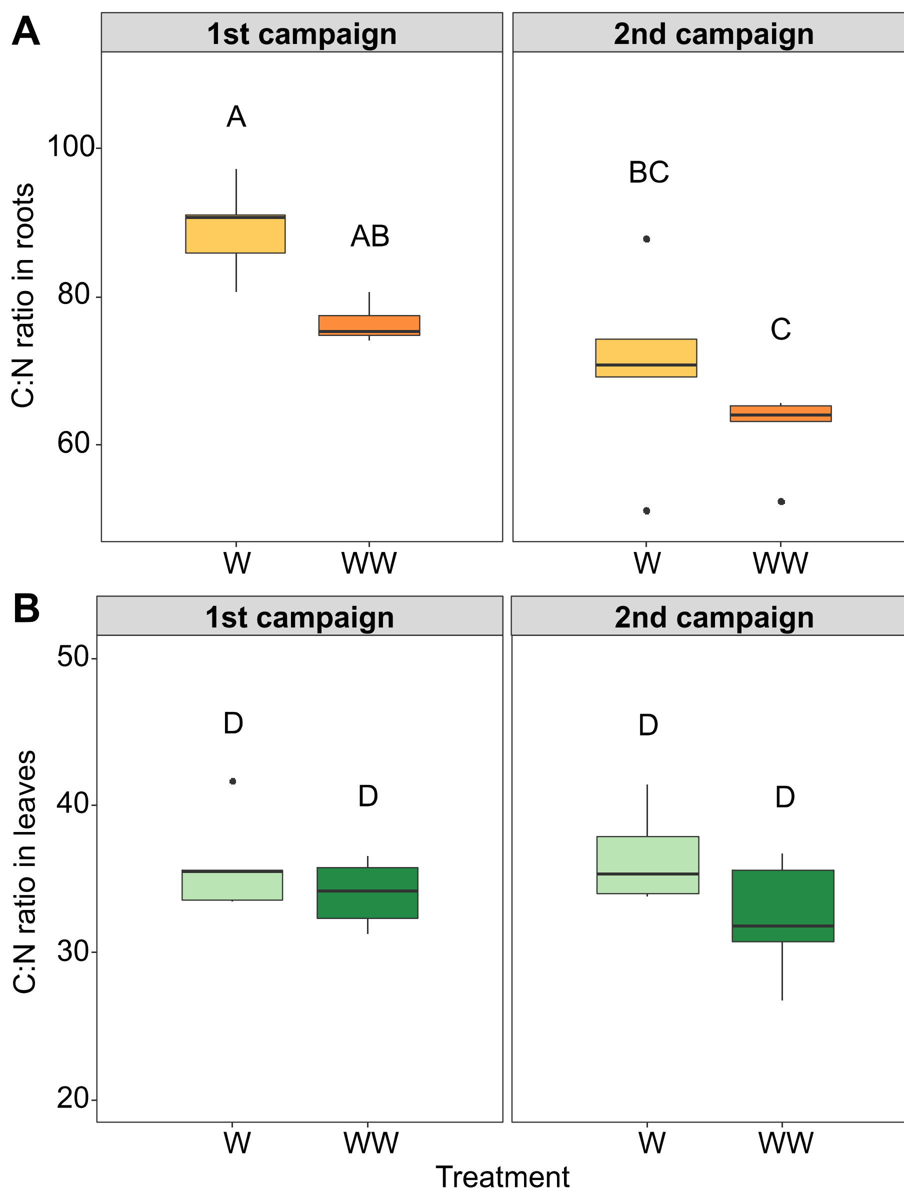

Fig. 2. C:N ratio in lettuce roots (A) and lettuce leaves (B) from planted soil microcosms irrigated with either water (W) and wastewater (WW) collected at the end of the first and second cultivation. Each value is the mean of five replicates. Standard deviations are indicated by error bars. ANOVA followed by Tukey's test was performed. Values indicated by different letters are significantly different.

the end of the second campaign than at the end of the first one. However, it is noteworthy that only the concentration of valsartan acid, one of the three metabolites, significantly increased ($p = 0.006$) in the roots of lettuce irrigated with spiked wastewater than in those irrigated with spiked water.

In leaves, differences ranged from $-67.7 \pm 8.0\%$ for acesulfame in the second campaign to $205.0 \pm 220.1\%$ for sulfamethoxazole in the first campaign. At the end of the first campaign, the concentration of clarithromycin and climbazole was significantly decreased in lettuce irrigated with spiked wastewater as compared to those irrigated with spiked water ($p < 0.02$). At the end of the second campaign, the concentrations of carbamazepine, citalopram, metoprolol and sucralose increased significantly ($p < 0.04$) in the leaves of lettuce irrigated with spiked wastewater as compared to those irrigated with spiked water.

3.3. Bacterial community analyses

3.3.1. Total bacterial abundance

The total bacterial abundance ranged from 2.9×10^7 to 5×10^7 copies of 16S rRNA per g of roots and was not significantly affected by any of the treatments applied (type of irrigation water (water vs. wastewater) nor concentration of PPCPs (10 μg/L or 100 μg/L) ($p > 0.55$) (Fig. S3).

3.3.2. Bacterial diversity

16S rRNA amplicons generated from extracted root DNA were sequenced to calculate a range of bacterial α-diversity indices pertaining richness (Chao1) and evenness (Shannon). At the end of the first campaign, bacterial α-diversity indices seemed to be not affected by any of the treatments (Fig. S4A). In contrast, at the end of the second campaign, Chao1 and Shannon indices were significantly decreased in roots of lettuce irrigated with wastewater ($p = 0.015$ and $p = 0.02$). In addition, a significant increase in the Chao1 index was observed in the roots of lettuce irrigated with 10 μg/L spiked water ($p = 0.028$) as compared to those irrigated with water (Fig. S4B).

Non-metric multidimensional scaling (NMDS) of β-diversity based on Bray-Curtis dissimilarities and PERMANOVA Adonis testing revealed a highly significant difference in the bacterial diversity in roots of lettuce plants irrigated with either water or wastewater no matter of the cultivation campaign ($p = 0.001$) (Fig. 4A, Table 1). For the first campaign, irrigation

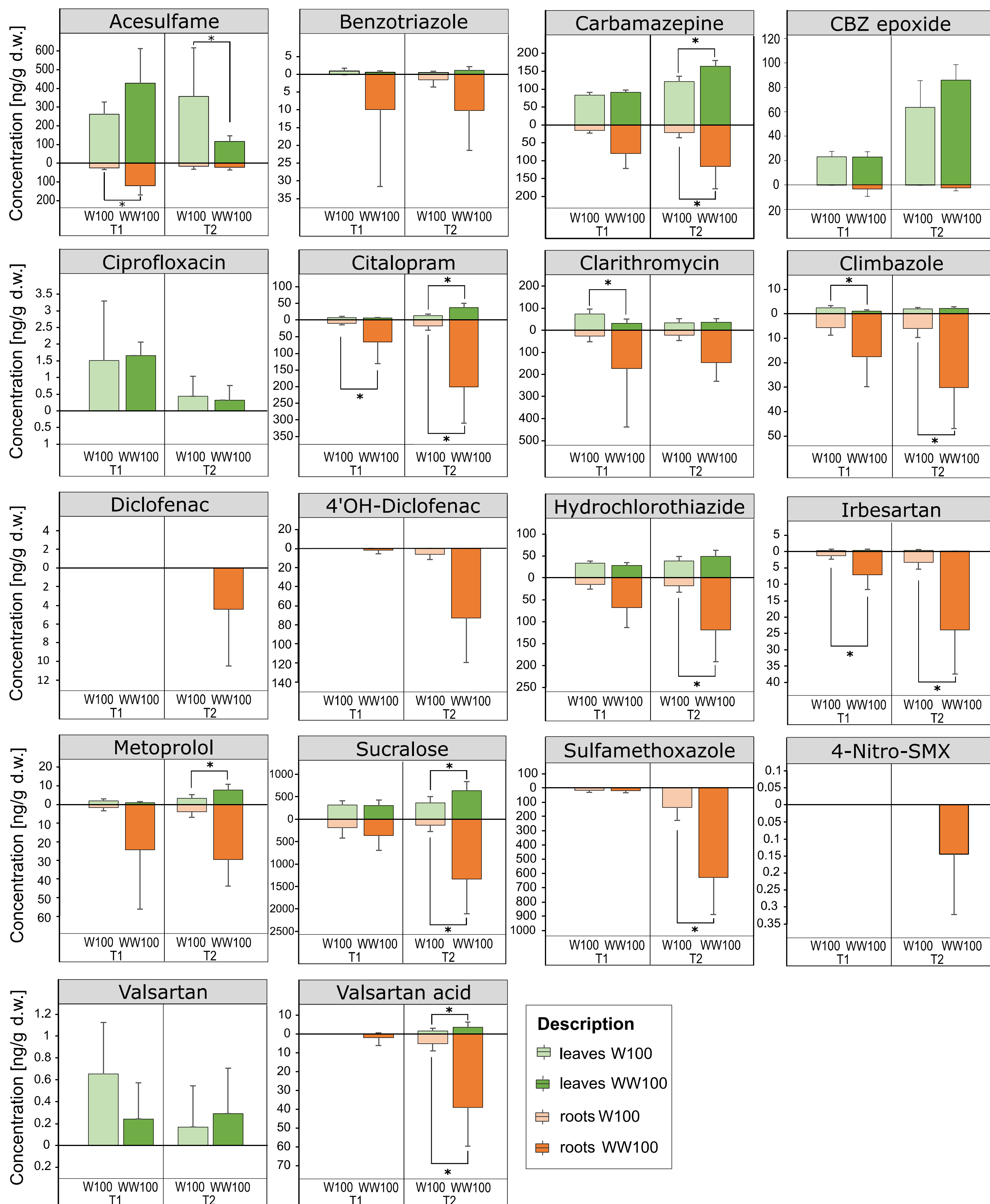

Fig. 3. Concentration [ng/g d.w.] of PPCPs in lettuce roots and leaves, derived from samples irrigated with water 100 μg/L PPCPs (W100) and wastewater 100 μg/L PPCPs (WW100) collected at the end of the first (T1) and second (T2) cultivation campaign. Significant differences between the concentration of PPCPs measured in water compared to wastewater irrigated samples are indicated as "*" for *p*-value ≤ 0.05. CBZ: carbamazepine; SMX: sulfamethoxazole.

with water or treated wastewater explained 8.5% of the variance in the dataset. The concentration of PPCPs and the interaction between the irrigation type (water vs. wastewater) and the PPCP concentration accounted for 8% and 7.8% but were not statistically significant. For the second campaign, the type of irrigation applied to grow the lettuce plants significantly affected the bacterial community structure ($p = 0.001$) with 19.7% of the variance explained by this factor (Fig. 4B, Table 1). In addition, at the end of the second campaign, both, the PPCP concentration and the interaction between the irrigation type and the PPCP concentration significantly influenced the lettuce-associated bacterial community structure with

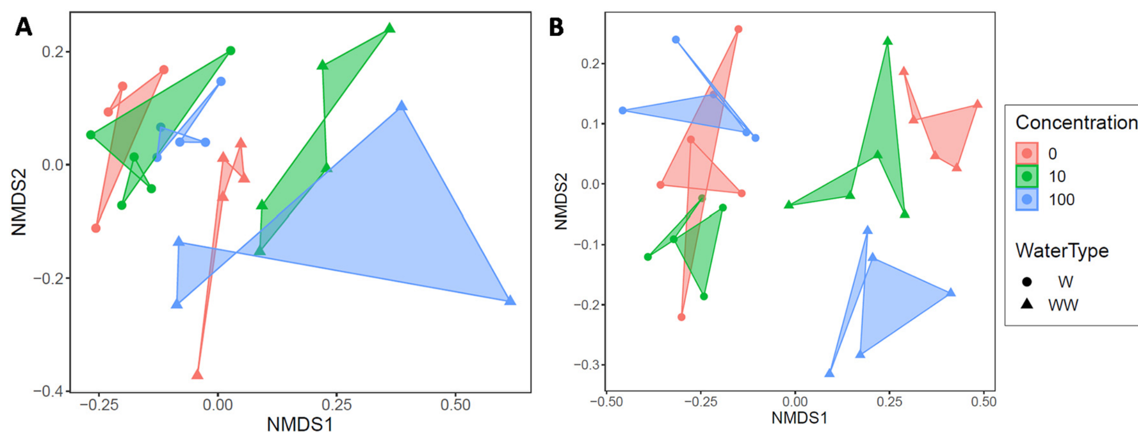

Fig. 4. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of the datasets of the first campaign (A) and the second campaign (B). "W" refers to samples irrigated with water shown as circles; "WW" indicates the wastewater-irrigated samples shown by triangles. Different colors correspond to different concentrations of spiked PPCPS (red: without, green: 10 μg/L, and blue: 100 μg/L PPCPs). For 1st campaign water without PPCPs, and 1st campaign wastewater 100 μg/L, n = 4.

10.2% and 8.1% of the variance explained, respectively. To further confirm the significance of effects detected by PERMANOVA Adonis, a permutation test for homogeneity of multivariate dispersions was performed. All observed significant effects could be clearly explained by the location and not by a multivariate dispersion of the values around the centroid (Table S7).

3.3.3. Bacterial community composition

The bacterial community in the roots of lettuce plants was dominated by six major phyla: Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, Spirochaetes and Acidobacteria (Fig. S5). Proteobacteria was the prominent one with relative abundance comprised between 77.5% and 88.0%. ASVs related to these six phyla represented up to 99% of all the ASVs. The relative abundance of these major phyla remained constant in all treatments and in both campaigns. However, at lower taxonomic level (families and genera), significant changes between the treatments were observed (Figs. S6 and S7). At the end of the first campaign, the relative abundance of ASVs related to five bacterial families changed significantly in response to the type of irrigation water (Fig. S6A). The relative abundance of the two families *Burkholderiaceae* (p = 0.0009) and *Chitinophagaceae* (p = 0.013) significantly decreased, whereas the relative abundance of *Enterobacteriaceae* (p = 0.02), *Ilumatobacteraceae* (p = 0.04) and *Pseudomonadaceae* (p = 0.02) significantly increased in roots of lettuce plants irrigated with treated wastewater as compared to water. At the end of the second campaign, fourteen families were significantly affected by the different treatments (Fig. S6B). Among them, four (*Beijerinckiaceae*, *Dongiaceae*, *Haliangiaceae* and *Rhizobiaceae*) were identified to differ between the different PPCP concentrations, while the remaining ones were affected by the type of irrigation water or one by both, the PPCP concentration and the type of irrigation water (*Spirochaetaceae*). At the genus level, twenty-five genera significantly differed between the different irrigation treatments at the end of the second campaign (Fig. S7). Only six genera whose relative abundance was higher than 2% were further analyzed (Fig. 5). The relative abundance of the clade *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was significantly increased in response to the PPCP exposure in the roots of lettuce plants irrigated with water (from a relative abundance of 2.9 ± 0.8% to 5.7% ± 1.5%) and wastewater (2.9 ± 0.4% to 4.5% ± 1.5%) (p = 0.008). *Haliangium* was significantly reduced in response to increasing PPCP exposure (p = 0.004) in the roots of lettuce plants irrigated with water and wastewater spiked with the highest concentration of PPCPs (100 μg/L each) (Fig. 5A). Four genera were significantly affected by the type of irrigation water (Fig. 5B), where two of those increased (*Cellvibrio and Hydrogenophaga*) and two decreased (*Caulobacter* and *Rhizobacter*) in the roots of lettuce plants irrigated with wastewater. The relative abundance of the genus *Cellvibrio* was twice as high in the roots of lettuce plants irrigated with wastewater (17.2 ± 4.6%) than in those irrigated with water (8.1 ± 2.9%) (p = 0.002). Similarly, the relative abundance of *Hydrogenophaga*, a genus belonging to the family *Burkholderiaceae* was five times higher in the roots of lettuce plants irrigated with wastewater (8.24 ± 4.82%) than in those irrigated with water (1.5 ± 1.9%) (p = 0.004). In contrast, the relative abundance of the genera *Rhizobacter* (p = 0.002) and *Caulobacter* (p = 0.004) was found to be significantly lower in the roots of lettuce plants irrigated with wastewater than in those irrigated with water.

Table 1
PERMANOVA adonis analyses of bacterial distribution among samples. Significant differences are indicated as "*" for 0.01 ≤ p-value ≤ 0.05, "**" for 0.001 ≤ p-value ≤ 0.01, and "***" for p-value ≤ 0.001.

Dataset	Factor(s)	n	Df	F	R^2	p-Value
Campaign T1	WaterType	28	1	2.4693	0.085	0.001***
	PPCP concentration		2	1.1659	0.080	0.108
	Interaction		2	1.1298	0.078	0.159
Campaign T2	WaterType	30	1	7.6339	0.197	0.001***
	PPCP concentration		2	1.9726	0.102	0.004**
	Interaction		2	1.5776	0.081	0.025*

4. Discussion

The uptake and translocation of organic contaminants in plants depend on various factors: the physico-chemical properties of the molecules, the soil/rhizosphere characteristics and the plant composition and physiology (lipid and protein content, biotransformation, sequestration capacities and transpiration rate) (Bigott et al., 2021b). Besides these parameters that account for the passive uptake of micropollutants via diffusion, they can also be translocated via active transporters (Bigott et al., 2021b; Eggen et al., 2011).

As reported in previous studies, the irrigation with treated wastewater had a positive effect on the growth of lettuce plants (Singh et al., 2012; Urbano et al., 2017). This effect was especially visible at the end of the second campaign, when the total plant biomass was significantly higher in treated wastewater-irrigated lettuce plants as compared to water-irrigated ones. No matter the treatment considered, lettuce fresh weight was significantly lower at the end of the second campaign as compared to that of the first campaign possibly because of the fertilization treatment, which did not entirely cover the nutrient depletion due to repeated plant cultivation. Although the C:N ratio, which is a good indicator under different stress and nutrient conditions (Royer et al., 2013; Li et al., 2016a, b) did not change significantly in roots and leaves within the two campaigns, the nitrogen

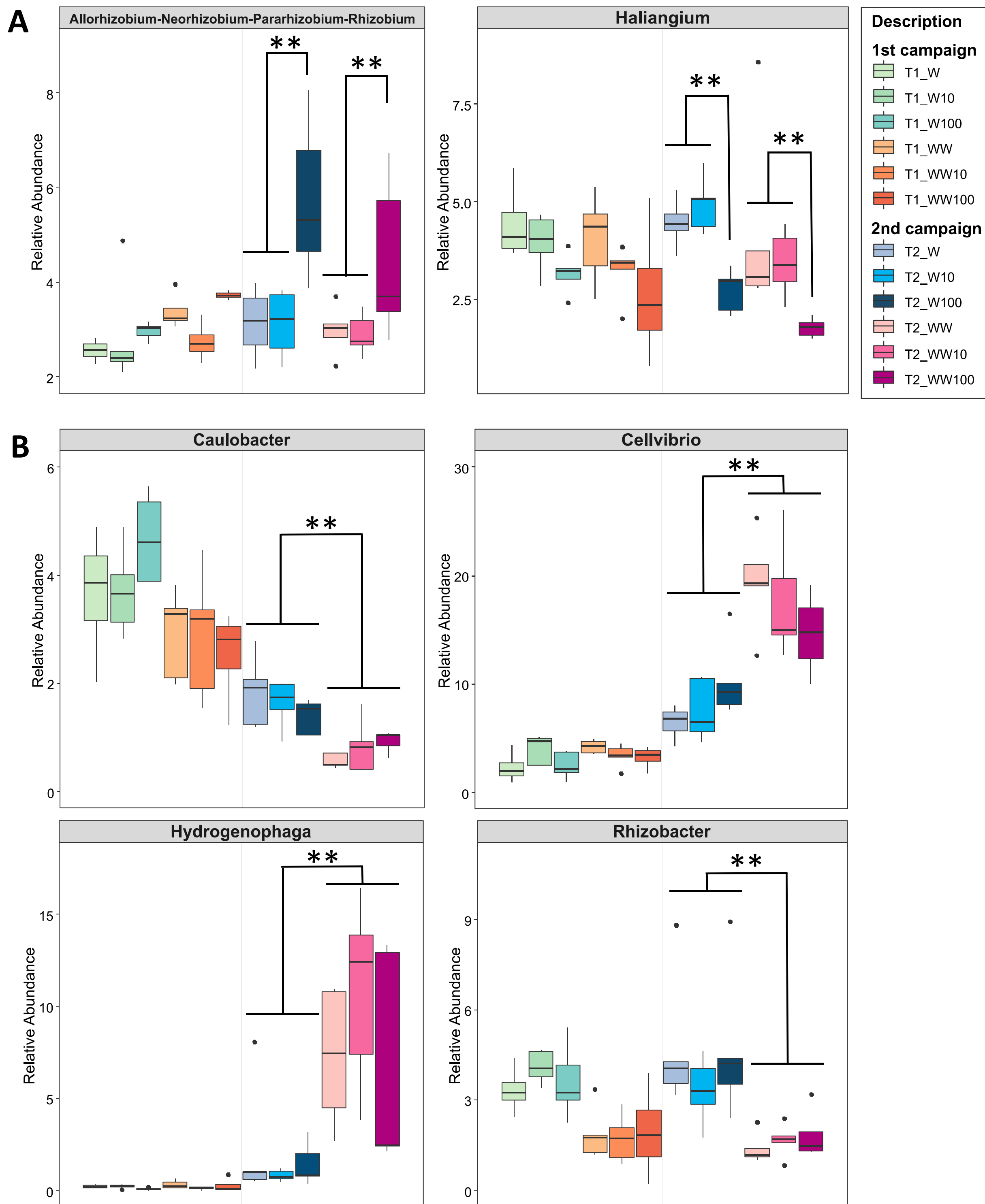

Fig. 5. Relative abundance of significant different genera affected by the PPCP concentration (A) and by the type of irrigation water (B) with relative abundance >2%. "W" refers to samples irrigated with water; "WW" indicates the wastewater-irrigated samples. For 1st campaign water without PPCPs, and 1st campaign wastewater 100 μg/L, n = 4. Significant differences are indicated as "**" for *p*-value ≤0.01.

content in roots significantly increased at the end of the first campaign while the carbon content significantly decreased at the end of the second campaign in wastewater-irrigated samples. Treated wastewater is known to be a good source of nitrogen (Montemurro et al., 2017a) especially in its organic and ammoniacal forms. Our observation is in accordance with Mañas et al. (2009) who reported significant increase of the nitrogen level in lettuce after irrigation with treated wastewater. In addition, fertilization treatment and additional nitrogen input may have led to a plant-induced shift of the microbial community in the root zone as shown in a previous study (Chen et al., 2019; Haichar et al., 2008), which would explain the observed reduction in the carbon content in treated wastewater-irrigated lettuce roots at the end of the second campaign. Chen and co- authors postulate that plants might adjust the quantity and composition of root exudates like organic acids as an adaption strategy to

recruit beneficial plant growth-promoting microbes in the rhizosphere to manage the high N input (Chen et al., 2019). For the synthesis of these organic acids, carbon is essential and might be reduced in the lettuce roots at the end of the second campaign.

Following the irrigation of lettuce with treated wastewater spiked with fourteen PPCPs, twelve PPCPs were detected in roots and thirteen in leaves. The highest concentrations were measured for neutral (sucralose, sulfamethoxazole, carbamazepine and clarithromycin) or positively charged PPCPs (citalopram) mainly in roots but also in leaves. Acesulfame (negatively charged) was also detected in leaves at high concentrations.

Neutral organic compounds at the pH of the experiment (pH 8.2) like benzotriazole, carbamazepine, hydrochlorothiazide, sucralose and sulfamethoxazole have been previously detected and quantified in different plant tissues at relatively high concentrations (Calderón-Preciado et al., 2011; Chuang et al., 2019). Because of its high photodegradation (Hem et al., 2003) and phytotransformation (Castro et al., 2003) benzotriazole was detected in soil, roots and leaves only in very low concentrations. Carbamazepine was detected in high concentrations in leaves, as previously observed by other authors (Montemurro et al., 2017b; Riemenschneider et al., 2017; Shenker et al., 2011). Its intermediate hydrophobicity (log K_{OW} 3.64) and uncharged ionization status may have favored its translocation from roots to the aerial part of the plants by xylem flow (Malchi et al., 2014; Shenker et al., 2011). Interestingly, carbamazepine epoxide, a transformation product (TP) of carbamazepine, was detected in high concentrations in lettuce leaves, which indicates it was metabolized by plants as shown previously (Dordio et al., 2011; Kodešová et al., 2019). In roots, carbamazepine concentrations were similar to those reported by Montemurro et al. (2020) and similar to the concentrations of the hydrochlorothiazide. Lower concentrations of hydrochorothiazide were translocated to the aerial part, in agreement with Manasfi et al. (2020). In the same study, high sucralose uptake and translocation to lettuce leaves was observed because of the structural similarity to sucrose which is an easily transported plant sugar. Indeed, the aquatic plant *Lemna* spp. was shown to assimilate carbon from sucralose (Amy-Sagers et al., 2017). Although not detected in soil, high concentrations of sucralose were observed in lettuce roots and leaves in agreement with Manasfi et al. (2020). Sulfamethoxazole was quantified in higher concentrations in the roots than in the soil. Sulfamethoxazole is generally present as an anionic compound but it also exists in non-ionic form. At the given soil pH, the charge of the substance was estimated to be -0.05 by SPARC (SPARC Performs Automated reasoning) based on the molecule's pK_a and structure. At this charge, sulfamethoxazole can be considered almost neutral which might explain its high accumulation in lettuce roots. In contrast, at pH 7.2 within the plant cytosol sulfamethoxazole has been hypothesized to be trapped as an anionic species in the roots resulting in an absent translocation to the leaves (Chuang et al., 2019). Earlier studies had also detected high accumulation in lettuce roots and reduced translocation to leaves in lettuce, cucumber and tomatoes (Ahmed et al., 2015). Additionally, 4-nitro-sulfamethoxazole, a phototransformation product of sulfamethoxazole (Su et al., 2016) was hardly detected in soil and roots, and it was not detected in leaves.

Positively charged molecules at pH 8.2 were citalopram, clarithromycin, climbazole and metoprolol. Citalopram was found to be taken up in much higher concentrations than metoprolol in lettuce roots, in line with Montemurro et al. (2020). A possible explanation can be that citalopram, clarithromycin and climbazole accumulated in higher concentrations in soil than metoprolol, which could then result in higher plant uptake. The concentrations of citalopram, clarithromycin, climbazole and metoprolol were lower in lettuce leaves as compared to roots. Experiments done by Tian et al. (2019) in lettuce grown in nutrient solution containing clarithromycin showed a high metabolization of clarithromycin in the plant tissue, which may explain our results. Metoprolol was shown to accumulate in carrot and sweet potato roots but not in leaves (Malchi et al., 2014). The high metoprolol concentrations used in our study could explain why in our study it was also detected in leaves.

Negatively charged molecules at pH 8.2 were acesulfame, diclofenac and valsartan. Acesulfame was found at very low concentrations in soil but at higher concentrations in lettuce leaves than in roots, confirming previous studies done in various vegetable species like cabbage, carrot, eggplant, lettuce, parsley, pepper, potato, rucola, tomato and zucchini (Manasfi et al., 2020; Riemenschneider et al., 2016). Diclofenac and valsartan were barely present in soil, lettuce roots and leaves. One possible explanation could be their rapid dissipation in soil (Al-Rajab et al., 2010; Gallego et al., 2021b; Helbling et al., 2010) and rapid metabolization of diclofenac in plants (Bartha et al., 2014; Fu et al., 2017; Huber et al., 2012). Indeed, 4-OH-diclofenac and valsartan acid, which are main TPs of diclofenac and valsartan were barely detected in soil, roots and leaves, which could indicate that subsequent TPs were formed during their transformation.

Ciprofloxacin and irbesartan are zwitterionic molecules at pH 8.2. Ciprofloxacin was not detected in lettuce roots and only at very low concentration in soil and lettuce leaves probably due to its high metabolization by plants with a potential role of root-associated microorganisms (Panja et al., 2019). Irbesartan was detected at very low concentrations in soil and lettuce leaves but at higher levels in roots. The accumulation of irbesartan in plants was firstly reported by Montemurro et al. (2020).

The concentration of eight out of the twelve spiked PPCPs (acesulfame, carbamazepine, citalopram, climbazole, hydrochlorothiazide, irbesartan, sucralose and sulfamethoxazole) significantly increased in lettuce roots irrigated with treated wastewater as compared to plants irrigated with spiked water. Similar results were obtained by Goldstein et al. (2014), who detected a trend of higher concentrations of carbamazepine and lamotrigine in leaves of cucumber plants irrigated with spiked treated wastewater as compared to plants irrigated with spiked water. However, in leaves of tomato plants this trend was only observed for carbamazepine. The concentration of the PPCPs in lettuce roots was higher at the end of the second campaign than at the end of the first campaign (7 vs. 3 significant different PPCPs). On the one hand, one can hypothesize that the metabolization of the PPCPs was slowed down in the plants irrigated with spiked treated wastewater due to the inhibition of detoxification enzymes (Dordio et al., 2011). On the other hand, this trend might result from higher uptake rates of the PPCPs due to interactions between PPCPs and other wastewater borne micro- and macroelements. Papaioannou et al. (2020) identified synergistic interactions between different PPCPs leading to higher PPCP accumulations in beet. However, in another study, the interactions of PPCPs with heavy metals and micro- and macroelements resulted in a decreased uptake of specific PPCPs in beet (i.e. phosphate and metoprolol) (Papaioannou et al., 2019). One exception was the soil nickel concentration, which correlated positively with sulfamethoxazole in beet. Heavy metals like nickel were shown to be significantly enriched in soils irrigated with treated wastewater in previous studies (Mkhinini et al., 2020). Therefore, the higher concentrations of PPCPs (i.e. sulfamethoxazole) detected in treated wastewater-irrigated samples might be partly explained by synergistic effects between the PPCPs or other wastewater borne micro- and macronutrients.

Interestingly, all the PPCPs that significantly increased in wastewater-irrigated samples as compared to water-irrigated samples at the end of the second campaign were either neutral, cationic or zwitterionic compounds. Plants possess a variety of transporters for the uptake and distribution of different structurally diverse substances like secondary compounds (Eggen and Lillo, 2015). Among them, organic cation transporters (OCT) and nitrate and peptide transporters are involved in the sensing and uptake of nitrogen-containing compounds and amino acids in plants (Eggen and Lillo, 2015; Tsay et al., 2007). Given the high nitrogen concentrations in the wastewater, it could be hypothesized that PPCPs were transported together. In this regard, a putative involvement of OCTs for the uptake of the pharmaceutical tramadol by plants was recently postulated by Khalaf et al. (2021).

The effects of the irrigation of lettuce with treated wastewater or water spiked or not with a mixture of PCCPs under a real and worst-case scenario on the community composition and diversity of lettuce-associated bacteria were assessed. The abundance of the total bacterial community did not

change in response to the different irrigation regimes applied. Changes in relative abundance observed in response to the irrigation can be considered as real changes in the community composition and not as loss of entire taxa or of lower absolute abundance within the root-associated bacteria. None of the irrigation regimes had an effect on the bacterial richness and evenness at the end of the first campaign. However, a significant impact was observed in Chao1 and Shannon indices in treated wastewater-irrigated samples at the end of the second campaign. In accordance to our results, Shen and co-workers (Shen et al., 2019) observed a reduced α-diversity in lettuce root, shoot and soil samples after irrigation with a fertilizer solution spiked with a mixture of pharmaceuticals. The significant impacts on the structure of the microbial community were mostly driven by the treated wastewater but also due to the PPCPs. These effects were more pronounced in the second campaign, probably due to the accumulation of PPCPs in the soil (see Gallego et al., 2021a) or due to the depletion in soil nutrients because of repeated culture.

The community composition of lettuce-associated root bacteria was also significantly affected by irrigation with treated wastewater and the concentration of PPCPs. During the first cultivation campaign, the bacterial taxonomic families *Burkholderiaceae* and *Chitinophagaceae* significantly decreased in the treated wastewater-irrigated samples, while the *Enterobacteriaceae*, *Ilumatobacteraceae* and *Pseudomonadaceae* families significantly increased. Members of *Burkholderiaceae*, *Chitinophagaceae*, *Enterobacteriaceae and Pseudomonadaceae* were shown to possess plant growth-promoting activities (Armanhi et al., 2018; Guo et al., 2020; Kuklinsky-Sobral et al., 2004; Ogbo and Okonkwo, 2012; Roquigny et al., 2017). Plant growth-promoting activities can help the plant withstand several biotic and abiotic stresses (Berg, 2009; Goswami and Deka, 2020; Reinhold-Hurek and Hurek, 2011). Additionally, certain plant growth promoting microorganisms can help degrade organic contaminants or enhance plant metabolization capacities (Sauvêtre et al., 2018, 2020b; Shahpoury et al., 2021). The increase in the relative abundance of the family *Ilumatobacteraceae* is consistent with a previous study of Coll and coworkers (Coll et al., 2020), who observed an enrichment of this family in microcosms filled with sediment sampled downstream of a wastewater treatment plant discharge and a mixture of PPCPs. Among the 14 different families that were significantly affected during the second cultivation campaign, members of the *Methylomonaceae* family have been identified as methylotrophs. Methylotrophs, such as *Methylocaldum*, *Methylomonas*, *Methylosinus* and *Methylotenera* have been shown to increase in the rhizosphere of constructed wetlands planted with *C. alternifolius*, *Cyperus papyrus* or *Juncus effuse* and exposed to wastewater containing sulfonamides (Man et al., 2020).

At genus level, the clade *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was increased in response to the exposure to the highest concentration of PPCPs. This agrees with a previous study done on soils contaminated with the plasticizer DEHP (Bai et al., 2020) and with observations from Guo and Chi (2014) in cadmium polluted soil. The plant growth-promoting clade *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* thus might be resistant against abiotic stress triggered by various contaminants and as a result have an advantage over other competing bacteria. In contrast, the bacterial genus *Haliangium* was decreased in response to the exposure to the highest concentration of PPCPs. The genus *Haliangium* was shown to be sensitive to veterinary antibiotics in plant–soil systems (Uddin et al., 2019), which may explain our findings. At the end of the second campaign, the genera *Cellvibrio* and *Hydrogenophaga* were enriched in the roots of plants irrigated with wastewater. These two genera were described as keystone denitrifiers responsible for nitrogen removal processes in a constructed wetland (Li et al., 2019). In contrast, *Caulobacter* and *Rhizobacter* significantly decreased in the roots of plants irrigated with treated wastewater. The order *Caulobacterales* was shown to decrease with increasing concentrations of nitrate (Hester et al., 2018). In our study, the relatively high ammonium content brought by the wastewater used might have supported the growth of ammonium oxidizers with a subsequent nitrate production, favoring the growth of genera such as *Cellvibrio* and *Hydrogenophaga* but limiting that of the *Caulobacter* and *Rhizobacter*.

5. Conclusions

A multiple approach was used to monitor the soil-plant fate of each element of a complex mixture of PPCPs brought either by water or by treated wastewater into a soil-plant experiment carried out in two successive campaigns and to investigate the effects of different irrigation regimes on root-associated bacteria. Higher uptake rates of PPCPs were found especially in roots of lettuce irrigated with treated wastewater under the tested worst-case scenario of exposure (irrigation with a mixture of 14 PPCPs at 100 μg/L each). Irrigation with treated wastewater had bigger influence on the root-associated bacterial diversity and community composition than the PPCPs, even under the worst-case scenario.

CRediT authorship contribution statement

Yvonne Bigott: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **Sara Gallego:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **Nicola Montemurro:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Marie-Christine Breuil:** Formal analysis. **Sandra Pérez:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Antonios Michas:** Formal analysis, Data curation. **Fabrice Martin-Laurent:** Conceptualization, Investigation, Writing – review & editing, Supervision, Funding acquisition. **Peter Schröder:** Conceptualization, Investigation, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154674.

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Appendix: Publication II – Supplementary Material

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Supplementary Materials

Fate and impact of wastewater-borne micropollutants in lettuce and the root-associated bacteria

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1. Supplementary Materials

1.1 Soil and wastewater characteristics

Soil (loam, pH 8.2, soil organic matter 3.68%, total organic carbon 2.13%, total nitrogen 0.201%) was collected from the experimental fields of IRSTEA at Montpellier (Lavalette, France, 43.64682 N, 3.87418 E). Secondary treated domestic wastewater (pH 7.1, WHC 71.91%; COD 200 mg/L, total suspended solids 58 mg/L, total organic carbon 56.8 mg/L, N-NH_4 29 mg/L, N-NO_3^- <0.22 mg/L) was collected from the wastewater lagoon at Murviel-les-Montpellier (Hérault, France, 43.605034 N, 3.757292 E) and stored at 4ºC until use. Additional details on soil and wastewater physico-chemical characteristics are described in Gallego et al., 2021.

1.2 Chemicals

Highly pure analytical reference standards (acesulfame (ASF), benzotriazole (BNZ), carbamazepine (CBZ), carbamazepine-10,11-epoxide (CBZ-EPX), ciprofloxacin (CIP), citalopram (CTP), clarithromycin (CLT), climbazole (CLB), diclofenac (DCF), 4'-hydroxydiclofenac (4’-OH-DCF), hydrochlorotiazide (HCT), irbesartan (IRB), metropolol (MTP), sucralose (SUC), sulfamethoxazole (SMX), 4-nitro-sulfamethoxazole (4-nitro-SMX), valsartan (VAL), valsartan acid (VAL-AC) were obtained from Sigma Aldrich (St. Louis, MO, U.S.). Whereas, isotopically labelled standards for quantitation purpose (acesulfame-d4, benzotriazole-d4, carbamazepine-d10, ciprofloxacin-d8, citalopram-d6, climbazole-d4, diclofenac-13C6, hydrochlorothiazide-d2, irbesartan-d6, metoprolol-d7, sucralose-d6, sulfamethoxazole-d4, valsartan acid-d4, and valsartan-d3) were purchased from Cerilliant (Sigma Aldrich, St. Louis, MO, U.S.) and Toronto Research Chemicals (Toronto, ON, Canada). LC-MS grade solvents (Acetone, acetonitrile (≥ 99.9%), methanol (≥ 99.9%), dimethyl sulfoxide (≥ 99.9%), and HPLC water were purchased from Merck (Darmstadt, Germany). All the above-mentioned reference standards were prepared individually in either LC-MS grade

acetone, acetonitrile (ACN, ≥ 99.9%), methanol (MeOH, ≥ 99.9%), dimethyl sulfoxide (DMSO, ≥ 99.9%), or HPLC water according to compounds solubility and stored at −20°C. The most relevant physicochemical properties of each compound are reported elsewhere (Montemurro et al., 2021). Commercially available Original QuEChERS (OR) extraction salts kit (4 g $MgSO_4$ + 1 g NaCl) and dispersive solid phase extraction (dSPE) clean-up mixture (150 mg PSA (primary secondary amine), 150 mg of C18-bonded silica, and 900 mg $MgSO_4$) were obtained from BEKOlut GmbH & Co. KG (Hauptstuhl, Germany). Disodium hydrogenphosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), citric acid monohydrate and anhydrous ethylenediamine tetraacetic acid (EDTA) (≥99%) for preparation of the EDTA-McIlvaine buffer (pH 4) (Montemurro et al., 2021) were obtained from Sigma Aldrich (St. Louis, MO, U.S).

1.3 Soil chemical extraction

For the extraction of the fourteen compounds and their main metabolites and transformation products from soil, 10 g of air-dried soil sample were added to a 50-mL polypropylene centrifuge tube and 3 mL of acetone were added followed by 50 µL of isotopically labeled compounds mixture (2 µg/mL). The tubes were then vortexed for 2 min at 2500 rpm using a BenchMixer XLQ QuEChERS Vortexer (Benchmark Scientific, Sayreville NJ, US) and left overnight under the hood at room temperature. Next, 8 mL of EDTA-Mcllvaine buffer were added to rehydrate the samples and then vortexed and left to stand for one hour prior to the extraction step. When 80% hydration was achieved, 10 mL of acetonitrile was added to the sample and vortexed again. To promote salting out, the QuEChERS original salt kit was emptied into the extraction tube and the resulting mixture was immediately shaken by hand for one minute to avoid salt agglomeration and then vortexed for another minute. Finally, the tube was centrifuged for 10 min at 4000 rpm and 4 ºC and 1 mL of the obtained supernatant was evaporated under a gentle nitrogen stream at room temperature to total dryness. Lastly, the

samples were reconstituted with 1 mL of water/10% methanol solution and injected for LC-MS/MS analysis (Manasfi et al., 2022. in preparation).

1.4 Lettuce chemical extraction

Pharmaceuticals from lettuce leaves were extracted according to Montemurro et al., 2020. Briefly, 1 g of freeze-dried leaves were placed in a 50-mL centrifuge tube and hydrated with 9 mL HPLC water. After 1-hour rest, the tubes were spiked with a proper volume of deuterated mix, vortexed and extracted by adding 10 mL of acidified acetonitrile (0.5% formic acid) followed by the OR QuEChERS salts content. The tubes were immediately hand shaken, vortexed, and centrifuged for 10 min at 4°C and at 4000 rpm. Before the clean-up step, the obtained supernatants were frozen overnight at -20°C to allow fatty acids and lipids precipitation. On the following day 6 mL of the organic phase supernatant were transferred to the dSPE tube for further extract clean-up. The tube was immediately hand shaken, vortexed, and centrifuged for 10 min at 4°C and at 4000rpm. Finally, 1 mL of the supernatant was evaporated under gentle nitrogen flow at room temperature until total dryness and reconstituted with 1 mL of water/MeOH (90/10, v/v) for injection. For the extraction of the PPCPs in roots, 1 g of freeze-dried plant material were hydrated with 9 mL of EDTA-Mcllvaine buffer (See Manasfi et al. 2022 in preparation) and extracted by adding 10 mL of acetonitrile followed by the OR QuEChERS salts kit. The tubes were immediately hand shaken, vortexed, and centrifuged for 10 min at 4°C and at 4000 rpm. No clean up step was performed for root extracts.

1.5 Statistical analyses of sequencing data

Originally, 4,466,919 reads (Table S2) were present in all samples. After denoising, rarefying and the removal of negative and blank extraction controls and two samples, which were not considered in downstream analysis due to contamination with *Enterobacteriaceae*, 45.34% of the reads were remaining. The rarefaction curves (Fig. S1) reached a plateau before the cutoff of 34,920 reads per sample, confirming that the sampling depth was sufficient. In total 22,961

different ASVs were detected and used for bacterial diversity and community composition analysis. To compensate the unequal sampling depth the datasets were subsampled to the minimum number of reads per sample (34,920 reads) using the vegan package (version 2.5-6; Oksanen et al., 2018).

Statistical data analysis and visualization was performed using the statistical program R (version 3.6.2). The normality of the data and residuals was checked using Shapiro Wilk's test ($p>0.05$) and the homogeneity of variance was verified using Levene's test ($p>0.05$). Inverse, root and log-10 transformations of the data were performed when necessary. For parametric distributions two-way ANOVA followed by Tukey's test (using time and treatments as factors) and pairwise Student's t-test were used to determine differences. For non-parametric distributions, data was compared using Kruskal Wallis test. The sample ordination by non-metric multidimensional scaling (NMDS) on the Bray-Curtis dissimilarity and the stacked barplot were generated with the phyloseq package (version 1.28.0; (McMurdie and Holmes, 2013). A PERMANOVA was then performed on the Bray-Curtis dissimilarity using the Adonis function also from the vegan package. The mvabund package (version 4.0.1; (Wang et al., 2012) was used for calculating significant differences among the relative abundance of ASVs between the treatments. ASVs with significantly different relative abundance were graphically displayed using boxplots and heatmaps. All data was visualized by phyloseq and ggplot2 (version 3.2.1; Wickham, 2009). Differences were considered as significant in all tests when the p-value was ≤ 0.05.

Supplementary Table S1. Source sequence of the qPCR linear standard.

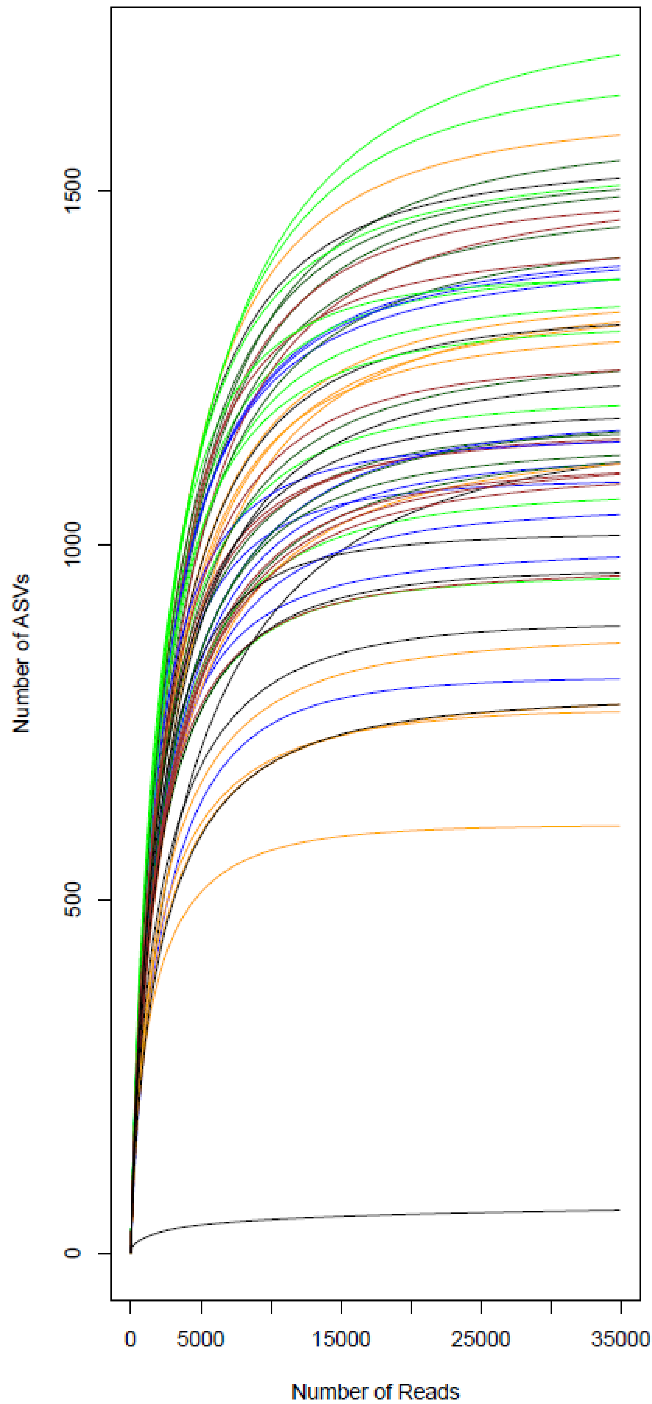
Gene	Sequence	Originating organism
16S rRNA	5’-CAGACTCCTACGGGAGGCAGCAGTGGGGAATATT GGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCG TGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAA GTTGGGAGGAAGGGTAGTAACCTAATACGTTGCTACT TTGACGTTACCGACAGAATAAGCACCGGCTAACTTCG TGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGT TAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTG GTTCAGTAAGTTGGAAGTGAAATCCCCGGGCTCAACC TGGGAACTGCTTTCAAAACTGCTGAGCTAGAGTACGG TAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATG CGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGA CCACCTGGACTGATACTGACACTGAGGTGCGAAAGC GTGGGGAGCAAACAGGATTAGATA-3’	*Pseudomonas anguilliseptica* strain YSH-14

Supplementary Table S2. Number of 16S rRNA sequences obtained per sample through Illumina MiSeq from lettuce roots planted in soil microcosms irrigated with either water, 10μg/L or 100μg/L spiked water, wastewater or 10μg/L or 100μg/L spiked wastewater and collected at the end of the first and second cultivation campaign.

Sample	Timepoint	Treatment	Sequences	Replicate	Number of different ASVs
1	1st campaign	water	66584	1	1414
2	1st campaign	water*	56343	2	822
3	1st campaign	water	54325	3	998
4	1st campaign	water	115872	4	1432
5	1st campaign	water	52741	5	1103
6	1st campaign	water 10μg/L	67709	1	1358
7	1st campaign	water 10μg/L	58818	2	1402
8	1st campaign	water 10μg/L	51893	3	968
9	1st campaign	water 10μg/L	48311	4	1211
10	1st campaign	water 10μg/L	62011	5	1089
11	1st campaign	water 100μg/L	56679	1	1172
12	1st campaign	water 100μg/L	94687	2	1585
13	1st campaign	water 100μg/L	76072	3	1477
14	1st campaign	water 100μg/L	84228	4	1540
15	1st campaign	water 100μg/L	94910	5	1449
16	1st campaign	wastewater	92711	1	1362
17	1st campaign	wastewater	85556	2	1358
18	1st campaign	wastewater	189565	3	1669
19	1st campaign	wastewater	102616	4	1335
20	1st campaign	wastewater	86489	5	1366
21	1st campaign	wastewater 10μg/L	87398	1	1433
22	1st campaign	wastewater 10μg/L	84075	2	1192
23	1st campaign	wastewater 10μg/L	86460	3	1181
24	1st campaign	wastewater 10μg/L	109081	4	1515
25	1st campaign	wastewater 10μg/L	98604	5	1499
26	1st campaign	wastewater 100μg/L	100424	1	1560
27	1st campaign	wastewater 100μg/L*	74981	2	63
28	1st campaign	wastewater 100μg/L	73791	3	1251
29	1st campaign	wastewater 100μg/L	71084	4	1343
30	1st campaign	wastewater 100μg/L	97388	5	1157
31	2nd campaign	water	66832	1	1060
32	2nd campaign	water	49912	2	1162
33	2nd campaign	water	73786	3	1132
34	2nd campaign	water	63847	4	1183
35	2nd campaign	water	80986	5	1426
36	2nd campaign	water 10μg/L	80290	1	1670
37	2nd campaign	water 10μg/L	79083	2	1541
38	2nd campaign	water 10μg/L	99483	3	1755
39	2nd campaign	water 10μg/L	76950	4	1392
40	2nd campaign	water 10μg/L	65630	5	1326

Sample	Timepoint	Treatment	Sequences	Replicate	Number of different ASVs
41	2nd campaign	water 100µg/L	81266	1	1278
42	2nd campaign	water 100µg/L	70610	2	1154
43	2nd campaign	water 100µg/L	62981	3	1184
44	2nd campaign	water 100µg/L	88302	4	1154
45	2nd campaign	water 100µg/L	77847	5	1542
46	2nd campaign	wastewater	74199	1	1153
47	2nd campaign	wastewater	43076	2	772
48	2nd campaign	wastewater	34948	3	613
49	2nd campaign	wastewater	56681	4	790
50	2nd campaign	wastewater	51852	5	877
51	2nd campaign	wastewater 10µg/L	63727	1	1126
52	2nd campaign	wastewater 10µg/L	63209	2	1106
53	2nd campaign	wastewater 10µg/L	63922	3	1261
54	2nd campaign	wastewater 10µg/L	64864	4	1125
55	2nd campaign	wastewater 10µg/L	64694	5	976
56	2nd campaign	wastewater 100µg/L	59167	1	794
57	2nd campaign	wastewater 100µg/L	56508	2	1200
58	2nd campaign	wastewater 100µg/L	56284	3	908
59	2nd campaign	wastewater 100µg/L	50820	4	1025
60	2nd campaign	wastewater 100µg/L	61048	5	975

*Samples not considered in downstream analysis



Supplementary Figure S1. Rarefaction curves of 16S rRNA sequences obtained from the sequencing analysis (n=60) before subsampling and removal of samples, which were not considered in downstream analysis.

Supplementary Table S3. Total nitrogen and carbon (in percentage) in lettuce roots and leaves planted in soil microcosms irrigated with either water and wastewater collected at the end of the first and second cultivation. Each value is the mean of five replicates. ANOVA followed by Tukey's test was performed. Values indicated by different letters are significantly different.

		roots		**leaves**	
		carbon	**nitrogen**	**carbon**	**nitrogen**
1st campaign	**water**	43.24 ± 0.16ab	0.48 ± 0.04a	39.97 ± 0.87ab	1.12 ± 0.09a
	wastewater	43.66 ± 0.14a	0.57 ± 0.02b	39.62 ± 0.31b	1.17 ± 0.08a
2nd campaign	**water**	43.00 ± 0.51b	0.63 ± 0.14b	41.47 ± 0.31c	1.13 ± 0.10a
	wastewater	42.31 ± 0.87c	0.68 ± 0.06b	40.60 ± 0.68ac	1.26 ± 0.15a

Supplementary Table S4. Concentration of the spiked products and major metabolites [ng/g d.w.] in soil from lettuce planted soil microcosms irrigated with either spiked water (100µg/L) or spiked wastewater (100µg/L) and collected at the end of the first and second cultivation campaign. BLOQ: Bellow limit of quantification; N.D.: Not detected.

Timepoint	Treatment	ASF	BNZ	CBZ	CBZ-EPX	CIP	CTP	CLT	CLB	DCF	4`OH-DCF	HCT	IRB	MTP	SUC	SMX	4-nitro-SMX	VAL	VAL-AC
1st campaign	**water**	0.28	0.57	3.65	0.72	0.16	4.02	230.35	2.57	N.D.	N.D.	25.80	1.17	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ	N.D.
		0.60	0.47	3.29	0.69	BLOQ	5.35	193.17	2.90	N.D.	N.D.	23.46	0.87	0.07	BLOQ	BLOQ	BLOQ	BLOQ	BLOQ
		0.28	0.56	3.55	0.76	0.15	4.93	180.52	2.61	N.D.	N.D.	29.56	0.86	BLOQ	2.13	BLOQ	BLOQ	BLOQ	BLOQ
		0.15	0.58	3.42	0.63	0.32	5.06	189.87	3.11	N.D.	N.D.	33.09	0.72	0.11	BLOQ	0.23	BLOQ	BLOQ	N.D.
		0.32	1.09	3.83	0.76	0.25	7.09	299.47	3.31	N.D.	N.D.	21.35	1.30	0.10	BLOQ	BLOQ	BLOQ	BLOQ	N.D.
	wastewater	0.40	1.01	4.24	0.92	1.20	9.10	316.72	5.90	BLOQ	N.D.	29.60	1.42	0.22	BLOQ	0.23	BLOQ	BLOQ	N.D.
		0.14	0.85	3.76	0.65	0.38	5.32	211.32	4.04	N.D.	N.D.	26.41	1.09	0.08	BLOQ	0.31	BLOQ	BLOQ	N.D.
		0.21	0.66	3.59	0.59	1.06	5.52	181.19	3.22	N.D.	N.D.	27.48	1.20	0.17	BLOQ	0.24	BLOQ	BLOQ	N.D.
		0.23	0.91	4.12	0.85	0.51	8.74	286.56	5.10	N.D.	N.D.	32.80	1.19	BLOQ	1.09	BLOQ	BLOQ	BLOQ	BLOQ
		0.24	0.78	3.69	0.61	0.60	5.23	181.54	4.11	N.D.	N.D.	25.30	0.84	0.10	BLOQ	BLOQ	BLOQ	BLOQ	N.D.
2nd campaign	**water**	0.47	0.98	5.55	1.70	BLOQ	10.92	396.70	5.50	N.D.	N.D.	31.30	1.18	0.12	BLOQ	0.37	BLOQ	BLOQ	BLOQ
		BLOQ	1.23	5.74	2.25	5.50	10.58	348.70	5.85	N.D.	N.D.	33.39	1.08	0.10	BLOQ	0.26	BLOQ	BLOQ	BLOQ
		BLOQ	1.11	5.81	2.62	N.D.	12.59	359.30	6.53	N.D.	N.D.	30.98	1.16	0.08	BLOQ	0.34	BLOQ	BLOQ	N.D.
		0.67	1.08	5.72	2.63	0.81	14.65	307.14	6.29	N.D.	N.D.	36.77	1.11	0.19	BLOQ	0.24	BLOQ	BLOQ	N.D.
		0.46	1.35	6.10	2.04	2.01	10.69	373.08	5.22	N.D.	N.D.	31.67	1.13	0.20	BLOQ	0.33	BLOQ	BLOQ	N.D.
	wastewater	BLOQ	1.22	6.73	2.30	1.67	12.32	319.70	8.59	0.14	N.D.	33.14	1.81	0.23	BLOQ	BLOQ	BLOQ	BLOQ	0.09
		BLOQ	0.87	6.28	1.74	2.34	10.70	369.30	8.88	0.29	N.D.	34.94	1.85	0.22	BLOQ	0.42	BLOQ	BLOQ	0.14
		BLOQ	0.96	6.38	2.77	0.94	13.57	324.10	8.28	0.14	N.D.	28.15	2.17	0.29	BLOQ	0.30	BLOQ	BLOQ	0.27
		BLOQ	0.90	6.64	2.41	0.59	13.40	294.69	8.16	0.13	N.D.	27.92	2.03	0.14	BLOQ	0.33	BLOQ	BLOQ	0.15

The MQL (minimum quantification level) [ng/g d.w.] are ASF= 0.01, CIP= 0.03, DCF= 0.16, MTP= 0.01, SUC= 0.05, SMX= 0.03, 4-nitro-SMX= 0.07, VAL= 0.03 and VAL-AC= 0.12.

Supplementary Table S5. Concentration of the spiked products and major metabolites [ng/g d.w.] in lettuce roots planted in soil microcosms irrigated with either spiked water (100µg/L) or spiked wastewater (100µg/L) and collected at the end of the first and second cultivation campaign. BLOQ: Bellow limit of quantification; N.D.: Not detected.

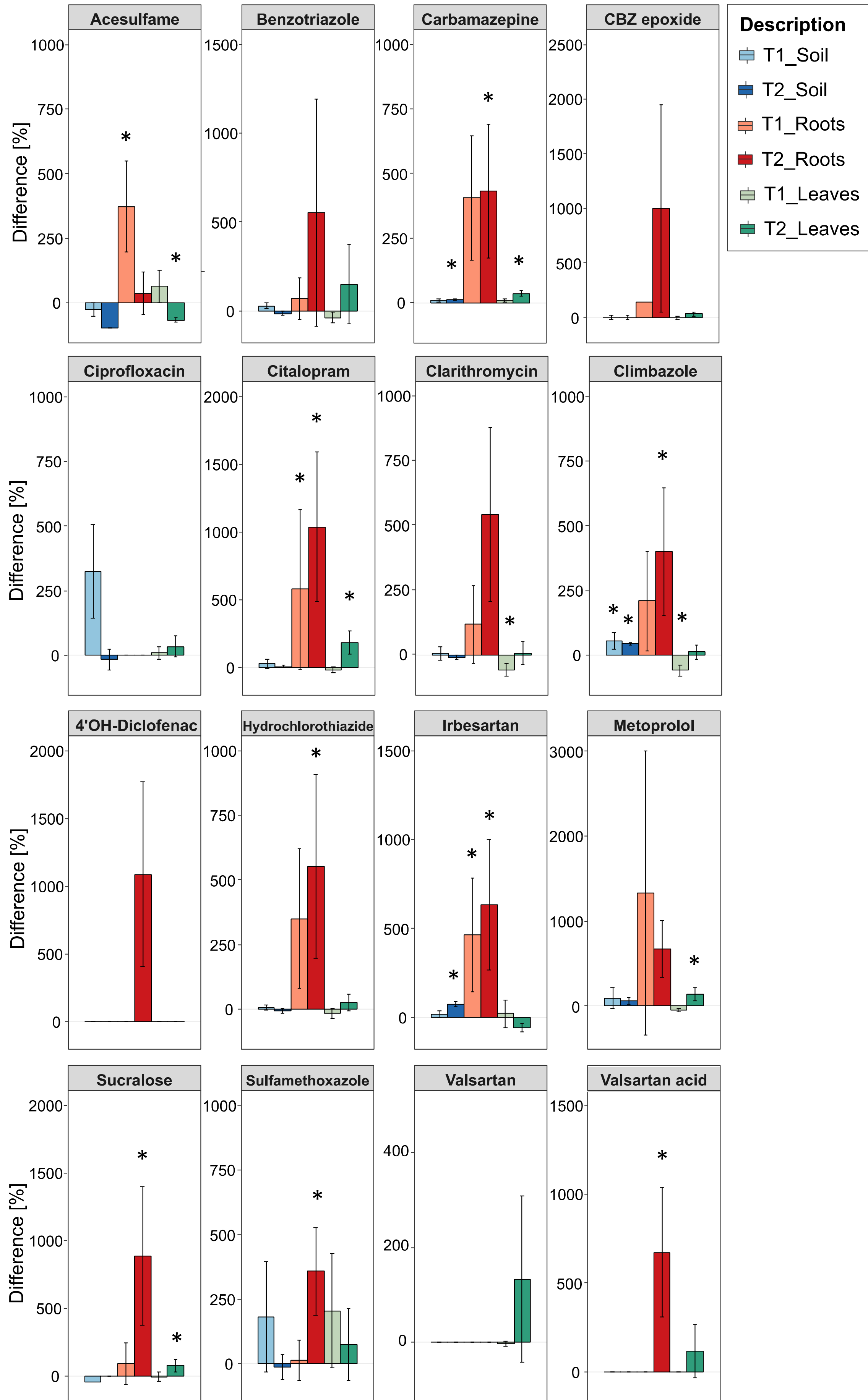
Timepoint	Treatment	ASF	BNZ	CBZ	CBZ-EPX	CIP	CTP	CLT	CLB	DCF	4`OH-DCF	HCT	IRB	MTP	SUC	SMX	4-nitro-SMX	VAL	VAL-AC
1st campaign	**water**	14.54	BLOQ	7.45	BLOQ	N.D.	6.40	5.85	4.22	N.D.	N.D.	12.16	1.57	1.87	BLOQ	28.77	N.D.	N.D.	N.D.
		33.78	BLOQ	21.20	BLOQ	N.D.	14.95	68.74	8.18	N.D.	N.D.	31.71	2.87	4.29	139.54	6.09	N.D.	N.D.	N.D.
		29.58	BLOQ	24.44	BLOQ	N.D.	14.79	36.84	9.71	N.D.	N.D.	15.37	0.68	1.62	601.70	32.13	N.D.	N.D.	N.D.
		31.11	BLOQ	17.48	BLOQ	N.D.	7.06	7.93	3.70	N.D.	N.D.	11.73	N.D.	BLOQ	113.59	3.87	N.D.	N.D.	N.D.
		18.91	BLOQ	8.40	BLOQ	N.D.	5.64	9.24	2.57	N.D.	N.D.	4.55	1.17	0.60	98.70	9.78	N.D.	N.D.	N.D.
	wastewater	53.53	BLOQ	21.76	BLOQ	N.D.	13.10	8.65	5.99	N.D.	N.D.	17.77	2.06	0.76	44.02	10.59	N.D.	N.D.	N.D.
		193.6	48.49	138.08	14.07	N.D.	163.3	643.44	32.90	N.D.	N.D.	141.75	12.22	63.44	734.69	18.43	N.D.	N.D.	N.D.
		109.2	0.65	73.73	BLOQ	N.D.	30.75	92.60	11.47	N.D.	N.D.	51.26	4.38	2.11	711.55	41.94	N.D.	N.D.	N.D.
		113.5	BLOQ	68.84	BLOQ	N.D.	22.46	28.03	8.89	N.D.	N.D.	60.13	5.41	0.82	175.20	13.45	N.D.	N.D.	N.D.
		134.5	BLOQ	96.89	1.53	N.D.	101.0	95.85	28.48	N.D.	7.94	69.32	11.34	54.48	160.83	5.95	N.D.	N.D.	9.50
2nd campaign	**water**	11.13	3.86	36.46	BLOQ	N.D.	29.93	48.66	8.96	N.D.	11.80	33.77	5.16	6.38	280.53	228.81	N.D.	N.D.	9.23
		34.42	0.66	21.63	BLOQ	N.D.	18.09	17.59	7.13	N.D.	5.94	15.65	3.46	4.70	125.02	131.25	N.D.	N.D.	4.37
		4.04	BLOQ	7.20	BLOQ	N.D.	5.05	2.27	2.08	N.D.	0.66	5.19	1.18	0.42	BLOQ	51.43	N.D.	N.D.	1.57
	wastewater	25.50	20.67	137.28	3.74	N.D.	265.4	191.71	37.65	N.D.	115.61	200.66	35.38	34.37	2137.0	955.08	N.D.	N.D.	53.35
		45.96	23.61	198.51	5.79	N.D.	325.0	209.90	54.30	11.67	83.21	173.95	38.38	32.40	1945.8	738.99	N.D.	N.D.	59.36
		19.50	6.01	129.28	2.37	N.D.	183.5	210.18	22.80	N.D.	52.45	122.82	20.03	35.55	1054.7	666.24	N.D.	N.D.	29.41
		4.17	0.67	28.94	BLOQ	N.D.	33.97	13.57	9.85	N.D.	2.08	23.37	5.02	4.70	197.03	254.97	0.22	N.D.	8.13
		18.13	0.08	85.69	BLOQ	N.D.	197.5	108.47	26.52	10.51	110.15	73.77	20.78	40.66	1343.4	526.08	0.36	N.D.	44.72

The MQL (minimum quantification level) [ng/g d.w.] are BNZ= 0.35, CBZ-EPX= 0.45, MTP= 0.23, SUC= 0.68.

Supplementary Table S6. Concentration of the spiked products and major metabolites [ng/g d.w.] in lettuce leaves planted in soil microcosms irrigated with either spiked water (100µg/L) or spiked wastewater (100µg/L) and collected at the end of the first and second cultivation campaign. BLOQ: Bellow limit of quantification; N.D.: Not detected.

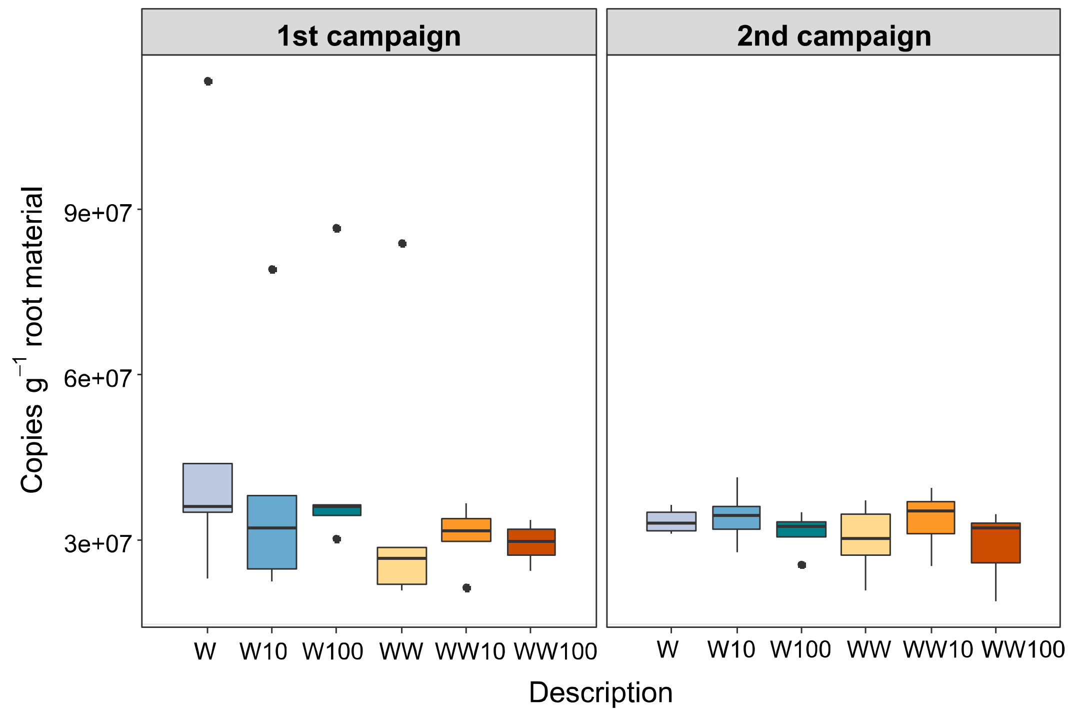
Timepoint	Treatment	ASF	BNZ	CBZ	CBZ-EPX	CIP	CTP	CLT	CLB	DCF	4`OH-DCF	HCT	IRB	MTP	SUC	SMX	4-nitro-SMX	VAL	VAL-AC
1st campaign	**water**	312.20	0.52	83.03	25.26	3.46	11.87	99.56	3.64	N.D.	N.D.	29.93	0.38	2.60	305.70	N.D.	N.D.	1.11	N.D.
		291.00	BLOQ	92.06	27.42	N.D.	6.36	48.53	1.91	N.D.	N.D.	36.06	BLOQ	1.61	399.80	N.D.	N.D.	N.D.	N.D.
		166.60	1.43	75.42	17.40	N.D.	4.05	62.79	1.83	N.D.	N.D.	29.21	BLOQ	0.84	187.60	0.32	N.D.	0.79	N.D.
		275.18	1.57	81.37	22.40	2.57	7.57	80.86	2.25	N.D.	N.D.	38.52	0.90	2.76	370.76	N.D.	N.D.	0.72	N.D.
	wastewater	748.80	0.80	100.60	30.18	1.90	8.72	23.28	1.63	N.D.	N.D.	36.41	0.22	1.71	378.70	0.32	N.D.	0.63	N.D.
		312.63	0.56	84.00	21.65	1.16	7.03	62.16	1.59	N.D.	N.D.	34.20	0.88	0.98	396.68	N.D.	N.D.	N.D.	N.D.
		357.84	0.85	88.12	19.25	1.82	4.89	38.95	0.47	N.D.	N.D.	24.36	0.21	0.53	144.53	N.D.	N.D.	N.D.	N.D.
		415.17	BLOQ	90.80	20.67	2.11	5.63	16.51	0.77	N.D.	N.D.	24.29	0.49	1.03	402.47	3.38	N.D.	0.57	0.98
		305.63	0.55	90.75	22.85	1.28	4.14	13.27	0.56	N.D.	N.D.	20.55	0.24	0.77	174.12	0.51	N.D.	N.D.	N.D.
2nd campaign	**water**	209.44	BLOQ	127.88	89.30	N.D.	12.27	9.88	1.34	N.D.	N.D.	34.77	BLOQ	2.31	317.69	0.80	N.D.	N.D.	2.97
		210.92	0.56	119.46	56.22	N.D.	12.55	19.00	2.22	N.D.	N.D.	29.72	0.35	2.83	166.12	0.22	N.D.	N.D.	N.D.
		220.58	0.95	104.13	44.60	1.14	9.64	50.55	2.12	N.D.	N.D.	34.01	0.40	2.35	545.60	N.D.	N.D.	N.D.	N.D.
		816.08	0.50	143.22	84.70	N.D.	21.76	31.63	1.54	N.D.	N.D.	56.97	0.25	6.81	383.90	N.D.	N.D.	N.D.	2.70
		324.56	BLOQ	107.92	42.91	1.03	9.45	54.03	2.69	N.D.	N.D.	36.87	0.72	2.11	397.19	1.04	N.D.	0.84	2.63
	wastewater	165.85	0.87	185.03	99.29	0.84	55.28	53.70	2.55	N.D.	N.D.	69.18	0.27	11.04	818.45	1.62	N.D.	N.D.	8.22
		127.68	2.01	176.93	100.01	0.74	45.11	24.14	1.45	N.D.	N.D.	55.50	BLOQ	10.01	797.90	1.07	N.D.	N.D.	0.99
		94.31	2.44	154.40	78.34	N.D.	29.27	52.87	1.70	N.D.	N.D.	47.32	0.16	8.02	678.70	0.61	N.D.	0.90	2.54
		95.59	BLOQ	150.79	77.93	N.D.	24.47	21.23	2.40	N.D.	N.D.	37.83	0.16	3.90	343.91	0.13	N.D.	N.D.	2.26
		91.53	BLOQ	153.61	74.31	N.D.	32.59	26.02	2.95	N.D.	N.D.	34.74	0.10	5.93	543.86	0.15	N.D.	0.55	3.92

The MQL (minimum quantification level) are [ng/g d.w.] BNZ= 0.18, IRB = 0.08.

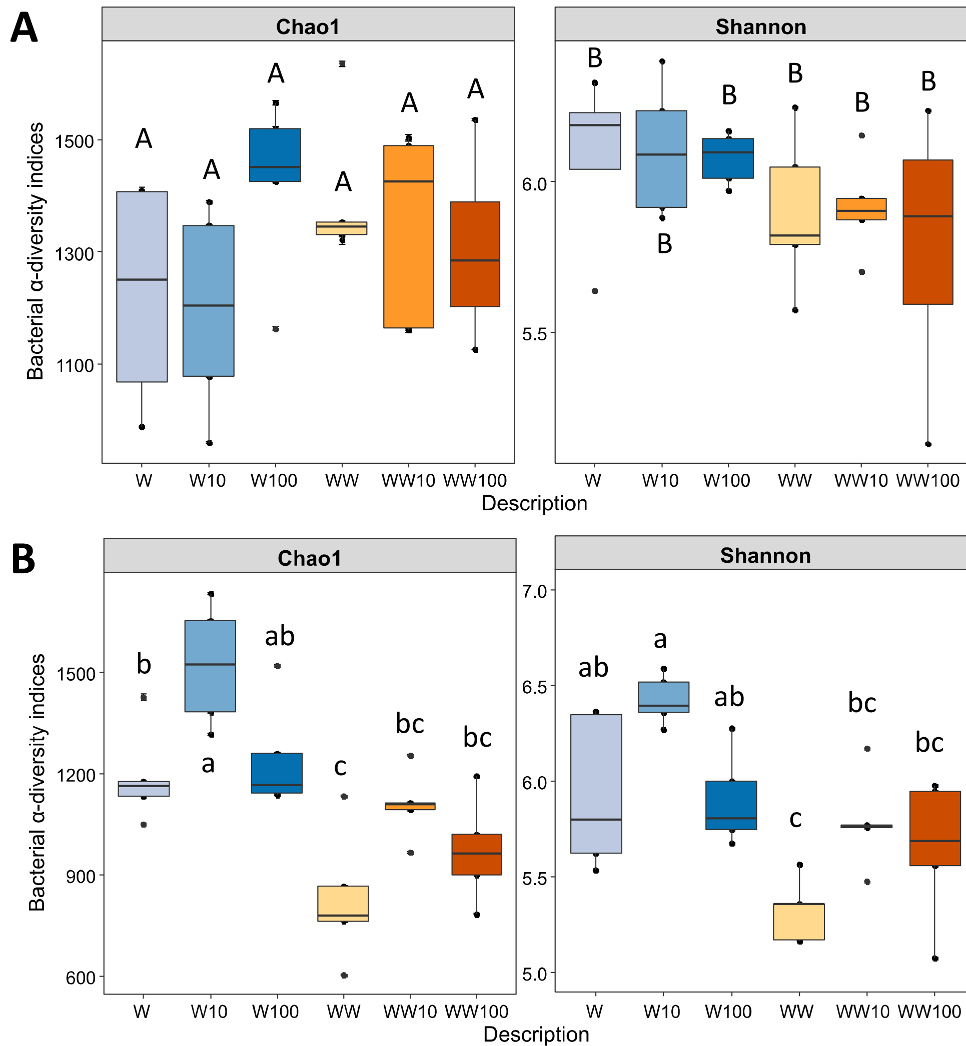


Acesulfame
Benzotriazole
Carbamazepine
CBZ epoxide
Ciprofloxacin
Citalopram
Clarithromycin
Climbazole
4'OH-Diclofenac
Hydrochlorothiazide
Irbesartan
Metoprolol
Sucralose
Sulfamethoxazole
Valsartan
Valsartan acid
Difference [%]
Description
T1_Soil
T2_Soil
T1_Roots
T2_Roots
T1_Leaves
T2_Leaves

Supplementary Figure S2. Difference [%] in the concentration of PPCPs in soil/plant tissue, derived from samples irrigated with wastewater 100µg/L PPCPs compared to water 100µg/L PPCPs. Significant differences between the concentration of PPCPs measured in water compared to wastewater irrigated samples are indicated as "*" for p–value ≤ 0.05.



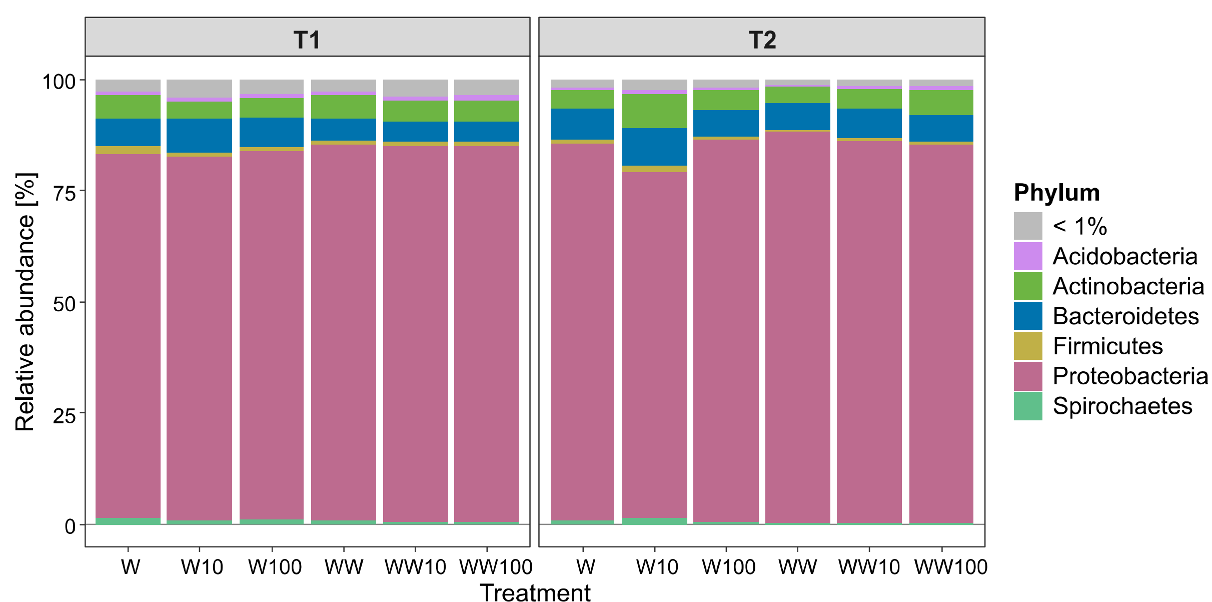
Supplementary Figure S3. Copy numbers of bacterial 16S rRNA. "W", "W10", and "W100" refer to samples irrigated with water, or water spiked with 10µg/L or 100µg/L PPCPs; "WW0", "WW10", and "WW100" indicate the corresponding wastewater-irrigated samples. No significant difference was detected by ANOVA and Tukey's post hoc testing.



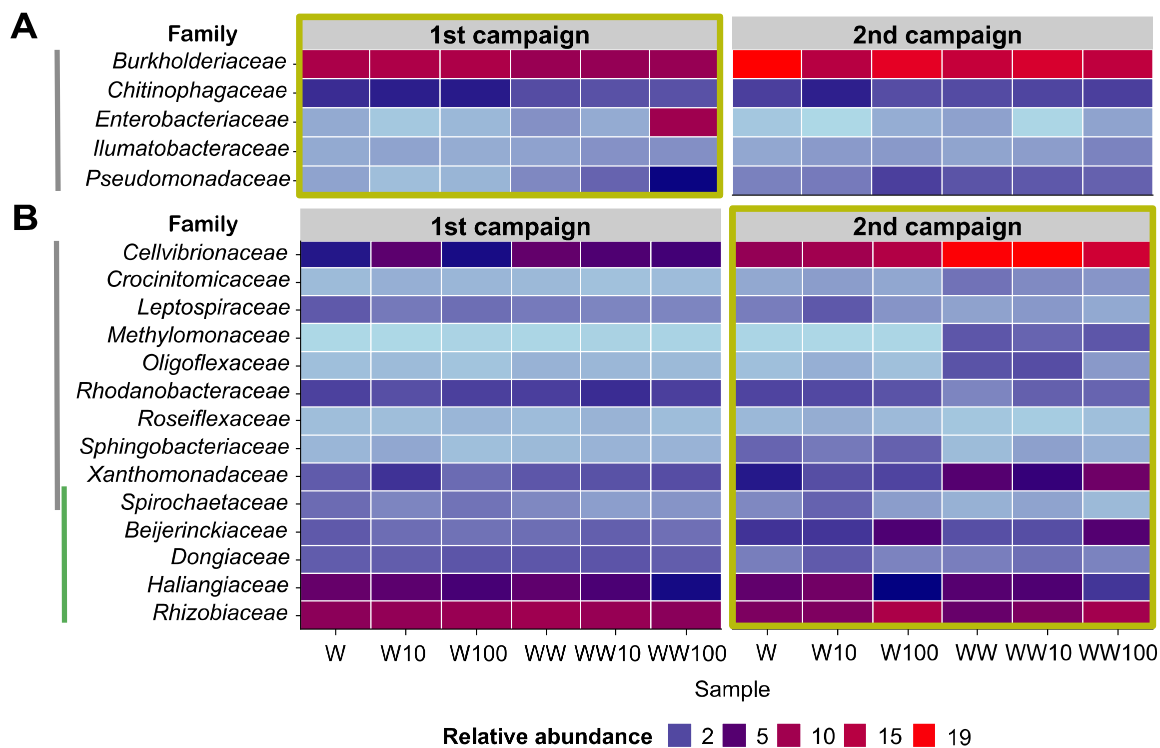
Supplementary Figure S4. Alpha-diversity and richness of root-associated bacteria in fresh soil (1st campaign, **A**) and contaminated soil (2nd campaign, **B**) irrigated with water or treated wastewater and different concentrations of PPCPs. "W", "W10", and "W100" refer to samples irrigated with water, or water spiked with 10μg/L or 100μg/L PPCPs; "WW0", "WW10", and "WW100" indicate the corresponding wastewater-irrigated samples. Standard deviations are indicated by error bars. ANOVA followed by Tukey's test was performed. Significant differences is indicated as "**" for N.D.1 ≤ p–value ≤ 0.01. For 1st campaign water without PPCPs, and 1st campaign wastewater 100μg/L, n=4.

Supplementary Table S7. Permutation analysis for the homogeneity of multivariate dispersions among samples (permutations = 1000).

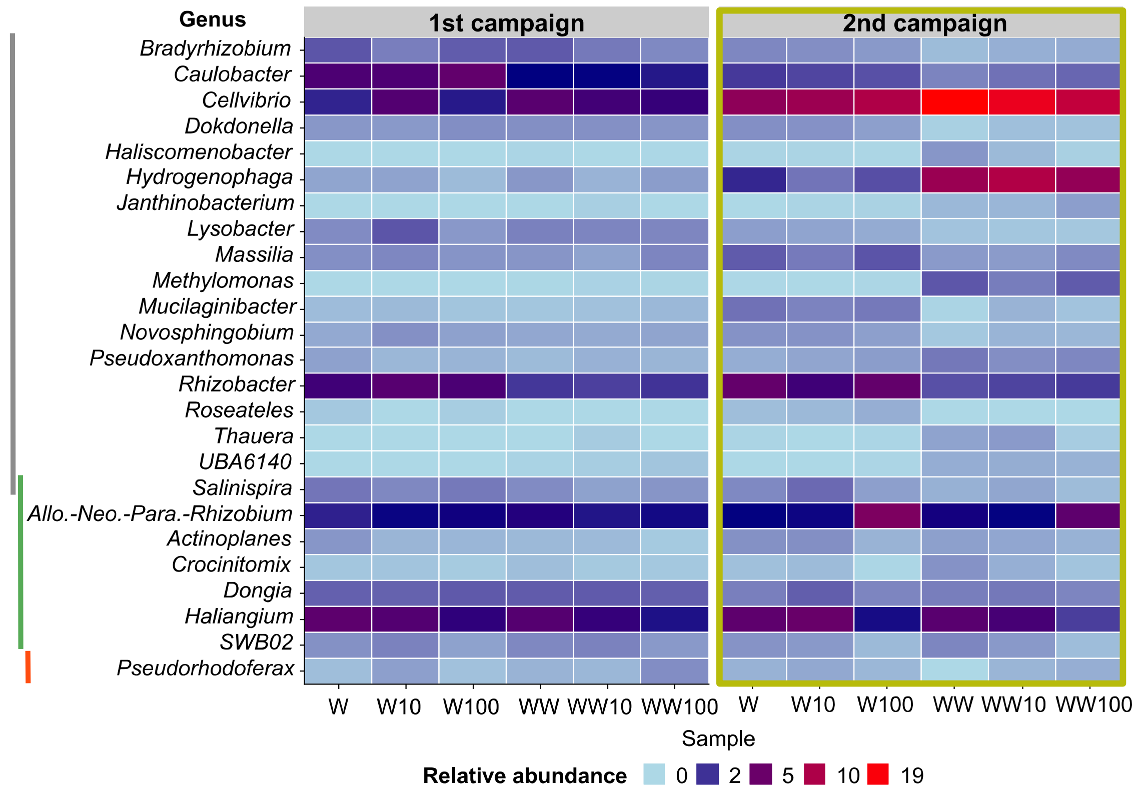
Dataset	Factor(s)	Df	p-value
1st Campaign	WaterType	1	0.2218
	PPCP Concentration	2	0.4885
2nd Campaign	WaterType	1	0.3207
	PPCP Concentration	2	0.6993



Supplementary Figure S5. Relative abundance [%] of bacterial phyla based on the taxonomic assignments of the amplified 16S rRNA genes. Only phyla with abundance higher than 1% are shown. For 1st campaign water without PPCPs, and 1st campaign wastewater 100µg/L, n=4.



Supplementary Figure S6. Heatmap showing relative abundance of significantly different bacterial families in the first (A) and in the second (B) campaign. Grey bars indicate bacterial families significantly affected by the WaterType, green bars the significantly affected by the Concentration of pharmaceuticals and personal care products. For 1st campaign water without PPCPs, and 1st campaign wastewater 100µg/L, n=4.



Supplementary Figure S7. Heatmap showing relative abundance of significantly different bacterial genera in the second campaign. Grey bar indicates bacterial genera significantly affected by the WaterType, green bar the significantly affected ones by the concentration of PPCPs and the orange bar the ones affected by the interaction of WaterType:Concentration. For 1st campaign water without PPCPs, and 1st campaign wastewater 100µg/L, n=4. *Allo.-Neo.-Para.-Rhizobium* = *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*.

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Appendix: Publication III – Peer-reviewed Book Chapter

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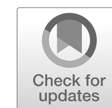
Uptake and Translocation of Pharmaceuticals in Plants: Principles and Data Analysis



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Abstract Pharmaceuticals originating from reclaimed wastewater or biosolid-, livestock manure- or sewage sludge-amended soils can enter crops by irrigation and fertilization. Generally, the putative uptake occurs through the plants' roots and

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can lead to the bioaccumulation in different plant parts. The uptake and translocation therefore is dependent on multiple parameters, i.e. physicochemical properties of compounds, plant physiology and environmental factors. This book chapter combines a theoretical background on the main principles of uptake and translocation of pharmaceuticals by plants and a critical evaluation of current available literature, by analysing studies for the bioconcentration and translocation factors of different pharmaceutical groups in several plant species. Thereby, interesting results were obtained by looking at the translocation of various pharmaceuticals in radish and at cationic compounds in soil studies. Comparing the different studies, the relevance of testing not only high but also real environmental concentrations became obvious, since for some pharmaceuticals, higher uptake and translocation ratios were achieved with lower applied concentrations. Basic guidelines could provide a possibility to make scientific data more comparable and reliable and to avoid the exclusion of potential reasons for the missing uptake or translocation of pharmaceuticals. This book chapter provides recommendations for future research studies to generate more valid conclusions within the scientific community.

Keywords Bioconcentration factor, Hydroponic studies, Ionic compounds, Sequestration, Soil studies, Translocation factor

1 Background

Ecosystems are often exposed to natural or synthetic substances that have no direct nutritional value or significance for metabolism but can have a negative impact on the function and performance of biota. Commonly, these substances enter the aquatic environments through wastewater treatment plant effluents as a consequence of partial and/or inefficient removal during wastewater treatment processes. Recent studies, supported by powerful analytical screening analyses, described a high number of emerging pollutants in those effluents; they can range from pesticides, pharmaceuticals and personal care products (PPCPs), illicit drugs, endocrine disruptive compounds, flame retardants, food additives, disinfection by-products through all possible metabolites and transformation products (TPs) [1, 2]. Although only low concentrations (ng/L–μg/L) of these organic molecules were frequently found in surface and groundwater, they can be considered as ‘pseudo-persistent’ because of their continuous discharge and deposition into the environment [3]. These substances can also enter the terrestrial environment by agricultural practices, i.e. the irrigation of plants with treated wastewater or fertilization with manure; after their exposure to agricultural soils, compounds can be taken up by crops and therefore enter the food chain. In case of pharmaceuticals, long-term exposure to low concentration levels can induce toxic or metabolic dysregulation in terrestrial and aquatic organisms [4, 5].

Due to their chemical properties, a topic that will be also discussed further in this chapter, pharmaceutical residues, metabolites and TPs might be adsorbed to soil particles and taken up by plants [6]. In order to be able to estimate the effects not only on biota but also on human health, an understanding of the absorption and transport processes in plants is of ample relevance.

This chapter will provide readers with an overview of the most important uptake mechanisms in plants, in addition to the transport of pharmaceutical compounds through the plant vascular system. Concepts will be resumed from soil and chemical properties ending up in plant biotransformation and sequestration mechanisms and environmental factors that can influence the pharmaceuticals' uptake.

This article will cover the main pharmaceutical groups, i.e. antibiotics, hormones, analgesics, anti-inflammatory, lipid regulator agents, antidiabetic, anticonvulsants, stimulants, psychotropic drugs and antihypertensives (e.g. beta-blockers, calcium channel- or angiotensin receptor blockers) since these compound classes are in continuous debit into the environment and due to their chemical characteristics that make them prone to plant uptake.

Data on pharmaceutical uptake and translocation published from year 2013 on were analysed to take conclusions based on different experiments and conditions.

2 Which Factors Can Influence the Uptake of Pharmaceuticals by Plant Roots?

Soil properties, like ionic strength, pH and organic matter (OM) content, are determining factors in the fate of emergent compounds (as pharmaceuticals) in soil-plant systems. OM is an important sorbent for pharmaceuticals, which changes their bioavailability/bioaccessibility for root uptake [7, 8] (see Fig. 1). According to Miller and co-authors [9], polar and ionizable pharmaceuticals can engage in interactions beyond hydrophobic partitioning, including electron donor-acceptor interactions, cation and anion exchange, protonation, water bridging, cation bridging and surface complexation. Moreover, for ionizable compounds, several physico-chemical properties strongly influence the degree of association with soil particles.

Abiotic transformations like redox reactions may occur in the clay fraction through reactive mineral phases and influence the molecule's integrity. Photolysis can likewise be involved in processes close to soil surfaces, but it has a lower relevance due to strong light attenuation deeper in soils [9].

Synergistic effects between different pharmaceuticals can also play an important role. Especially, when crops are irrigated with treated wastewater, plants are not only exposed to one but to a cocktail of pharmaceuticals and other compounds. The co-occurrence of carbamazepine and lamotrigine in crops showed that synergistic effects enhanced the uptake of lamotrigine when carbamazepine was present, but the uptake of carbamazepine was not affected in presence of lamotrigine in cucumber plants (*Cucumis sativus*) grown under hydroponic conditions [10]. Moreover, the

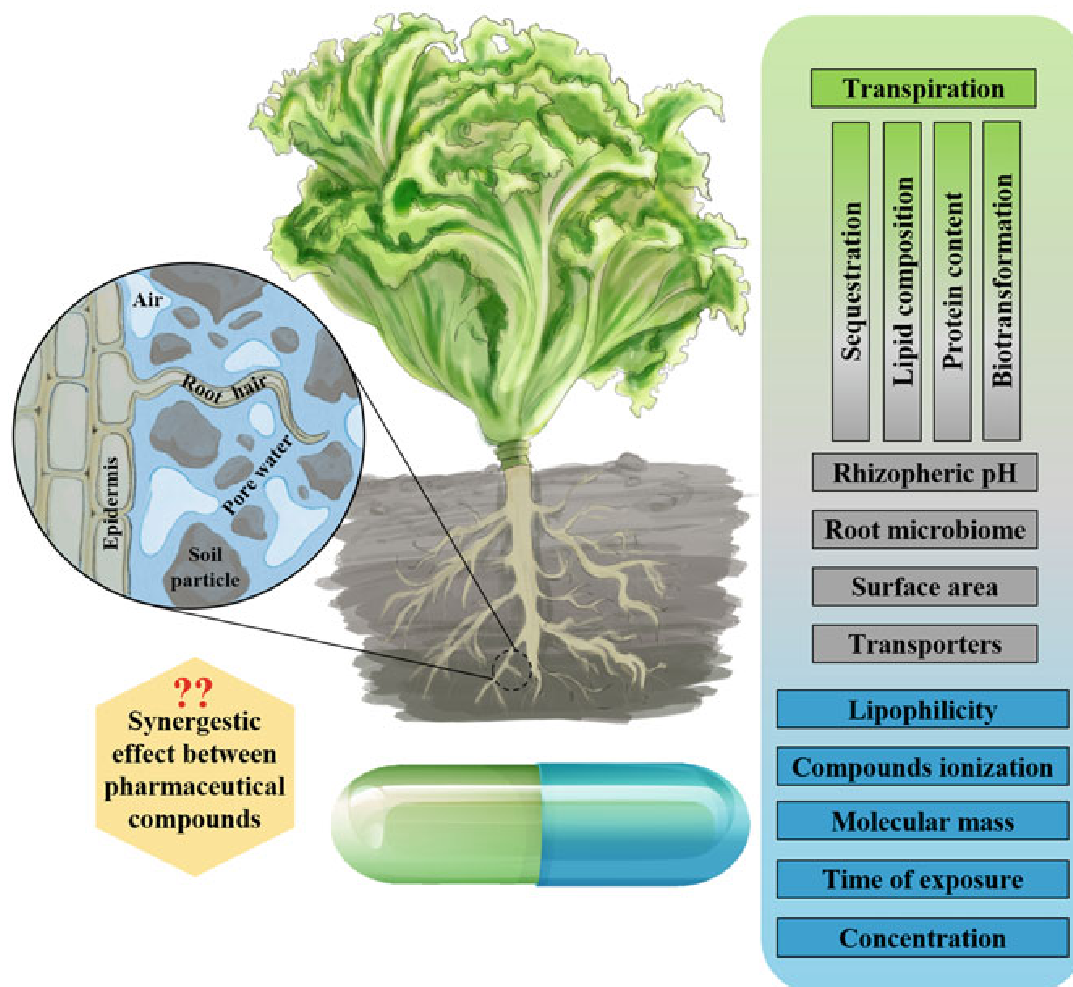

Fig. 1 Multiple parameters, which play a critical role on plants' uptake of pharmaceuticals along with their distribution among different plant organs

uptake of pharmaceuticals, when applied in a mixture compared to single compound exposure, also differed between plant species. The concentration of atenolol was higher during the single compound exposure in the roots of lamb's lettuce (*Valerianella locusta* L.) whereas on arugula (*Eruca sativa* L.) and radish (*Raphanus sativus* L.) did not show higher values compared to the mixture application. The uptake and translocation of other substances were in contrast similar between plant species in the single and mixture application of pharmaceuticals [11]. However, as this study was performed in soil, the additional soil effects might influence the uptake of these pharmaceuticals, which was also shown in the same study. Furthermore, interactions between pharmaceuticals, heavy metals and metalloids were detected in beet root (*Beta vulgaris* L.). The concentration of sulfamethoxazole in beet root increased with increasing concentration of a mixture of heavy metals (Mn, Zn, Cu, Cd, CO, Cr, Ni and Pb). In contrast, the accumulation of metoprolol decreased with increasing heavy metal concentration. For other compounds, the changes were negligible or no clear trend was observed [12]. To conclude,

interactions between different pharmaceuticals but also pharmaceuticals and heavy metals could be observed, which are not always favouring an increased or decreased accumulation in plants. This uptake is rather influenced by additional parameters like physicochemical properties of the compound, plant physiology or soil composition.

Biodegradation is considered the most important process for eliminating the majority of xenobiotics (e.g. pharmaceuticals), where microorganisms – as important degraders – provide products to other organisms in the food web. However, these processes are only significant when the molecules' toxicity does not inhibit microbial activity. Although, known for a long time, the biodegradation of drugs and their effects on ecological processes driven by microorganisms is quite scarce but may be also too complex to be fully addressed in this book chapter [13]. Besides the potential transformation of pharmaceuticals by soil organisms, their bioavailability might also be reduced by the microbial communities at root surfaces – so-called rhizobacteria – which can act as enhancers of phytoremediation efficiency; the same concept has been proposed for endophytic bacteria inhabiting root tissue. Moreover, the latter can interact closely with their host plant boosting the degradation pathways and metabolic activities and then decreasing both phytotoxicity and evapotranspiration of volatile organic compounds [14–16].

Various microbial species and strains may perform differently under different environmental and growth conditions, determining their efficiency and hence their usefulness [17, 18]. Although many microbial species are still unidentified, Agrawal and co-authors [17] listed a wide range of pollutant-degrading microorganisms that have been spotted by culture-independent techniques and could be harboured in the root environment of various plant species. The full metabolic capacity of the plant associated bacteria (plant endophytes and rhizosphere bacteria) has not been completely resolved yet, although first experiments indicate that microbial activities can have a strong influence on biotransformation processes of pharmaceuticals [15, 19] (more details are provided in chapter "Impact of PhACs on Soil Microorganisms").

Another factor that has been mostly neglected is the direct availability of active metabolites that may be excreted from animals or humans. Generally, it is assumed that 90% of an active compound are metabolized from a mammalian body within 48 h, after treatment. In any case, the availability of parent compounds and major metabolites will be decisive for their further fate in plants.

2.1 Compounds Properties

One of the primary criteria that influences uptake into roots and translocation in plant tissue is the *molar mass* of the pharmaceuticals [20]. Low-molar mass organic compounds can easily enter the soft rhizodermis and move through the porous mesh of the cell wall. Hence, organic substances with molar mass <1,000 g/mol are easily absorbed by the apical sections of plant roots [21]. However according to Chuang and co-workers [20], only molecules below 300 g/mol can, in general, enter

the roots easily, when compared to large-sized pharmaceuticals (molar mass >400 g/mol).

In the living parenchymal tissue deeper inside the root, and towards the delicate younger apical roots, the cell wall and the biomembrane (plasmalemma) may function as filters (*membrane permeation*) limiting the uptake or movement of organic molecules based on their size.

Besides that, physicochemical properties of the molecules, like *lipophilicity* and *ionic strength* (polarity H bonding), will dictate their fate, even before uptake and transport into the plant vascular translocation system (xylem and phloem) occur. A significant proportion of pharmaceuticals are ionizable meaning that they can assume neutral, cationic, anionic or zwitterionic form under different pH conditions [22]. This means that the difference in lipophilicity between the neutral and ionic forms varies within compounds and is difficult to predict. Usually, a single log K_{OW} value (also called P) is determined, reflecting only the lipophilicity of neutral species [23]. So, it has been discussed that for ionic forms, log D_{OW} seems to be more appropriate to express the lipophilicity of these molecules because it accounts for pH dependence (i.e. acid dissociation constant (pKa)) of a molecule in aqueous solution [24].

In early research on the topic, Briggs and co-workers [25] established a linear relationship between K_{OW} of non-ionized chemicals and the observed root concentration. Albeit only shown for industrial pollutants and herbicides [26], this relationship seems to hold true also for other synthetic molecules like pharmaceuticals. It is crucial to consider that pharmaceuticals have been specifically designed to penetrate through biological borders and membranes, to ensure their rapid delivery at the site of action. Wild and co-workers [27] pointed out that non-ionic organic chemicals with log $K_{OW} > 4$ seem to exhibit high retention in plant roots, while Cousins and Mackay [28] suggested that for organic chemicals with log $K_{OW} < 2$ and a Henry's Law constant of less than 100 $cm^3\ cm^{-3}$, the water filled intercellular space seemed to be the main storage compartment [29]; the topic has been extensively covered by Schröder and Collins [30].

2.2 Uptake of Pharmaceuticals by Plant Roots

In the first step, compounds from the surrounding medium or pore water (usable water in soil for plants) become available for root uptake by diffusion, where compounds properties like *solubility*, lipophilicity, molar mass, *compound concentration* and characteristics from the surrounding environment as temperature and soil humidity (if the case) will influence the uptake performance [21] (Fig. 1). Here, soils with high proportions of clay minerals might be a significant temporary sink for charged molecules and build up local hotspots of organic pollutants. In a second phase, compounds are available to root uptake: due to a negative water potential in soils at field capacity, a net movement of pharmaceutics towards plant rhizospheres might prevail. The root surface and its extensions are key compartments for uptake

of organic compounds: roots of perennial plants (except monocots) typically develop a rigid protective structure called periderm (replaces the normal rhizoderm), which comprises a large component of bark and the most outer layer called phellem, consisting of suberized-dead cells [31]. These bark-like materials contain accumulations of lipophilic substances and may hence act as a sink for lipophilic compounds. In this context, the role of the protective root cap and its mucilage has not been investigated as sink in depth.

Although chemical features of a molecule may be important predictors for the uptake, the physiology of the plant root itself and its composition can also have significant influence. Trapp and Pussemeir [32] critically reviewed the relationship derived by Briggs and co-workers [25] as an overestimate of the uptake of some herbicides by common bean (*Phaseolus vulgaris*) [33]. We are still lacking knowledge about the factors determining such differences.

Among all biological factors, root extractable lipid content seems to have the strongest influence on the emerging compounds' uptake [34]. Either way, lipophilic compounds are expected to partition to root lipids (membrane and storage lipids) and thus concentrate in roots, until an equilibrium between the chemical concentration in the aqueous phase within the plant root and the external solution is reached. The strong affinity of charged compounds or their metabolites in roots retards pharmaceutical transport to shoots and results in a significant accumulation in roots, making tuberous vegetables critical sources of food and fodder [35]. However, protein content was found to have a greater influence on the prediction of uptake than the lipid content as described by González García and co-authors [36]. For weak acids like ibuprofen, ketoprofen and naproxen, higher concentrations in roots than in leaves were quantified, suggesting the adsorption to proteins and consequently retention in roots, which supported their model.

Once a solute enters the root – through the growing tip of the root hair epidermis passing by cortex, endodermis and pericycle, ending up with the entrance into the vascular tissue – it can take two pathways to reach the xylem, along which it is transported to the aerial plant parts:

In the *apoplastic pathway*, the solute travels along cell walls through the intercellular space of the epidermis and cortex region of the root and across cell membranes at the endodermis. Non-ionic pharmaceutics are able to cross cell membranes easily and thus have higher potential to be taken up by the roots due to their higher lipophilicity [37]. However, compounds taken up exclusively by the apoplastic route cannot cross the Casparian strip; that is, they must cross at least one lipid bilayer to enter the xylem or phloem; if not, they tend to accumulate in roots [9]. Little research has been directed towards elucidating xenobiotic uptake mechanisms and pathways, knowledge that is needed to develop models to predict uptake and accumulation. Chemical sorption to lipophilic root structures may be a significant factor influencing the available concentration.

In the *symplastic pathway*, the solute crosses cell membranes of root hairs, epidermis and cortex and moves to the vascular cylinders by the plasmodesmata and/or by membrane permeation [38], which means that only a small fraction of the compounds is transported via the symplastic movement into cellular vacuoles

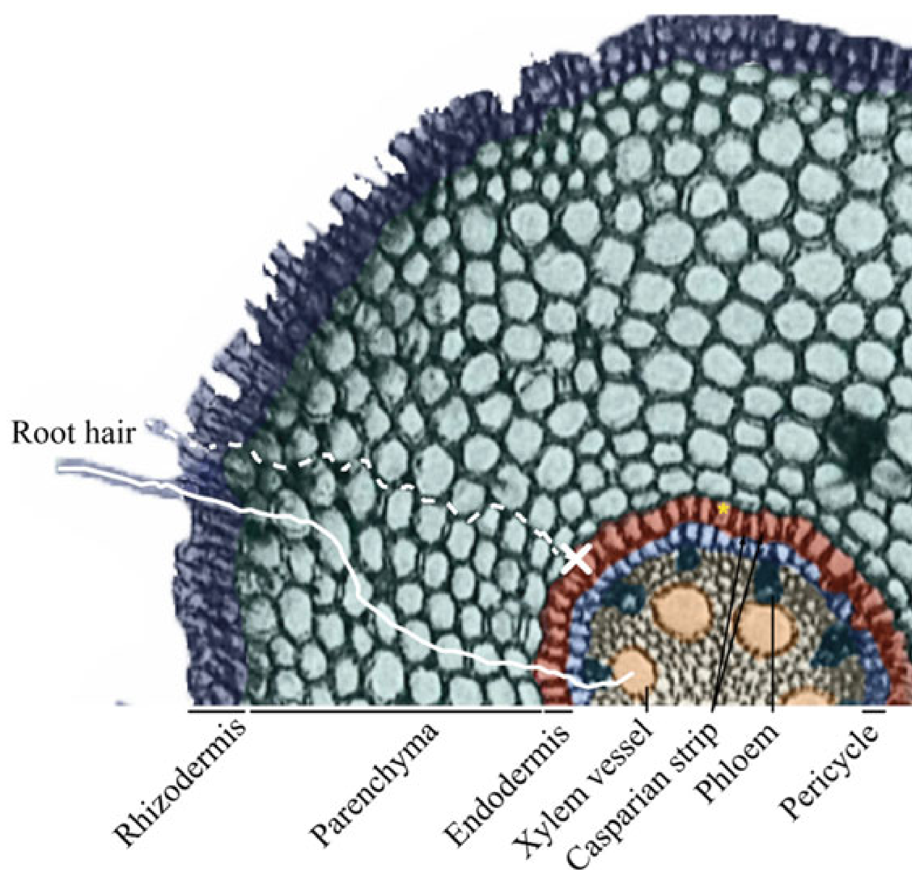

Fig. 2 Cross section of an iris (*Iris pseudacorus*) root. Diffusive uptake of chemicals can occur via the apoplast, i.e. through the cell wall continuum (dotted line). However, at the endodermis with its thickened suberized cell walls (Casparian strip; red), diffusive apoplastic transfer is stopped. This mechanism is responsible for the accumulation of various pollutants in the root. Chemicals can only penetrate into the central tissues after active passage to the symplast, i.e. the continuum of living cells (solid line). Their passage into the central cylinder with its access to vessels is facilitated by passage cells (asterisk in yellow) lacking the suberized wall deposits

[39]. Once in the symplast, these compounds can move through the xylem in the direction of the transpiration stream and accumulated mostly in transpiring organs (i.e. leaves) [8, 37]. Ionizable compounds may be subject to additional processes such as ion trapping and electrostatic interactions with cell walls [9] (see Fig. 2).

As large numbers of pharmaceuticals, as well as endogenous metabolites, are organic ions, it seems that uptake, distribution and sequestration of these compounds highly correlates with the expression of the transport system [40]. It is well known that the major facilitator superfamily (MFS) and/or ATP-binding cassette (ABC) transporters are responsible of conveying organic compounds (like sugars or amino acids) throughout the plant [41]. Members of solute carrier 22 family (SLC22), which have been initially found in animals [42], are plasma membrane transporters that belong to the MFS and strongly contribute to organic ions homeostasis. The SLC22 family encompasses organic cation transporters (OCTs), organic cation/zwitterions transporters (OCTNs) and organic anion transporters (OATs) [43]. Transporters of multidrug and toxic compound extrusion (MATE) are cation antiporters,

which are considered as one of the major transporter families in plants [44]. It has been reported that the first isolated MATE transporters from in plants (specifically in *Arabidopsis*) were involved in the detoxification of xenobiotics [45, 46]. Li and co-authors [46] succeeded to characterize the first multi-specific MATE transporter and named it AtDTX1 (for *Arabidopsis thaliana* detoxification 1). Moreover, they demonstrated that AtDTX1 serves as an efflux carrier for the antibiotic norfloxacin during functional screening with *Escherichia coli* KAM3 mutant. Furthermore, they suggested that AtDTX1 is localized in the plasma membrane and consequently will mediate the efflux of exogenous or plant-derived toxic compounds from the cytoplasm. PvOCT1 is the first protein linked to the SLC22 family and has been identified in *Phaseolus vulgaris* [47]. The expression of PvOCT1 is upregulated after exposure to the drought stress, and this presumes that it plays a role in stress adaption. In 2007, Lelandais-Briere and co-workers [48] discovered AtOCT1 (a PvOCT1 homologous) that is localized in the plasma membrane of *Arabidopsis* and can be characterized as carnitine transporter. The other five members of *A. thaliana* OCT family (AtOCT2-AtOCT6) are localized in the tonoplast, and their functions are still unknown; nevertheless, the expression of these genes was upregulated during the exposure of *Arabidopsis* plants to drought, cold and salt stress [49]. In a recent study, it was suggested that OCTs might provide an important route for delivery of the antidiabetic drug metformin (MET) [50], showing that MET transport was significantly affected in common cattail (*Typha latifolia*) roots after addition of quinidine (OCTs inhibitor in mammals).

2.3 Translocation of Pharmaceuticals Within Different Plant Parts

After organic contaminants (e.g. pharmaceuticals) entered the root, translocation might occur to the aerial part of the plant via the vascular tissue. These compounds can be transported upwards with water and other solutes by *transpiration* through vessels and tracheids in the xylem (Fig. 2). Transpiration flow, driven by root pressure and transpirational pulling, was shown to be the main driving force of the translocation of pharmaceuticals [51].

During photosynthesis and to protect plants from overheating, stomatal apparatus – specific ventilation pores – mostly present on the abaxial side of the leaf are open for gas exchange or evaporative cooling. Mesophyllic cells located above the stomata are transpiring water, leading to water deficiency and increased negative water potential. To compensate this effect, the cell takes away water from neighbouring cells, which results in a spreading suction force towards leaf vessels, to xylem tracheids and finally to roots to take up water from the surrounding environment. A high *light intensity* (higher photosynthesis rates), *warm temperature* (which increase saturation level of water vapour within leaves) and *dry air or wind* enhance transpiration rates. Transpiration rates determine the flux of water and solutes and

depend on plant species and shoot height. Environmental factors are also influencing the daily transpiration rates. As it has been mentioned before, the molecular size of pharmaceuticals can determine their diffusion rate through root cell membranes. A good example of a pharmaceutical being translocated by xylem flow is carbamazepine. The uncharged compound with intermediate hydrophobicity (log K_{OW} 3.64) is known to be frequently detected in higher concentrations in aerial parts rather than in roots [20, 52, 53]. Moreover, carbamazepine was detected through the whole plant in xylem sap and even found in transpiration waters in the ambient air [10, 54].

Pharmaceuticals could be also transported via sieve tubes of the phloem, as shown already for several herbicides [55, 56]. Compared to the unidirectional flow from roots to leaves in the xylem, compounds in phloem can be translocated in two directions: together with photosynthates (photosynthetically derived carbohydrates) from leaves to the plant below (branch, shoot, root) and above (young developing leaves, apical meristem, fruits). As generally alleged, phloem mass flow is driven by an osmotically generated pressure gradient by the accumulation (active loading) of sugars in the photosynthetically active leaves (source) and their deliverance (unloading) to the place of consumption (sink). Therefore, it is hypothesized that neutral compounds, which are mainly translocated by water flow (xylem), can be generally found in higher concentrations in mature leaves [53], in contrast to xenobiotics being transported via phloem to younger leaves, as suggested by Hsu and Kleier [57]. In this respect the abovementioned carbamazepine, which is known to be transported by xylem, was detected in higher concentrations in old leaves compared to young leaves of cucumber plants. In contrast, the anionic antibiotic tetracycline was quantified in similar concentrations in both kinds of leaves [58].

However, for non-ionic compounds, like the insecticide fipronil or some neonicotinoids, the ion trap theory does not apply, and the active ingredient can move freely between phloem and xylem according to its membrane permeability [59]. Herbicides with high ability to cross membranes may equilibrate between phloem and xylem but are preferentially transported by xylem because of the higher water flow [60]. Although only described for agrochemicals, this concept may as well influence the pharmaceutical compounds transport in plants.

The *transpiration stream concentration factor* (TSCF) is a descriptor for the quantitative uptake of contaminants. It is defined as a ratio of contaminant concentration in the xylem to the concentration in nutrient media, and this ratio varies between 0 and 1 [61]. The hydrophilic compound caffeine had a higher TSCF value than the more hydrophobic compounds triclocarban or endosulfan in zucchini (*Cucurbita pepo* ssp. *pepo*), soybean (*Glycine max* L.) and squash (*Cucurbita pepo* ssp. *ovifera*). Hence, hydrophilic pharmaceuticals, after passing the Casparian strip, seem to be translocated faster than hydrophobic ones [62]. The TSCF can give useful information about the translocation of compounds although not many studies exist measuring the pharmaceutical concentrations in xylem sap. Thus, the *translocation factor* (TF) describing the ratio between the pharmaceutical concentrations in the leaf compared to the root is often used to characterize the translocation of compounds. However, it is not taken into account if compounds are translocated by xylem or phloem.

Another difference between xylem and phloem, which influence the translocation of environmental contaminants (e.g. pharmaceuticals), is the *pH*. Phloem juice is about 8.0, which is similar to cytoplasmic pH (6.9–7.6), but inside xylem vessels, and also in the apoplast and intracellular spaces, the pH is about 5.0 [63]. Translocation of emerging contaminants is also interlinked to physical and chemical properties of the organic compounds. p*K*a values, influencing the charge of some pharmaceuticals at a specific pH is highly relevant (see previous section about root uptake). Accumulation of lamotrigine in leaves correlated with uncharged lamotrigine in pore water; thus, the pH-dependent charge of the molecule in the soil had an impact on its translocation to aerial parts of durum weed (*Triticum durum*) [64]. Such as the p*K*a, also the *lipophilicity* of compounds plays a crucial role, as moderately lipophilic neutral substances, with log K_{OW} (1–3.5) or log D_{OW} (0.5–3), are preferably translocated [65, 66]. Collins and co-workers [33] pointed out that for some uptake models, the lipid content (in their case, of the leaves) represents the most sensitive input parameter for lipophilic chemicals. It has not yet been investigated whether this is also valid for the root compartment, although several experimental studies showed missing or very low translocation of lipophilic compounds to aboveground parts [67, 68], but an exception exists. Astonishingly, zucchini is able to take up and translocate different highly hydrophobic polychlorinated dibenzodioxins and furans (PCDD/F) congeners to leaves and to the entire fruit, whereas for pumpkin and cucumber, contaminants were shown to be restricted to the outer part of the fruit [69]. It was hypothesized that zucchini might release a binding substance for PCDD/Fs with root exudates, which forms a hydrophilic complex with the pollutant to enable the uptake by the plants' roots. Furthermore, molecules in leaf extracts and in the xylem sap of zucchini and melon (*Cucumis melo* L.) were detected with the ability to increase the apparent aqueous solubility of tetrachlorodibenzodioxin (TCDD) by forming a reversible binding [70]. More recently, 17-kD proteins (probably major latex-like proteins (MLPs)) in xylem sap of zucchini were suggested to influence the translocation of hydrophobic organic contaminants, as the expression of the *MLP-GR3* gene in *C. pepo* cultivars correlated positively with the presence of the 17-kD proteins and BCFs of dioxins and dioxin-like compounds [71]. The translocation of hydrophobic pharmaceuticals to shoots was as well enhanced in zucchini plants compared to soybean and closely related squash. Additionally, higher xylem sap solubilities of these chemicals were detected in zucchini, leading to the hypothesis of an involvement of xylem sap proteins in the enhanced translocation of pharmaceuticals to aerial tissues like for other ECs [62].

Dilution by growth is another factor influencing the concentration in plant parts, which is especially important for the prediction of the foliar uptake of organic compounds [29]. The resulting increased plant biomass leads to a potential dilution of the pharmaceutical concentration relative to the flux of their uptake. In contrast, expanded plant leaf area provides a larger surface for the *foliar uptake* of emerging contaminants from ambient air [30, 33]. The uptake of organic contaminants by aerial tissues was shown for many pesticides, polycyclic aromatic hydrocarbons (PAHs) or polychlorinated contaminants [72–75]. To enter the leaf, chemicals have

to either cross the cuticle or to enter through the stomata. Therefore, cutin, cuticular waxes and other cellular lipids act as a lipophilic barrier that might absorb different substances. A correlation could furthermore be detected between the surface wax concentration and the resistance to foliar penetration [76]. Although spray irrigation with treated wastewater contaminated with pharmaceuticals serves the possibility that these molecules are deposited on plants' leaves and could therefore be taken up, we are not aware of studies about the leaf penetration of these chemical contaminants. Some hints for the possibility of pharmaceuticals uptake by leaves are given [77]. Comparing the bioaccumulation in roots and leaves of a submerged and a free-floating plant species, differences in allocation of several pharmaceuticals could be detected. Highest concentrations of these chemicals were found in the plant tissue, which was exposed to the contaminated environment. Free-floating common water hyacinth (*Eichhornia crassipes*) having their roots exposed to different pharmaceuticals in water exhibited a higher concentration in the roots rather than leaves, except for carbamazepine which is known to be translocated to the leaves very fast [20]. For the submerged plant, burhead (*Echinodorus horemanii*), where leaves are surrounded by contaminated water, the tested compounds accumulated in the leaves in a higher proportion compared to roots. Even though submerged plants show differences compared to higher terrestrial plants (e.g. no transpiration, reduced xylem, thin cuticle), this study gives useful initial information about the possible uptake of pharmaceuticals by plant leaves.

Many pharmaceuticals are susceptible to *photodegradation*, which is an advantage in the wastewater treatment process to degrade them by UV treatment [78, 79]. As leaves are exposed to intensive light intensities, photodegradation within plants is theoretically possible, although no evidence about photodegradation of pharmaceuticals in plants is available till now.

2.4 Role of Biotransformation in the Translocation of Pharmaceuticals

The *biotransformation* of pharmaceuticals plays an important role in their translocation and risk assessment. From the intensive research about herbicide resistance in weeds, herbicide detoxification in crops and the removal of organic xenobiotics by phytoremediation, it has been known that plants possess an elaborate detoxification system for organic xenobiotics and agrochemicals, comprising of a metabolic cascade proceeding in three phases [80–82] (see Fig. 3). During phase I, xenobiotics can be activated by oxidation, reduction or hydrolysis depending on their molecule structure. The activated molecules can be conjugated to reactive groups, such as amino acids, glutathione or sugars by specific enzymes like glutathione *S*-transferases or glycosyltransferases to reduce the compounds reactivity and increase their water solubility during the consecutive phase II. Conjugated metabolites can afterwards be sequestered in vacuoles during phase III (*vacuolar sequestration*) or form

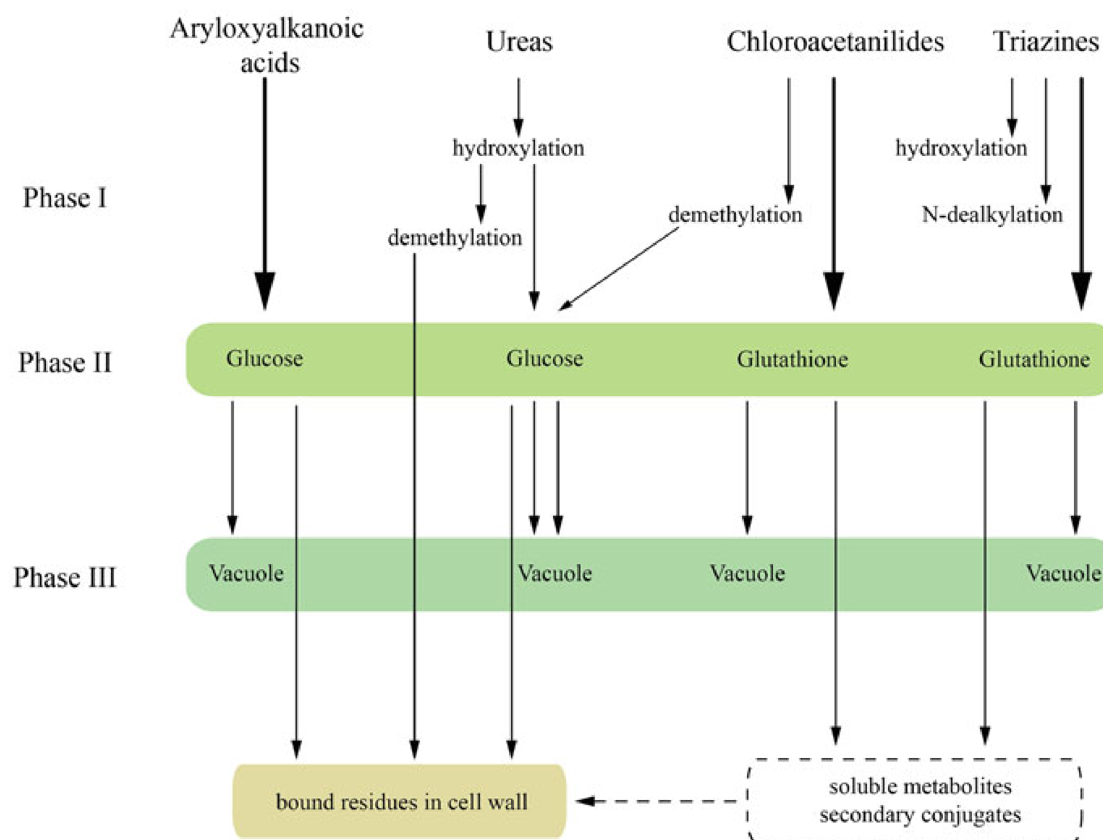

Fig. 3 The metabolic cascade of the green liver concept implies three phases for the fate of herbicides and foreign compounds in plants. It can be assumed that pharmaceuticals follow the same routes. While many compounds are finally bound to cell wall material to form insoluble residues, other xenobiotics may be stored in the vacuole as "soluble residues" and undergo further metabolism (adapted from [83])

insoluble residues in the cell wall (*bound residues*) [80] chapter "Metabolism of Pharmaceuticals in Plants and their Associated Microbiota". Several studies showed that this detoxification mechanism is also applicable for the metabolization of pharmaceuticals in plants [84–86]. The metabolization of particular pharmaceuticals can be differentially pronounced in plant tissues. Therefore, the metabolization of the anticonvulsant carbamazepine was noticeably higher in shoots than in roots, which might suggest a higher metabolism occurring in the leaves. However, one should bear in mind the fast translocation and the subsequent higher concentration of carbamazepine in shoots compared to roots [10]. Supporting this hypothesis, the phase I and phase II metabolites 4′-OH diclofenac, 4-O-glucopyranosyloxydiclofenac and 4-OH-glutathionyl-diclofenac were present in much higher concentrations in roots than shoots of cattail. These conjugates all originated from diclofenac, a pharmaceutical known to accumulate in roots rather than to be translocated to shoots [84]. In light of current literature, it is also possible that partially metabolized compounds, at least after phase I reactions, or even as conjugates can be translocated in plants via the vascular tissue [87, 88]. Nonetheless,

many recently published studies about the uptake and translocation of environmental contaminants overlook the concentration of metabolites. Neglecting pharmaceutical metabolites in environmental studies might lead to a severe underestimation of the uptake and translocation of pharmaceuticals in plants and eventually to an underestimated human exposure to these contaminants in food [89]. Therefore, it is always necessary to perform mass balance analysis because only this can provide clear-cut information to evaluate the potential metabolic routes of pharmaceuticals in distinct plant tissues.

Figure 3 displays the current knowledge about the detoxification cascade for herbicides [90]. While the traditional scheme of herbicide detoxification concluded in a phase III leading to bound cell wall residues has been well accepted for agrochemicals as the concept of the "green liver" [80], information on the fate of non-herbicidal pollutants and pharmaceuticals in plants is only poor and scattered. However, it can be assumed that pharmaceuticals undergo exactly the same metabolic steps since they possess similar molecular properties and sometimes derive from identical chemical families (e.g. triazines, sulfonylureas). Since experimental evidence indicated that xenobiotic glutathione or glucosyl conjugates may inhibit cytosolic processes [91], it has generally been accepted that xenobiotic conjugates are sequestered from the cytosol in higher plants during phase III.

2.5 Vacuolar Transport and Sequestration

Considering now the central dogma of xenobiotic metabolism in plants as valid that conjugation of xenobiotics may not be the end point of metabolism, a deeper look should be taken into plant storage processes. In fact, it seems that storage may be only intermediary for many substances and that further breakdown of these polar derivatives can lead to a complex set of processing reactions (Fig. 3), both in the vacuole and in the cytoplasm [92, 93]. One of the best studied routes of xenobiotic conjugate catabolism relates to glutathionylated pesticides [94]. An early report followed a chloroacetamide herbicide in cereals that could be tracked into the vacuole, where the respective detoxification products, glutathione conjugates, were cleaved by a carboxypeptidase to produce γ-Glu-Cys-alachlor conjugates [95].

Hence, it is not unlikely that ABC and MATE transporters in plasmalemma and tonoplast may also be involved in the detoxification of organic compounds other than herbicides, since enzymes involved in the synthesis of secondary compounds may also recognize and modify potentially toxic molecules taken up by the plant. Subsequently, molecules can yield cell wall residues or be transported into the vacuole for final detoxification (Fig. 3). Evidence for this latter sequestration step has been presented for several species and seems to be ubiquitous [96]. In a recent paper, the uptake and metabolism of the sun shield, oxybenzone, has been followed in umbrella papyrus (*Cyperus alternifolius*). Uptake and phase I and II metabolism followed the green liver concept, and it seems likely that some member of the ABCC subfamily was responsible for vacuolar delivery of the glutathionated phase II

metabolite [85, 97]. This is an important finding, since so far only plasma membrane-localized MATEs had been found to be involved in detoxification (reviewed in [98]). It is likely that further studies will reveal a role for vacuolar MATEs in cellular detoxification. Sequestration of detoxified compounds seems beneficial for the living plant cell, and the vacuole might be regarded as final storage compartment. Break down to smaller metabolites [95] or adding a malonyl residue alters the molecule so that backflush through the ABC transporters is prevented and final storage in vacuoles occurs [99, 100]. Interestingly, in umbrella papyrus the oxybenzone conjugate also undergoes partial cleavage and subsequent malonylation [85].

The significance of such phase III sequestration mechanism for the uptake of xenobiotics may be understood from the membrane potential across the tonoplast, which is −30 to −40 mV, and maintained by the activity of ATPases [101]. Since most ABC transporters are antiporters, the extrusion of cations leads to the accumulation of organic anions by a factor of 3 or 4 [96]. Such an efficient flow of xenobiotic metabolites will lead to a diminished cytosolic concentration of the active parent compound and hence be a strong driver for further diffusive uptake into the cell.

3 Experimental Section

For a bibliographic online search (using the search engine Google Scholar) of the scientific literature on plant uptake of pharmaceutical compounds, crossing 7 years of publications, authors used a combination of keywords as “plant uptake + pharmaceutical group” or “plant uptake + compound name” to obtain the highest number of articles within the topic and pharmaceutical group. Parameters like concentration applied in the study, time of exposure, type of experiment (hydroponic, pot or plate experiment), final concentration in the plant or plant part with clear units and plant species were used to decide which articles would be part of the study.

Field and lysimeter studies were not included due to their complexity and the number of external factors that can influence the results and therefore may not be compatible with the other studies. Experiments with different time points where concentrations in nutrient media/soil were not mentioned for the middle time points were also excluded, since it was not possible to calculate bioconcentration factors for these cases. Moreover, when no numerical data was provided in the studies, approximate values were extracted from figures with support of ImageJ software (version 1.52a) using the tools “set scale” and “analyse”.

Chemical properties like molar mass (g/mol), logarithmic octanol-water partition coefficient (log K_{OW}) and water solubility (mg/L) were gathered from PubChem and/or DrugBank website, while the acid dissociation constant (pKa) and the logarithmic distribution coefficient (log D_{OW}) were calculated using the software SPARC Performs Automated Reasoning in Chemistry and values used according to the pH measured in each article.

The bioconcentration factor (BCF), which is the ratio of the concentration of a chemical in an organism to the concentration of the chemical in the surrounding environment [37], was calculated as:

$$\mathrm{BCF} = \frac{\mathrm{concentration_{root}}\ (\mathrm{ng/kg})}{\mathrm{concentration_{soil}}\ (\mathrm{ng/kg})} \quad \text{or}$$

$$\mathrm{BCF} = \frac{\mathrm{concentration_{root}}\ (\mathrm{ng/kg})}{\mathrm{concentration_{nutrient\ media}}\ (\mathrm{ng/L})}$$

The concentration in soil or nutrient media was applied as the difference between the spiked concentration and the concentration found at that particular time point; like this, a more realistic BCF can be obtained since it is only considered the concentration that was available for the plant.

The translocation factor (TF) or mobilization ratio was calculated to determine relative translocation from root to shoots (stem and/or leaves) [102]:

$$\mathrm{TF} = \frac{\mathrm{concentration}_{shoot}\ (\mathrm{ng/kg})}{\mathrm{concentration_{root}}\ (\mathrm{ng/kg})}$$

Therefore, $\mathrm{TF} > 1$ means that the target compound was effectively translocated from roots to shoots. In contrast, $\mathrm{TF} < 1$ highlights an accumulation in the roots rather than a translocation to shoots.

For BCFs and TFs, plant-to-soil/nutrient media or leaves-to-root concentrations were both expressed in fresh weight (FW/FW) or dry weight (DW/DW). If not, data would be converted using the percentage of dry weight for each plant species.

3.1 Data Collected

A total of 53 ISI scientific articles and one technical report were used in this study. From all covered years, 2016 and 2018 presented the highest number of articles ($n = 11$) published on the uptake and translocation of pharmaceuticals in plants. Antibiotics ($n = 19$) and the psychotropic drugs ($n = 14$) were the pharmaceutical classes with the highest number of different compounds studied. Furthermore, antibiotics was the most frequent pharmaceutical class addressed in several articles (27.6%), followed by anticonvulsants (15.6%) and anti-inflammatory drugs (14.6%), which is showing a special interest by the scientific community in these chemicals. These numbers illustrate also, to which extent scientists are concerned about the presence and the potential effects of antibiotics, anti-inflammatory drugs, anticonvulsants and psychotropic drugs in the environment and in a second baseline, the publics' concern.

Regarding antibiotics, the main concern is the propagation of multiresistant bacteria and as a consequence, the dispersal of genes related to resistance against

those agents. This issue creates two main lines of scientific work: phytoremediation and human health risk assessment. From the collected articles, only 12.5% of the studies focused on phytoremediation [103–105], which shows a trend towards a focus on edible plants for further human risk assessments. In that respect, the most studied plant of the analysed studies was lettuce (*Lactuca sativa*) (29.65%), followed by radish (12.96%) and cucumber (7.41%), which are all economically relevant crops.

The duration of exposure in the collected studies varied between 6 h and 98 days; some showed only single time point measurements ($n = 32$) and others a time course with multiple time points ($n = 22$). Considering only single time points studies, in 71.9% of the cases, they tested a duration of at least 21 days. As it was mention before, only studies with multiple collection time points with given concentrations in nutrient media or soil at tested time points were used, to avoid overestimations of BCFs. It is also necessary to be aware of studies where nutrient solutions or soils were replenished/irrigated with solutions containing pharmaceuticals during the time course of the experiment when no information about volume, concentration and frequency of the added solution were mentioned to calculate the correct BCF. The tested concentrations of pharmaceuticals varied between 100 ng/L and 200 mg/L. In some studies, a single concentration was used, while in others, like Adeel and co-workers [106], several concentrations were studied ranging from 100 ng/L to 10 mg/L.

Taking into account all conditions and limitations presented above, data from selected publications was grouped and expressed as BCF and TF, according to the chemical properties and the ionic status of the compounds and additionally separated into trials done as hydroponic (a) and soil (b) experiments (Tables 1, 2, 3, 4, 5, and 6). Information is presented like this, because most of the concepts in the first part of this chapter can only be directly related to experimental data with controlled and/or few external interferences, as the hydroponic experiments. With the soil experiments, factors like the percentage of OM and even the soil constituents will interfere in the analysis, especially when comparing different studies, but on the other hand, the results will be closer to a realistic scenario.

The boxplots (designed using GraphPad Prism software, v 6.01) in Figs. 4, 5 and 6, which are showing the BCFs and TFs of the distribution of observations from different studies as well as minimum, median and maximum values, were also separated according to the ionic status of the compounds and the type of study (hydroponic and soil experiments), as mentioned above. One study can include several observations (shown by dots) by testing various conditions like duration, concentration or pH. Therefore, boxplots (Figs. 4, 5 and 6) provide a detailed picture of summarized data in Tables 1, 2, 3, 4, 5 and 6, and exceptions can be detected easily and considered for discussion to secure the validity of BCF and TF average values.

For the uptake and translocation of organic compounds, the molar mass with high possibility only plays a role for big molecules with molar mass $\geq$1,000 g/mol [21] or as hypothesized for pharmaceuticals with molar mass $\geq$400 g/mol [20]. None of the studied pharmaceuticals was $\geq$1,000 g/mol, and only eight of them can be

Table 1 Chemical properties of neutral pharmaceuticals, as well as the average BCFs and TFs calculated per compound in hydroponic studies

Compounds	log K_{OW}	pH	p*K*a	log D_{OW}	BCF	TF	Authors
		Average values					
Analgesic							
Acetaminophen	0.46	5.60	0.00	0.09	1.43	0.49	[20, 58, 107–111]
Antibacterial							
Triclocarban	4.34	na	0.00	5.23	31.39	0.01	[109, 110]
Antibiotic							
Sulfamethoxazole	0.89	5.59	0.00	−0.06	0.55	0.13	[11, 109, 110, 112–114]
Sulfapyridine	0.35	6.53	0.00	4.21	3.29	0.03	[105]
Anticonvulsant							
Carbamazepine	2.45	6.23	0.00	3.64	0.93	2.05	[10, 11, 20, 110, 114–119]
Primidone	0.91	na	0.00	−1.23	1.61	0.17	[109]
Hormone							
17β-estradiol	0.20	na	0.00	4.33	2.01	1.11	[106]
17α-ethinylestradiol	3.67	5.30	0.00	4.94	0.98	1.04	[106, 112, 114]
Beta-estradiol	3.67	5.55	0.00	4.33	0.01	nd in leaf	[20, 114]
Estrone	3.13	5.55	0.00	4.23	0.13	0.07	[20, 114]
Levonorgestrel	3.48	na	0.00	4.27	17.26	nd in leaf	[101]
Lipid regulator							
Atorvastatin	6.36	na	0.00	2.38	0.48	0.26	[109]
Psychotropic drug							
Meprobamate	0.70	na	0.00	1.16	0.37	6.11	[109, 110]
Stimulant							
Caffeine	−0.07	5.68	0.00	0.95	0.32	12.06	[20, 109, 114, 116, 119, 120]

Symbols: na, means not available; *nd*, means not detected

considered as large-sized pharmaceuticals, as mentioned by Chuang and co-workers [20]. Of these pharmaceuticals, five were antibiotics (clarithromycin, streptomycin, oxytetracycline, tetracycline and lincomycin), two drugs against high blood pressure (verapamil and valsartan) and one lipid regulator (atorvastatin). All selected compounds can enter the roots, and only a minor amount of the tested pharmaceuticals could have difficulties to enter because of their high molar mass.

Table 2 Chemical properties of neutral pharmaceuticals, as well as the average BCFs and TFs calculated per compound in soil studies

Compounds	log K_{OW}	pH	pKa	log D_{OW}	BCF	TF	Authors
		Average values					
Analgesic							
Acetaminophen	0.46	8.10	0.00	0.09	0.01	0.34	[102]
Antibacterial							
Triclocarban	4.34	6.42	0.00	5.23	0.02	0.50	[121]
Antibiotic							
Sulfamethoxazole	0.89	5.67	0.00	−0.06	0.96	0.58	[11, 112, 122, 123]
Anticonvulsant							
Carbamazepine	2.45	7.03	0.00	3.64	0.62	3.57	[11, 102, 112, 123–128]
Hormone							
17α-ethinylestradiol	3.67	6.60	0.00	4.94	0.61	0.06	[129]
Estrone	3.13	8.10	0.00	4.23	0.00	2.45	[20]
Psychotropic drug							
Oxazepam	2.24	6.30	0.00	3.42	0.04	17.15	[130]
Temazepam	2.19	6.30	0.00	4.71	0.01	5.99	[130]
Stimulant							
Caffeine	−0.07	7.87	0.00	0.95	0.23	20.03	[102, 126, 127]

Symbols: na, means not available; *nd*, means not detected

3.2 *Data Analysis*

3.2.1 Neutral Compounds

Neutral organic compounds were identified as having higher membrane penetration than ionized substances [140]. Therefore, it is expected, that these molecules can be taken up and translocated easily by transpiration via the xylem [51], resulting in TFs > BCFs. For compounds like meprobamate, caffeine and carbamazepine and, additionally, estrone, oxazepam and temazepam (in soil assays), this pattern was observed (Tables 1 and 2). Figure 4 shows that many observations and studies were made on the uptake and translocation of caffeine and carbamazepine, reflecting a TF > BCF, which clearly underlines their validity. However, this pattern is not clearly detected for the whole group of compounds.

Looking at the data in detail, triclocarban (antibacterial) stands out with an average BCF of 31.4, as a result from data reported in Sun and co-authors [109] and Wu and co-authors [110] (Table 1). In total, these two studies had nine observations, and none of them had TF higher than 0.08 (Fig. 4). BCFs (18.9–32.4) obtained by Wu and co-workers [110] for spinach and lettuce, when exposed for 21 days at two different concentrations (5 and 0.5 μg/L), were similar, and also Sun and co-authors [109] observed a relatively high BCF (12.6) when cucumber was exposed for 7 days to a concentration of 5.0 μg/L. Therefore, the

Table 3 Chemical properties of anionic pharmaceuticals, as well as the average BCFs and TFs calculated per compound in hydroponic studies

Compounds	log K_{OW}	pH	pKa	log D_{OW}	BCF	TF	Authors
		Average values					
Antibacterial							
Triclosan	4.76	5.47	−0.01	5.42	1.50	0.20	[9, 19, 41, 55, 117]
Antibiotic							
Ofloxacin	−0.39	7.80	−0.16	0.74	0.01	nm in leaves	[131]
Oxytetracycline	−0.90	5.97	−0.15	−6.49	0.59	0.02	[25, 55]
Sulfadiazine	−0.09	6.81	−0.88	1.04	14.87	0.15	[46, 87]
Sulfamerazine	0.14	6.81	−0.58	3.54	25.98	0.03	[105]
Sulfamethazine	0.89	6.85	−0.23	4.39	9.13	0.02	[105, 116]
Sulfamethoxazole	0.89	6.85	−0.002	−0.06	16.61	0.02	[105]
Sulfapyridine	0.35	6.91	−0.01	4.21	11.78	0.03	[105]
Tetracycline	−1.30	na	−0.08	−5.44	0.15	3.18	[103]
Anticonvulsant							
Dilantin	2.47	na	−0.003	1.71	0.95	2.98	[41, 110]
Anti-inflammatory							
Diclofenac	4.51	6.61	−0.98	1.85	2.82	0.24	[43, 55, 56, 65, 101, 132]
Ibuprofen	3.97	5.48	−0.90	2.25	0.21	1.52	[109, 110, 116, 133–135]
Naproxen	3.18	5.65	−0.89	2.09	0.61	0.90	[109, 110, 114, 119]
Lipid regulator							
Clofibric acid	3.32	6.00	−0.99	1.20	1.37	1.59	[119]
Gemfibrozil	4.77	na	−1.00	2.92	4.03	0.04	[109]

Symbols: *na*, means not available; *nm,* means not measured

dynamic between BCF and TF reported in the two studies was the opposite of what was expected (i.e. BCF > TF) but might be explained due to the high lipophilicity of triclocarban (log K_{OW} = 4.34). Indeed, several models purposed different ranges during which translocation is favoured or not. All of these models predicted a low transfer for compounds with around log K_{OW} > 4 [25, 61, 140, 141].

Another neutral compound that stands out from the proposed observation was levonorgestrel (hormone). Li and co-workers [101] described a BCF average of 17.3, where no compound was detected in stems and leaves. Therefore, further investigation is needed to scrutinize these results.

Sulfamethoxazole was another pharmaceutical, which was studied intensively in hydroponic and soil experiments. This antibiotic and the analgesic acetaminophen, which is also studied on several hydroponic experiments, showed a slightly higher average BCF > TF. Moreover, as many observations were showing similar results for these two pharmaceuticals, the validity is high. The reason for this might be their

Table 4 Chemical properties of anionic pharmaceuticals, as well as the average BCFs and TFs calculated per compound in soil studies

Compounds	log K_{OW}	pH	pKa	log D_{OW}	BCF	TF	Authors
		Average values					
Antibacterial							
Triclosan	4.76	7.05	−0.11	5.33	1.09	1.06	[103, 116, 121, 124, 125, 129]
Antibiotic							
Amoxicillin	0.87	7.01	−0.40	−2.05	0.00	na	[132]
Oxytetracycline	−0.90	7.50	−0.91	−6.64	0.00	0.58	[102]
Sulfadiazine	−0.09	7.50	−0.98	1.02	0.65	1.72	[102, 136]
Sulfamethoxazole	0.89	7.68	−0.03	−0.06	0.27	0.77	[11, 102, 123]
Tetracycline	−1.30	7.28	−0.66	−5.59	0.00	na	[132]
Trimethoprim	0.91	8.10	−0.44	0.67	0.00	5.38	[102]
Anti-inflammatory							
Diclofenac	4.51	6.25	−0.98	2.13	2.02	2.43	[125, 126]
Ibuprofen	3.97	7.42	−0.97	2.08	2.51	1.38	[126, 127]
Naproxen	3.18	na	−0.95	1.86	0.24	0.51	[126]
Blood pressure							
Furosemide	2.03	7.42	−1.00	0.73	1.27	nd in leaves	[127]
Lipid regulator							
Clofibric acid	3.32	7.42	−1.00	1.20	1.11	0.04	[127]
Psychotropic drug							
Diazepam	2.82	6.30	−0.99	4.73	0.03	3.13	[130]

Symbols: *na*, means not available; *nd*, means not detected

metabolization by plants [105, 118]. As mentioned before, the fast biotransformation of some pharmaceuticals should not be neglected to not underestimate BCFs and TFs of the parent compound.

3.2.2 Anionic Compounds

Among the anionic compounds, antibiotics are represented by the largest group of studied substances in both hydroponic and soil experiments. Within antibiotics, the sulfonamides (SAs), i.e. sulfadiazine, sulfamerazine, sulfamethazine, sulfamethoxazole and sulfapyridine, display the largest group. They are widely used for the control of infectious diseases, in both human and livestock care, and due to their stability – with a half-life over 81 days [105] – they are ubiquitously present in wastewaters. Therefore, SAs receive a special attention by the researchers since they are prone to increase the resistance of pathogenic bacteria and boost the spread of antibiotic resistance, hostile to aquatic environments and human health. According to Wang and co-authors [142], the uptake process of these molecules might be

Table 5 Chemical properties of cationic pharmaceuticals, as well as the average BCFs and TFs calculated per compound in hydroponic studies

Compounds	log K_{OW}	pH	p*K*a	log D_{OW}	BCF	TF	Authors
		Average values					
Antibiotic							
Clarithromycin	3.16	na	1.00	−1.98	9.91	0.04	[137]
Lincomycin	0.20	5.80	1.00	−4.84	0.33	0.08	[20]
Trimethoprim	0.91	5.80	0.88	0.09	2.45	0.23	[20, 97, 110, 112]
Anticonvulsant							
Lamotrigine	2.57	6.05	0.65	2.49	7.86	0.12	[138]
Antidiabetic							
Metformin	−2.64	6.00	1.01	−2.56	32.14	0.02	[65]
Beta-blocker							
Atenolol	0.16	7.80	0.98	−2.23	0.21	2.79	[11, 109, 131]
Propranolol	3.48	na	1.00	0.15	0.92	0.22	[116]
Lipid regulator							
Gemfibrozil	4.77	5.30	0.81	3.56	–	0.06	[114]
Psychotropic drug							
Amitriptyline	4.92	7.00	1.00	3.60	29.85	1.11	[117]
Clomipramine	5.19	na	1.00	2.35	0.18	0.62	[139]
Diazepam	2.82	na	0.01	4.73	3.21	0.45	[109, 110]
Fluoxetine	4.05	7.00	1.00	1.05	13.96	1.03	[110, 117]
Sertraline	1.37	na	1.00	2.27	0.43	0.12	[139]
Trazodone	3.21	na	0.10	3.97	0.09	2.89	[139]

Symbols: na, means not available; – not possible to calculate

slower, when compared to cationic and neutral compounds due to electrostatic repulsion between root surface and anionic substances. However, looking at data from the hydroponic experiment of Tai and co-workers [105] (Table 3, Fig. 5a), high BCF ratios of SAs, ranging from 9.1 to 26.0, were quantified in two wetland plant species (Indian shot (*Canna indica*) and yellow iris (*Iris pseudacorus*)) in a 7-day trial. In this work, authors suggested that plants take up SAs via active processes. However, the high BCF values might be related to the plant lipid content, since it is considered as the main storage site for hydrophobic organic contaminants, as hypothesized by the same group. To support this hypothesis, a positive correlation between the obtained BCF and the respective log D_{OW}, for several nutrient media and soil articles (cited in Tables 3 and 4), was calculated (0.29 and 0.42, accordingly ($p > 0.05$)). Nonetheless, for a specific antibiotic (tetracycline), the results were the opposite (i.e. TF > BCF), meaning that this compound is rather translocated to the aerial parts than being stored in roots [104], which can be explained by its hydrophilic behaviour (log D_{OW} −5.44).

As observed for SAs, high average BCF > TF values for triclosan, diclofenac and gemfibrozil were registered in hydroponic experiments (see Table 3). Several studies focused on the antibacterial pharmaceutical triclosan, but only in some of them, high average BCFs were obtained. It can be highlighted that highest BCFs were

Table 6 Chemical properties of cationic pharmaceuticals, as well as the average BCFs and TFs calculated per compound in soil studies

Compounds	log K_{OW}	pH	p*K*a	log D_{OW}	BCF	*TF*	Authors
		Average values					
Antibiotic							
Lincomycin	0.20	7.58	0.75	−3.35	0.00	9.96	[102]
Anticonvulsant							
Lamotrigine	2.57	8.10	0.18	2.70	0.03	1.93	[102]
Antidiabetic							
Metformin	−2.64	na	1.01	−2.56	0.34	0.61	[126]
Beta-blocker							
Atenolol	0.16	6.96	0.99	−2.60	0.39	3.51	[124]
Propranolol	3.48	6.63	0.99	0.59	2.59	1.97	[125]
Psychotropic drug							
Chlordiazepoxide	2.44	6.30	0.43	−0.12	0.04	6.58	[130]
Clonazepam	2.41	6.30	0.01	3.56	0.01	16.82	[130]
Fluoxetine	4.05	6.25	1.00	1.07	0.04	0.24	[125]
Flurazepam	3.80	6.30	1.00	3.77	0.01	1.24	[130]

Symbols: *na*, means not available

calculated for several plant species (cucumber, lettuce, spinach (in hydroponic experiments) and for ryegrass and lettuce (in soil)), with a time exposure ranging from 7 to 40 days [109, 110, 125, 127]. For all the selected cases, the applied concentrations were relatively low (2.7–69.0 μg/L), when compared to the rest of the studies (5.0–758.0 μg/L), which might indicate a more efficient uptake for lower applied concentrations. For the well-studied anti-inflammatory drug diclofenac, ten times higher average BCF > TF values were detected in hydroponic experiments (Table 3); nonetheless, four of thirteen studies had higher BCFs (3.2–17.7) than the rest of the studies (BCF ≈ 0.5; Fig. 5a) [101, 111, 112, 126]. Several works therefore reported that this pattern is caused by the hydrophobicity of diclofenac [115, 119], but as for charged molecules, the log D_{OW} rather than the log K_{OW} should be considered. Since this compound has a log D_{OW} of 1.85 translocation should be favoured, however it is not the case. As mentioned in the first part of the chapter, the protein plant composition might play an important role on storage of anionic compounds in roots, as discussed by González García and co-authors [36].

The same pattern (BCF > TF) was also obtained for gemfibrozil (lipid regulator) in a 2-week study with old cucumber plants [109] (Table 3); this result might be related to the high metabolism of young plants, since for different type of compounds (neutral, anionic and cationic) BCF > TF were registered in this study. In any case, further investigation is needed to evaluate the uptake results according to rigorous pH measurements, since this molecule dramatically changes its ionization status (p*K*a 0.8 to −0.99) in a very short pH interval (5.3–6).

In contrast to the behaving of most of the anionic compounds, dilantin (anticonvulsant) presented a higher average TF (2.9) when compared to its BCF (0.9)

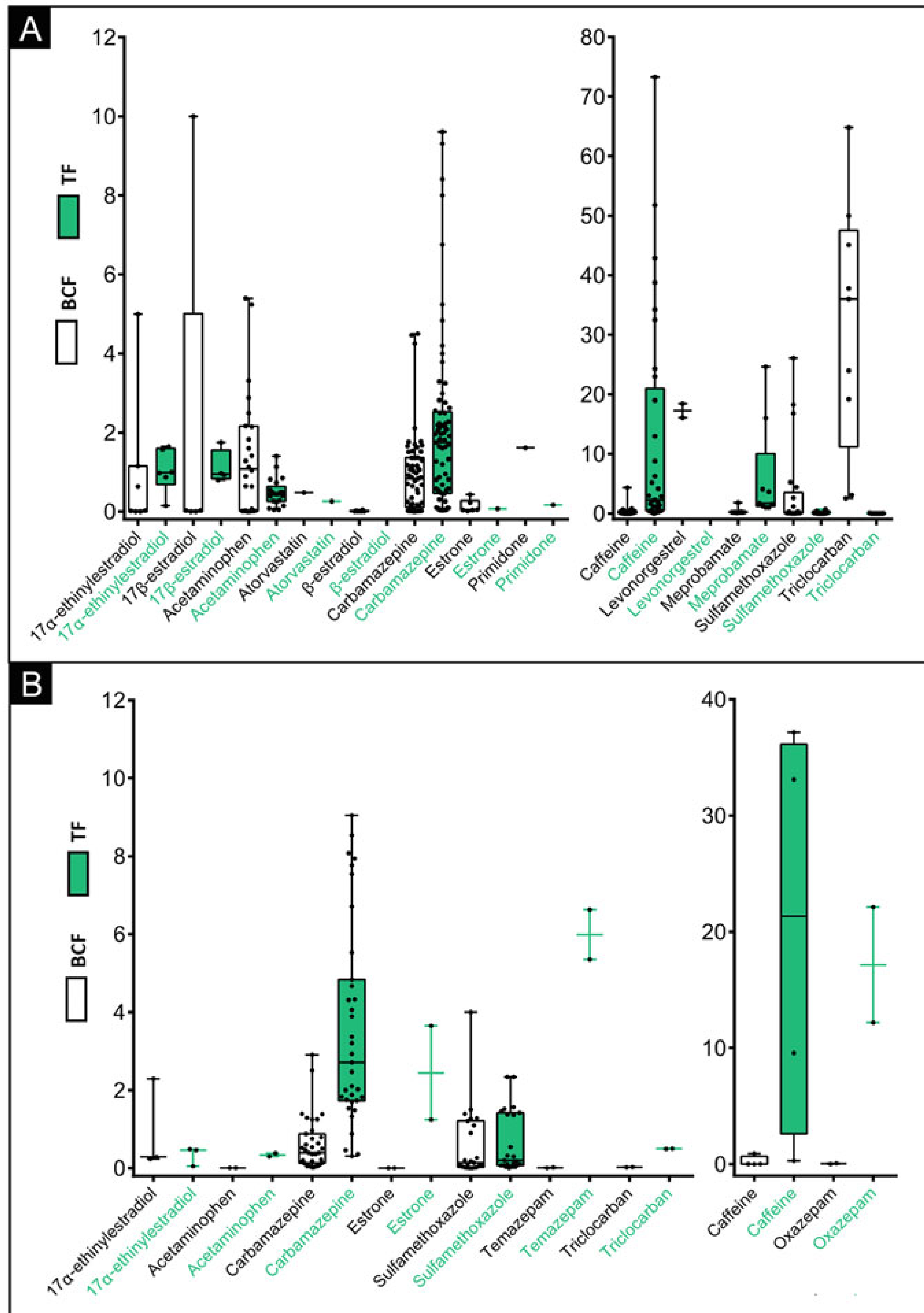

Fig. 4 Boxplot visualization of all BCF (black) and TF (green) values of several neutral compounds (every dot, represents an observation) from hydroponic (**a**) and soil studies (**b**); data references in Tables 1 and 2

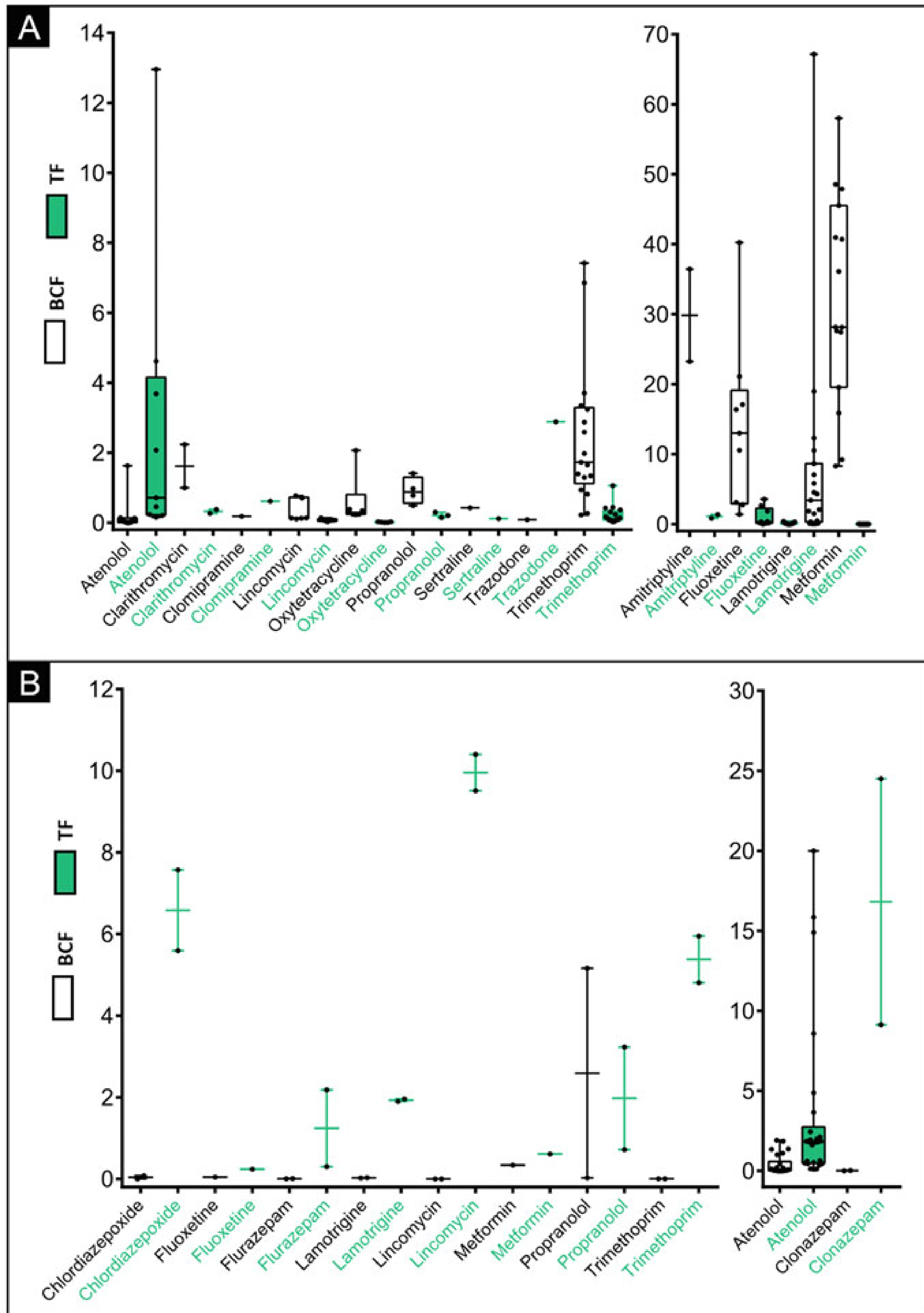

Fig. 5 Boxplot visualization of all BCF (black) and TF (green) values of several anionic compounds (every dot, represents an observation) from hydroponic (**a**) and soil studies (**b**); data references in Tables 3 and 4

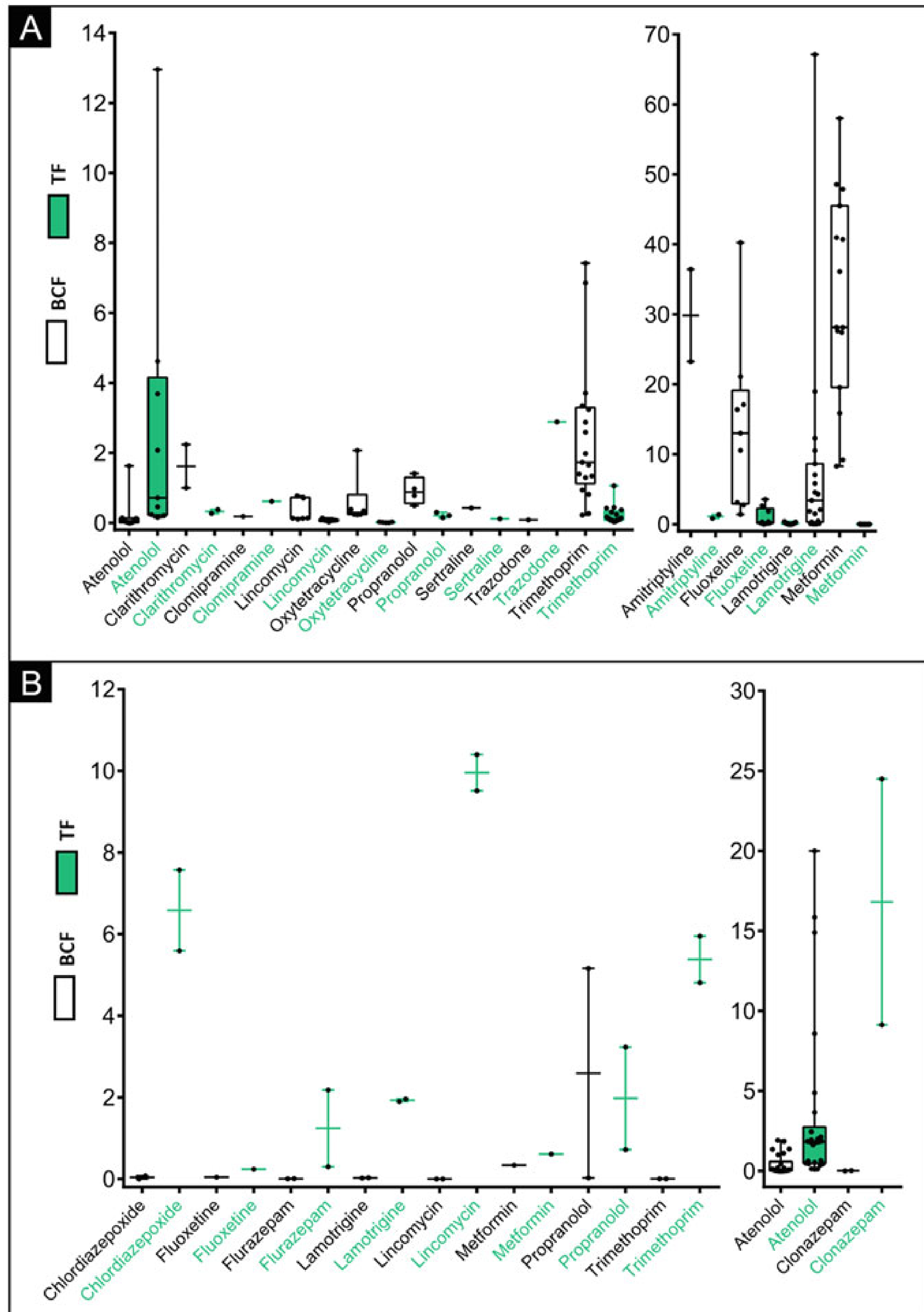

Fig. 6 Boxplot visualization of all BCF (black) and TF (green) values of several cationic compounds (every dot, represents an observation) from hydroponic (**a**) and soil studies (**b**); data references in Tables 5 and 6

(Table 3). These results are mainly represented by Wu and co-workers [110], where the highest translocations were observed for pepper plants (*Capsicum annuum*) even when exposed to different concentrations (0.5 and 5 μg/L), which might indicate a favoured translocation because of the plant species. Moreover, dilantin displays only a slightly negative p*K*a (−0.003), which could mean that its behaviour is more similar to a neutral compound, like carbamazepine, than to an anionic one.

The uptake of the psychotropic drug diazepam was studied in a radish experiment in soil [130]. As for all studied compounds on this crop, TF values were higher than the ones for BCF (Table 4, Fig. 5b), however according to its log D_{OW} (4.73), it would be expected the opposite, which might indicate the important role of this specific plant species [11, 102, 112, 121, 124]. This hypothesis is also supported by the higher BCF > TF values of diazepam in different other plants (cucumber, lettuce, pepper, spinach), which was tested in hydroponic experiments [109, 110].

Lastly, average TF values of ibuprofen (anti-inflammatory) were higher than average BCF values in hydroponic studies [109, 110, 126, 127, 135]. However, these differences are mainly caused by the presence of an outlier in TF observations (see Fig. 5a).

3.2.3 Cationic Compounds

In hydroponic studies with cationic compounds, generally higher BCFs>TFs were obtained (Tables 5 and 6, Fig. 6). The main reason behind this observation might be the fact that plant cell walls are negatively charged, due to their high concentration in uronic acids [142]. The electrostatic attraction between the root cell wall and the cationic compounds may facilitate adsorption to the root epidermis. Compounds that are positively charged at pH 4–6 can be trapped in the apoplast or root vacuoles (pH 5) [63]. Consequently, a reduced concentration can enter the vascular system for the translocation to aerial parts.

Among these cases, atenolol (beta-blocker) and trazodone (psychotropic drug) presented TFs > BCFs. For both compounds, this might be related to the high concentrations applied (830–1,000 and 10,000 μg/L, respectively) and to the plant species used [11, 139]. Kedosová and colleagues [11] registered higher atenolol concentrations in leaves of radish and spinach than in arugula and lamb's lettuce. Additionally, in the study of Reichl et al. [139], high amounts of trazodone in cress aerial tissues (*Lepidium sativum*) were registered, showing that uptake efficiency is dependent of the plant species used, and therefore, for studies of human health risk assessment, different plant species should be tested to estimate more reliable risks.

For soil data, when compared to BCFs values, higher TFs were calculated (Table 6). According to Miller and co-workers [9], some evidences were already demonstrated, that cationic compounds applied to soil have higher TF values than, for example, anionic ones. However, in our studies no correlation was found between TFs and the respective log D_{OW}, suggesting that other factors might be more relevant for the translocation of cationic compounds.

4 Recommendations and Outcomes from Data Analysis

When compiling data to do this analysis, it became obvious that some articles had to be omitted, because there was a lack of important information needed to compare data between studies. Basic guidelines for controlled uptake and translocation studies, including relevant properties of the compound, the plant and the environment, are crucial to produce valid results. Indeed, comparability and reliability of scientific data have become burning topics recently and therefore were discussed by many publishing and governmental agencies, which are concerned about data integrity and how data can be made "available" for all stakeholders. Accordingly, a resume of recommendations for future studies might be:

A crucial parameter is the *concentration applied* in water or soil at the beginning of each study as theoretical and analytical value. In several articles where both concentrations were provided, theoretical and practical concentrations varied significantly for specific compounds. In any case, similar *concentration units* (expressed in fresh weight or dry weight) should be provided, to better relate data expressed in the same units.

In case *additional irrigation or replenishment* of nutrient media is needed, during the time course of the experiment, authors should mention the volume of water added, frequency of occurrence and if irrigation water was previously spiked with pharmaceuticals. Additionally, the quantification of the spiked irrigation water is a crucial information to calculate the exact concentration to which the plant was exposed. This is very important when estimating the BCF, since the concentration in nutrient media/soil is always considered as a base. In many cases, authors only relate its value to the concentration at T0, which finally leads to an overestimation of BCFs. Also, if the nutrient media is completely renewed, the concentration before and after removal should be measured and mentioned. For *kinetic studies*, it is moreover important to quantify the concentration in the nutrient media/soil at each sampling time, in order to relate it to the concentration in the plant at that specific time point and avoid wrong BCF assumptions.

In all the cases, *pH* measurements – in nutrient media or in pore water and soil – are recommended at least for each time point of collection. Some *chemical properties* (i.e. p*K*a and log *D*ow) of selected compounds are dependent on the measured pH values; this is central when compounds change their ionic status easily in a very narrow pH range.

Moreover, authors should always consider using different *controls*, i.e. the inclusion of negative controls (where no plant is included in the spiked nutrient media/soil, which is used to evaluate the adsorption and potential degradation along the study) and the plant in a non-spiked situation (to evaluate the plant growth performance in normal conditions).

For soil studies, measuring soil properties besides pH, like *percentage of humidity* and *organic carbon content* plus the *soil porosity* and *texture*, is recommended to enable the comparison of studies and diminish the bias.

Another parameter influencing the uptake and translocation of pharmaceuticals is the *plant* per se. It is recommended to consider the plants' age (number of days after germination) and developmental stage (e.g. two-leaf stage, vegetative growth or flowering/fruiting) at the time point of exposure and during the study. The plant variety, the percentage of dry weight (root and aerial part) as well as the total lipid content should be provided, since this information is necessary to successfully indicate the differences on the uptake and translocation of especially lipophilic pharmaceuticals in different plant organs or varieties.

Analytically, the *extraction protocol* for target compounds in the different studied matrices should be provided along with the specific *limits of detection* and *quantification*. This is essential when authors cannot quantify a specific compound, so the readers can understand if this is due to an analytical limitation or if the compound is not present in that matrix. Furthermore, concentrations of pharmaceuticals in plant tissues can be easily underestimated when only parent compounds are quantified. As some pharmaceuticals can undergo a rapid metabolization within a few hours, it is recommended to consider the measurement of the main *metabolites*, if technically possible, to prove the uptake and translocation of such compounds.

4.1 Concluding Remarks

In many studies it became obvious that the concentration in nutrient media/soil does not correlate with the concentration in plants, and thus it is not easy to forecast transfer rates. Chemistry and plant physiology play important roles in the processes involved. Moreover, interactions with soil constituents, rhizosphere processes governed by microbes and the selective uptake mechanisms of several plant species may be decisive for the fate of PPCP as well. The concentration of pharmaceuticals applied in controlled experiments may affect in opposite way the BCF and TF ratio values, since in some studies higher uptake and translocation ratios were achieved with lower concentrations, which is highlighting the relevance of realistic environmental concentrations in uptake studies. Some plant species may also have special features, such as Cucurbitaceae, which is known to be the only family to take up and translocate hydrophobic PAHs. Interestingly, radish from the Brassicaceae family stands out with consistent higher translocations, for all pharmaceutical compounds in the analysed studies. Furthermore, it may hold true that most cationic pharmaceuticals show higher TFs in soil studies, but some will also undergo activation and metabolization on the way, which might change their behaviour and fate. As highlighted before, it is crucial to take all relevant plant and physicochemical properties into consideration through every step of the scientific process that starts with the experimental design and ends with data analysis and interpretation.

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