HelmholtzZentrum münchen
Deutsches Forschungszentrum für Gesundheit und Umwelt

Abschlussbericht zum Vorhaben

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Vorhabenbezeichnung: „Verbleib von Pestiziden und in Effluenten enthaltenen Schadstoffen in landwirtschaftlichen Systemen vor dem Hintergrund der möglichen Nutzung von Brauchwässern (AWARE)“ **-** Teilprojekt Helmholtz Zentrum München – Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH)	
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1. **Beitrag des Ergebnisses zu den förderpolitischen Zielen des BMEL**

Bis 2050 wird die Weltbevölkerung bei 9 Mrd. Menschen liegen. Damit steigt die Nachfrage nach Lebensmitteln und nachwachsenden Rohstoffen für die energetische und stoffliche Nutzung. Die Entwicklung und Aufrechterhaltung einer leistungsfähigen, effizienten und nachhaltigen Landwirtschaft im ländlichen Raum ist essenziell. Dazu müssen gesundheitlich unbedenkliche und nährstoffreiche Nahrungsmittel gesichert sein. Vor allem wird die zunehmende Verknappung von Boden, Wasser und Nährstoffen infolge des Klimawandels und der Verlust an Bodenfruchtbarkeit die Sicherung der Lebensqualität im ländlichen Raum beeinträchtigen. AWARE steht mit seinem Ziel, die schwindenden Wasserreserven zu bewahren und eine Kontamination von Nahrungsmitteln mit Pestiziden und Pharmazeutika zu verhindern, im Zentrum dieser Bemühungen und trägt wesentlich zur Umsetzung der forschungspolitischen Ziele des BLE bei.

AWARE hatte zum Ziel, Erkenntnisse zu gewinnen, Umsetzungsoptionen zu entwickeln und Bewertungsstandards zu erarbeiten, um die Produktion und Nutzung biogener Ressourcen nachhaltig, umweltgerecht und dem Vorsorgeprinzip entsprechend sicher auszugestalten.

2. Darstellung und Erläuterung zu:

a. **dem wissenschaftlichen und technischen Stand an den angeknüpft wurde**,

Behandelte Abwässer werden bereits heute für die Bewässerung landwirtschaftlich genutzter Flächen in den südlichen Ländern Europas verwendet. Dies birgt den großen Vorteil, dass solche Wässer nicht mit dem Trinkwasserbedarf um Frischwasserreserven konkurrieren. Durch die Nutzung der behandelten Abwässer besteht jedoch auch das Risiko des Eintrags von Arzneimitteln und Pflegeprodukten (PPCPs) in die landwirtschaftlich genutzten Böden und somit auch in Nutzpflanzen (Calderón-Preciado et al. 2013; Miller et al. 2016). Die von den Pflanzen aufgenommenen Substanzen können einerseits akkumulieren und nach der Ernte in der Nahrungskette durch den Menschen aufgenommen werden, andererseits können die sog. „*Emerging Contaminants*“ oder deren Stoffwechselmetabolite zu Veränderungen in der Pflanze und in den mit Pflanzen assoziierten Mikroorganismen führen. Solche molekularen, biochemischen, physiologischen und morphologischen Veränderungen als Reaktion auf abiotischen Stress können Wettbewerbsvorteile für die Pflanzen haben und somit überlebenswichtig sein (Pareek et al. 2009). Allerdings können diese Modulationen andererseits auch zu erhöhten Fitnesskosten führen oder Auswirkungen auf Qualitätsmerkmale des Ernteguts haben, wie dies bereits für Tomatenpflanzen gezeigt wurde (Christou et al. 2019).

b. **den wissenschaftlich-technischen Ergebnissen des Vorhabens im Vergleich zu ursprünglichen Zielen, erreichten Nebenergebnissen und wesentlichen Erfahrungen**

Das HMGU-Teilprojekt von AWARE zielte darauf ab, den Einfluss umweltrelevanter Konzentrationen von zwei hochgradig persistenten Pharmazeutika (Diclofenac und Lamotrigin) auf die Physiologie und Biochemie essbarer Pflanzen qualitativ und quantitativ zu erfassen. In

diesem Zusammenhang (1) wurden in unseren Laboratorien die Konzentrationen der beiden Pharmazeutika und ihrer Stoffwechselmetaboliten in Salatwurzeln und -blättern quantifiziert, um Aussagen über deren Aufnahme und Translokation in die Pflanzen machen zu können. Diese Ergebnisse setzten wir in Zusammenhang (2) mit der Analyse des oxidativen Stressniveaus in der Pflanze und (3) mit der Expression von Genen, die an der abiotischen Stressreaktion und der Metabolisierung von Xenobiotika beteiligt sind. Die in Kooperation mit den europäischen Partnern über drei Jahre gewonnen Daten sollen entscheidend zur Risikobewertung der Verwendung von Brauchwässern in der Landwirtschaft beitragen und so einen Einfluss auf die zukünftige europäische Gesetzgebung haben.
Mittelpunkt des deutschen Projektanteils war das integrale Experiment zum Verständnis der in Salatpflanzen durch die Arzneimittel Lamotrigin und Diclofenac ausgelösten Stressantworten.
Der Wirkstoff Diclofenac war bereits in der Vergangenheit von unserer Arbeitsgruppe auf seine Metaboliten in Pflanzen sowie dessen Einfluss in hohen Konzentrationen auf die Aktivität von Stressenzymen untersucht worden (Bartha et al. 2014; Huber et al. 2012). Zur Translokation, Metabolisierung und den von Lamotrigin ausgelösten Stressreaktionen in Pflanzen ist dagegen nur wenig bekannt. Es wird davon ausgegangen, dass Lamotrigin von den Wurzeln adsorbiert bzw. aufgrund seiner Ladung in den Vakuolen von Wurzelzellen zurückgehalten wird und deswegen nur in geringen Konzentrationen die Blätter erreicht (Goldstein et al. 2018). Auch wenn wir dies bestätigen können, sind noch weitere Studien notwendig, um den Verbleib und die Verstoffwechselung dieses Wirkstoffes in Nutzpflanzen zu beleuchten.

Ergebnisse der Arbeiten zu Aufnahme und Metabolisierung von Pharmazeutika

Nach der Inkubation des breitblättrigen Rohrkolbens (*Typha latifolia*) mit 1 mg L^{-1} Diclofenac konnte durch unsere Arbeitsgruppe bereits 2014 eine signifikante Erhöhung der Glutathion-*S*-Transferase (GST)-Aktivität gezeigt werden (Bartha et al. 2014). GSTs gehören zu den zentralen pflanzlichen Entgiftungsenzymen. Grundlegend laufen die Entgiftungsvorgänge von Xenobiotika in der Pflanze in 3 Phasen ab. Nach der Aufnahme und Erkennung folgt eine Aktivierung der Xenobiotika durch oxidative Abwehrenzyme, beispielsweise durch Cyotchrom P450 Oxidasen oder Hydrolasen (Phase I). In der zweiten Phase folgt dann die Konjugation des aktivierten Moleküls an biogene Schutzsubstanzen wie das Tripeptid Glutathion oder Glucose mittels der bereits erwähnten GSTs oder Glycosyltransferasen. Derart konjugierte Xenobiotika sind nun größer, wasserlöslicher, in einer für die Pflanze ungefährlicheren Form und können im dritten Schritt in Vakuolen oder in die Zellwand kompartimentiert und dort abgelagert werden (Chen et al. 2016). Parallel dazu entstehen sowohl während der enzymatischen Prozesse der ersten Phase als auch als eine der ersten pflanzlichen Stressantworten reaktive Sauerstoffspezies

(ROS). ROS haben eine wichtige Funktion in pflanzlichen Stressreaktionen, können jedoch in hohen Konzentrationen Schäden an Lipiden der Zellmembran, Proteinen oder DNS verursachen. Gene, die an abiotischen Stressreaktionen beteiligt sind, werden häufig in zirkadianen Rhythmen exprimiert (Covington et al. 2008, Wilkins et al. 2010). Mutationen wichtiger zeitregulierter Gene (clock-genes) verursachen bei *Arabidopsis thaliana* eine höhere Empfindlichkeit gegenüber Salz-, osmotischem und Hitze-Stress (Kant et al. 2008). Die Expression einer Peroxidase (*NtPXC8.1*), eines Cytochroms P450 (*NtCYP71D21*) und verschiedener anderer Gene, die am Metabolismus von xenobiotischen Verbindungen beteiligt sind, wurden in einer Wurzelkultur von *Nicotiana tabacum* unter Phenolbehandlung signifikant beeinflusst (Alderete et al. 2018). Der mutmaßliche Einfluss von Arzneimittelrückständen im Abwasser auf die Expression von zirkadian kontrollierten Genen, die für Stressenzyme in Pflanzen kodieren, wurde bisher jedoch nicht untersucht.

Versuchsaufbau

Kopfsalatsamen keimten auf einem mit Leitungswasser befeuchteten Filterpapier in einer Petri-Schale für drei Tage im Dunkeln. Danach wurden je fünf Keimlinge in ein hydroponisches System übertragen und für 30 Tage unter kontrollierten Bedingungen in einer Pflanzenkammer kultiviert (20ºC Temperatur, 50% Luftfeuchtigkeit, 16/8-h Tag/Nacht Zyklus). Als Nährmedium wurde 0,5 × Johnson`s Lösung verwendet, welches eine 20-fach erhöhte Konzentration an $FeSO_4 \times 4\ H_2O$ enthielt. Nach 30 Tagen wurden die ausgewachsenen Salatpflanzen dann in Triplikaten entweder mit Lamotrigin (60 µg/L), Diclofenac (20 µg/L) oder dem gleichen Volumen Ethanol (Kontrollen) behandelt, in dem die gelösten Wirkstoffe in das Medium hinzugegeben wurden. Zum Zeitpunkt 0,2, 6, 12, 24, 30, 36 und 48 Stunden wurde je eine Pflanze und 50 ml des Nährmediums als Probe entnommen. Die Pflanzenproben (aufgeteilt in Wurzeln und Blätter) wurden danach bis zur weiteren Verwendung kurzzeitig bei -80ºC gelagert.

Aufgrund der hohen Empfindlichkeit der RNA und Enzyme wurden die Wurzeln und Blätter unter flüssigem Stickstoff zu einem feinen Pulver zermörsert und aufgeteilt. Zirka 350 mg wurden für die Extraktion von RNA verwendet, die dann mittels des High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA) in cDNA umgeschrieben wurde. Anschließend folgte die Durchführung von qPCRs mit zwölf verschiedenen Primern (Tabelle 1), die an salatspezifische Stressgene binden sollen, und mit dem Power SYBR® Green PCR Master Mix. Weitere 3 g des gemörserten Pflanzenmaterials wurden verwendet, um einige für die Entgiftung von Xenobiotika relevanten Enzyme (GST, POX, APOX) zu extrahieren, auf ihre Aktivität zu testen, sowie um die zelluläre Konzentration an Wasserstoffperoxid, einem wichtigen Stressmarker, zu quantifizieren.

Tabelle 1: Liste der Gene, deren Expression mittels RT-qPCR ermittelt wurde. Es wurde ein Fokus auf pflanzliche Stressgene (Pathogen induziert, oxidativen Stress oder Detoxifikation von Xenobiotika) gelegt.

	Name of gene (Locus tag in *Arabidopsis thaliana*)	**Documented functions in *Arabidopsis thaliana***
1	*PR1* (AT2G14610)	Pathogenesis related protein 1, SA dependent expression, involved in resistance against broad spectrum of pathogen.
2	*PDF1.2* (AT5G44420)	Plant defensin factor involved in JA/ Et dependent pathogen defense response. Involved in ISR.
3	*LOX1*(AT1G55020)	Lipoxigenase; Upstream gene involved in the oxylipin metabolic pathway, being at the origin of many cell constituents and signalling molecules. Involved in the signalling of wounding response and JA induced defence against specific pathogens.
4	*WRKY70* (AT3G56400)	Transcription factor involved in both SA- and JA-mediated signal pathways. Also involved in abiotic stress signaling.
5	*WRKY25* (AT2G30250)	Negative regulator of SA-mediated defense responses, elevated expression in response to oxidative stress, heat stress or wounding.
6	*CAT1* (AT1G20630)	Catalase, induced by hydrogen peroxide, abscisic acid (ABA), drought, and salt stress.
7	*PER50* (AT4G37520)	Peroxidase; Response to environmental stresses such as wounding, pathogen attack and oxidative stress.
8	*GST6* (AT2G47730)	Glutathione S-transferase expressed in response to auxin, (SA) and hydrogen peroxide. JA independent induction by 12-oxo-Phytodienoic Acid (OPDA) in plant defense.
10	*MYB15* (AT3G23250)	ABA inducible abiotic stress regulator, upregulated in cold and drought stress.
11	*GST-U5*	Upregulated by Paracetamol Treatment in *A. thaliana* (Dissertation von Bernadette)
12	*GST-F6*	Upregulated by Paracetamol Treatment in *A. thaliana* (Dissertation von Bernadette)

Die Kultivierung und Behandlung des Kopfsalates wurde in einem getrennten Ansatz wiederholt und das gewonnene Pflanzenmaterial im Lyophilisator gefriergetrocknet, um es später beim Kooperationspartner IDAEA-CSIC in Barcelona bearbeiten zu können. Dafür wurden die Proben nach dem Protokoll unseres Projektpartners Nicola Montemurro (Montemurro et al., Manuskript in Vorbereitung) mittels des QuEChERS Original Extraktionskits (Bekolut, Hauptstuhl, Germany) extrahiert und am LC/QTOF-MS des IDAEA-CSIC analysiert. Es wurden sowohl die Wirkstoffe Diclofenac und Lamotrigin, als auch der Metabolit 4'-Hydroxydiclofenac in den Pflanzenproben analysiert. Zusätzlich wurden die Konzentrationen der Wirkstoffe im Nährstoffmedium am LC-MS/MS am Helmholtz Zentrum München untersucht. Dafür wurden die Proben 1:2 mit 200 mM 5-Sulfosalicylsäure gemischt, zentrifugiert und anschließend injiziert.

Sämtliche Statistikanalysen wurden mit der Software R, Version 3.6.1 durchgeführt, wobei in den meisten Fällen eine two-way ANOVA (Varianzanalyse) mit anschließender Bonferroni-Korrektur angewendet wurde. Lediglich für die statistische Analyse der RT-qPCR Ergebnisse, um die

Expressionen in den behandelten Proben mit den Kontrollen zu vergleichen, wurde ein one-way ANOVA mit anschließendem Tukey´s HSD Test durchgeführt.

Resultate

Die Analyse der Salatproben (Wurzeln und Blätter) zeigte, dass beide Pharmazeutika in die Pflanzen aufgenommen wurden, und dass die höchste Konzentration von Diclofenac in den Wurzelproben 6 Std. nach Exposition auftrat. Gleichzeitig konnte zu diesem Zeitpunkt 4'-Hydroxydiclofenac als erster pflanzlicher Metabolit detektiert werden (Abbildung 1).
Die Metabolisierung von Diclofenac verlief so schnell, dass nach 24 Std. die Konzentration des Metaboliten höher als die der Ausgangsverbindung war. Eine solche Hydroxylierung kann in Pflanzen nur durch Peroxidasen oder P-450-Monooxygenasen katalysiert werden. Beide Enzymklassen sind bekannt für ihre Rolle im Fremdstoff- und Herbizidmetabolismus (Huber et al. 2016). In den Blättern der Salatpflanzen konnten wir hingegen während der gesamten Versuchsdauer weder Diclofenac noch einen Metaboliten detektieren. Ähnliche Ergebnisse waren auch durch Bartha et al. (2014) gezeigt worden, denn nach 24 Std. Exposition mit einer sehr hohen, eingesetzten Ausgangskonzentration (1 mg L^{-1} Diclofenac) konnte in den Blättern von *Typha latifolia* nur 4% der in Wurzeln detektierbaren Menge gemessen werden.

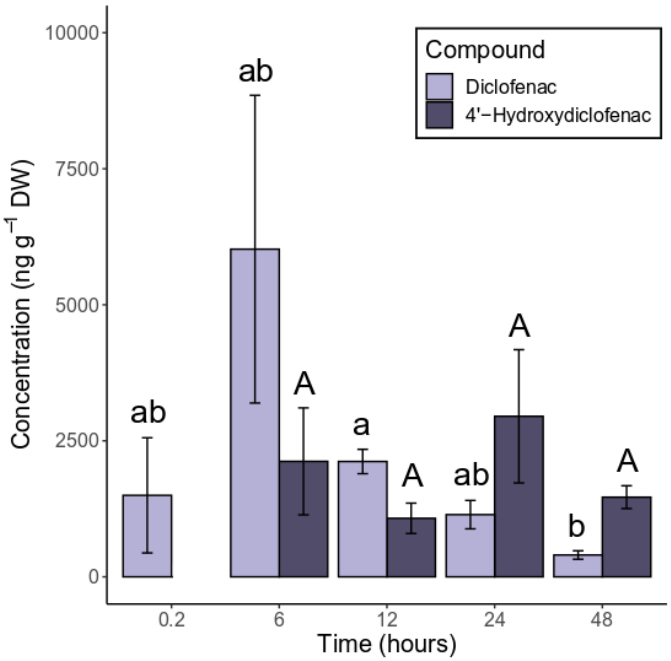

Abbildung 1: Konzentrationen von Diclofenac und seines Metaboliten 4'-Hydroxydiclofenac (ng g^{-1}) in Salatwurzeln. Die Daten zeigen die Mittelwerte der Konzentrationen pro Gramm Trockengewicht (dry weight, DW) ± Standardabweichung (n = 3). Die unterschiedlichen Buchstaben weisen auf statistische Signifikanzen zwischen den unterschiedlichen Zeitpunkten der Probenahme nach Exposition mit Diclofenac hin.

Im Gegensatz zu Diclofenac stieg die Konzentration von Lamotrigin in den Wurzelproben in den ersten 6 Std. an, blieb dann aber auf einem konstanten Level (durchschnittlich 2.14 ± 0.22 µg L^{-1}) (Abbildung 2A). Außerdem konnte Lamotrigin zusätzlich in den Blättern in geringen, aber ansteigenden Konzentrationen quantifiziert werden.

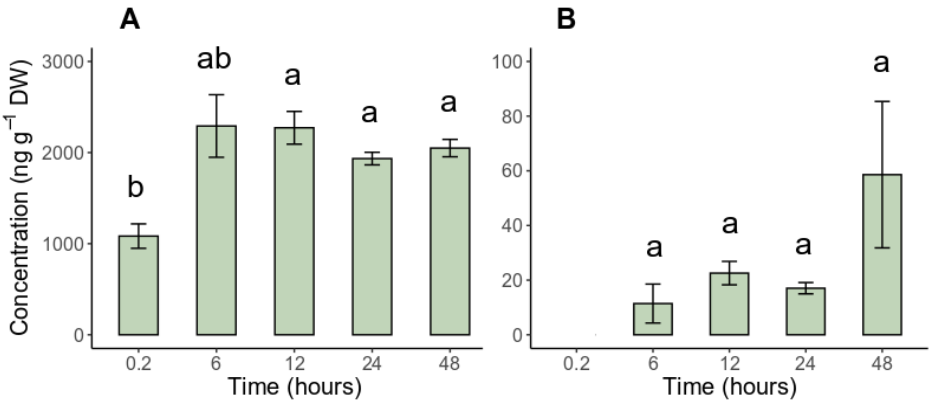

Abbildung 2: Konzentrationen des Wirkstoffs Lamotrigin (ng g^{-1}) in Salatwurzeln (A) und –blättern (B). Die Balken zeigen die Mittelwerte der Konzentrationen pro Gramm Trockengewicht (dry weight, DW) ± Standardabweichung (*n* = 3). Die unterschiedlichen Buchstaben weisen auf statistische Signifikanzen zwischen den unterschiedlichen Zeitpunkten der Probenahme nach Exposition mit Lamotrigin hin.

Zusätzlich zur Analyse der Wirkstoffe in Wurzeln und Blättern wurden diese auch im Nährmedium untersucht. In pflanzenlosen Kontrollen war die Konzentration von Lamotrigin stabil (Abbildung 3), was auf einen vernachlässigbaren Verlust des Wirkstoffes durch Absorption an das Gefäß, Perlit oder durch Photodegradation schließen lässt.

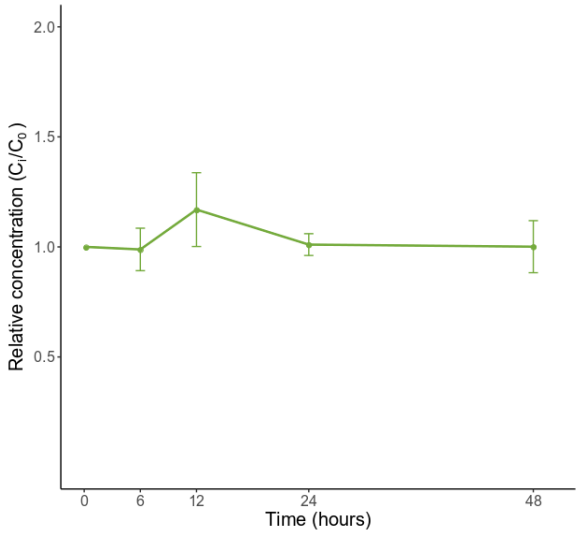

Abbildung 3: Relative Konzentration (C_i/C_0) von Lamotrigin in den pflanzenfreien Kontrollgruppen über die Zeit des Versuchs von 48 Stunden. C_i ist die gemessene Konzentration zu einem bestimmten Zeitpunkt, C_0 die Konzentration bei T0.

Wenn aber Salatpflanzen mit ihren Wurzeln in die Inkubationslösung ragten, sank die Anfangskonzentration von 58,32 ± 6,74 µg L^{-1} LMG im Nährmedium nach 48 h auf 45,48 ± 2,96 µg L^{-1} (Abbildung 4). Aufgrund technischer Probleme an der HPLC und dem Massenspektrometer lagen die entsprechenden Daten zum Ende des Berichtzeitraums leider noch nicht vor.

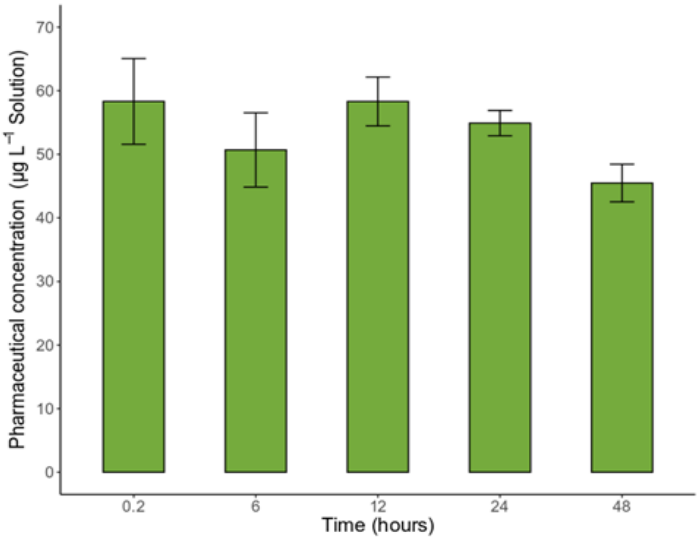

Abbildung 4: Konzentration von Lamotrigin (µg L^{-1}) im Nährmedium über die Zeit des Versuchs von 48 Stunden. Die Daten sind mittlere Konzentrationen ± Standardabweichung (n = 3).

Die Analyse des wichtigen Signalmoleküls Wasserstoffperoxid (H_2O_2) ergab, dass 12 Std. nach der Behandlung der Salatpflanzen mit Lamotrigin eine hoch-signifikant erhöhte H_2O_2-Konzentration (p-Werte ≤ 0.001) in Wurzeln und Blättern zur gleichen Zeit vorlag (Abbildung 5). Bei den anderen Zeitpunkten oder nach der Behandlung von Diclofenac konnte dies nicht festgestellt werden. Diese transiente Erhöhung der H_2O_2-Konzentration lässt auf einen durch Lamotrigin direkt oder indirekt ausgelösten oxidativen Burst schließen. Ein vergleichbarer oxidativer Burst konnte bereits in *Salvia officinalis* Blättern gezeigt werden, nachdem diese für 5 Std. mit Ozon behandelt worden waren (Marchica et al. 2019).

Unsere frühere Arbeit hatte bereits gezeigt, dass die beiden Gene, die für die Glutathion-*S*-Transferasen *GST-F6* und *GST-U5* kodieren, in *Brassica*-Wurzeln durch die Behandlung von Paracetamol induziert werden (Bartha et al. 2010). Die relative Genexpression von insgesamt zwölf Genen, die an verschieden Stressreaktion beteiligt sind, wurden daher auch im vorliegenden Projekt bestimmt. Bereits die Betrachtung der Zeitpunkte 0,2 , 6,12, 24 und 48 Std. legte einen Tagesverlauf in der Expression bei fast allen der getesteten Gene in den Kontrollen nahe. Daher wurden zusätzlich die Zeitpunkte 30 und 36 Std. untersucht, um den Verlauf von zwei ganzen Tagen abzubilden.

In einer Studie von Badawi et al. (2007) wurden Weizenpflanzen unter Langtagbedingungen (16/8-h Tag/Nacht Zyklus) kultiviert, um die relative Genexpression von kälteinduzierten Stressgenen (*TaCBF*) zu untersuchen.

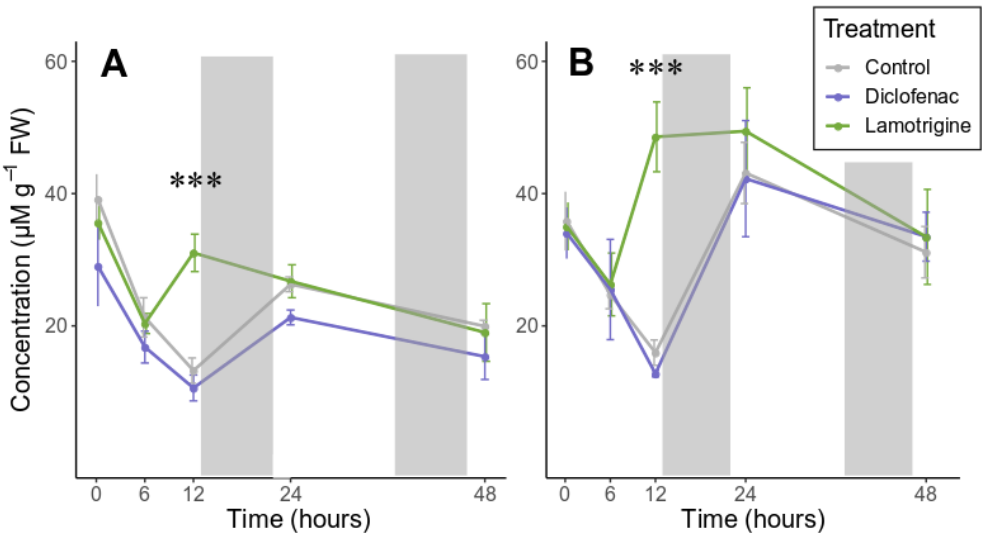

Abbildung 5: Wasserstoffperoxidkonzentrationen (µM g^{-1}) in Salatwurzeln (A) und –blättern (B) in Kontrollpflanzen (grau) und in mit Diclofenac (blau) oder Lamotrigin (grün) behandelten Gruppen. Die Daten zeigen Mittelwerte der Konzentration pro Gramm Frischgewicht (fresh weight, FW) ± Standardabweichung (n = 3).

Hierbei konnte ein Tagesverlauf mit der höchsten Transkriptmenge der verschiedenen *TaCBF* Genen in den Kontrollpflanzen zwischen 18:00 h und 22:00 h (Dämmerung bis Dunkelheit) festgestellt werden. In den Wurzeln von Salatpflanzen zeigten Gene, die für verschiedene am Metabolismus von Xenobiotika beteiligten Enzyme (*PER50*, *CAT1*, *GST-F6*, *GST-F8* und *GST-U5*) kodieren, höchste Expression ebenfalls kurz vor der Dämmerung (T12 und T36). In Blättern war die Expression der unterschiedlichen Gene zeitversetzt (Abbildung 6 A und B).

Alle getesteten Gene (*PER50*, *CAT1*, *GST-F6* und *GST-F8*), die in vorherigen Studien anderer Gruppen durch eine erhöhte Konzentration von H_2O_2 induziert worden waren (Guan et al. 2000, Wagner et al. 2002), hatten in unseren Experimenten ein ähnliches Expressionsmuster in Lamotrigin behandelten Salatwurzeln, das sich eindeutig von dem der Kontrollpflanzen unterschied (Abbildung 6 A und C). Generell konnte im vorliegenden Experiment eine Phasenverschiebung der Expression zirkadian exprimierter Gene beobachtet werden.

Es gab dabei interessanterweise einen Trend zu einer früheren, erhöhten Expression nach 6 Std. und einer erhöhten Expression über den Zeitverlauf für *PER50*, *CAT1* und *GST-F6* in Wurzeln. Die hohen und niedrigen Peaks in mit Lamotrigin behandelten Pflanzen waren für die meisten Gene verschoben, und die Expression bei T24, T36 und T48 unterschied sich signifikant von der in den Kontrollpflanzen in Wurzeln und Blättern (Abbildung 6 A-D).

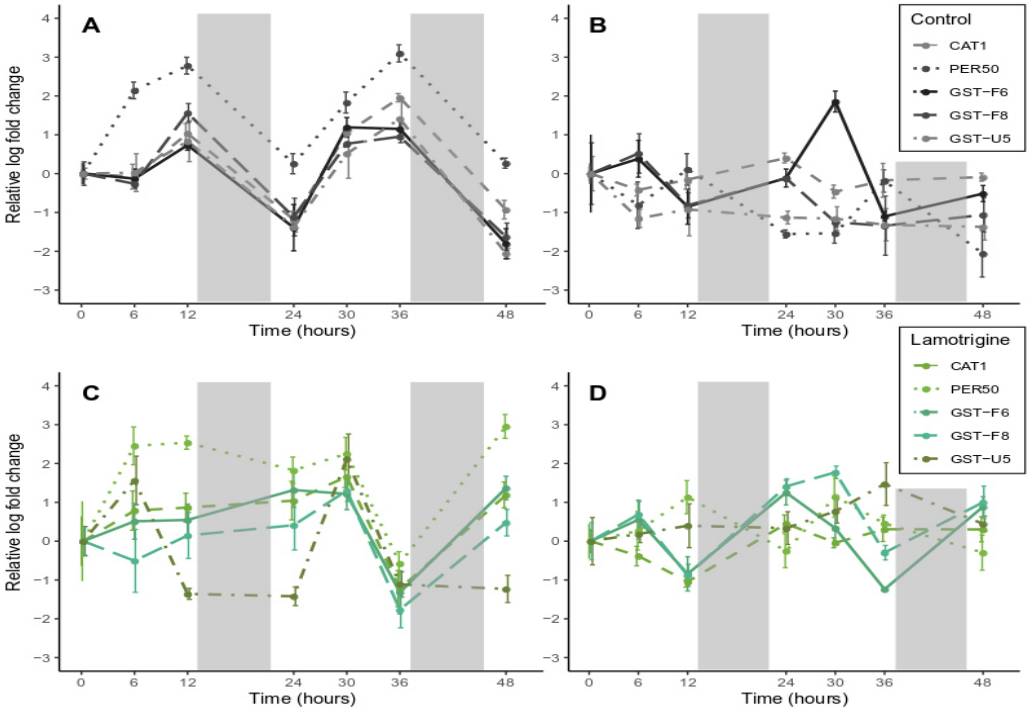

Abbildung 6: Relative Expression (log Fold Change) von drei Glutathion-*S*-Transferasen (*GST-F6*, *GST-F8* und *GST-U5*), einem Katalase- (*CAT1*) und einem Peroxidase- (*PER50*) Gen in der (A + B) Kontrolle und (C + D) in mit Lamotrigin behandeltem Salat in verschiedenen Pflanzengeweben ((A + C) Wurzeln und (B + D) Blätter). Fehlerbalken zeigen das 95% -Konfidenzintervall an. Es wurden signifikante Unterschiede im Expressionsmuster aller Gene im Vergleich zu Kontrollpflanzen zu verschiedenen Zeitpunkten beobachtet, die durch paarweise HSD-Tests von Tukey festgestellt wurden (Tabelle 2). Graue Balken: subjektive Nacht.

Tabelle 2: P-Werte, die aus dem Vergleich der Stressgenexpression in den mit Lamotrigin behandelten Salatgewebe ((A) Wurzeln, (B) Blätter) und den Kontrollpflanzen bei verschiedenen Zeitpunkten erhalten wurden. Unterschiedliche Signifikanzabstufungen werden durch unterschiedliche Farben als „hellgrün“ für 0,01 ≤ p-Wert ≤ 0,05, „hellrot“ für 0,001 ≤ p-Wert ≤ 0,01 und „dunkelrot“ für p-Wert ≤ 0,001 angezeigt.

A

Time [h]	*CAT1*	*PER50*	*GST-F8*	*GST-U5*	*GST-F6*
T6	0.0468	0.3169	0.5860	0.0191	0.0742
T12	0.5066	0.1589	0.0123	0.0014	0.4384
T24	0.0013	0.0020	0.0202	0.7355	0.0020
T30	0.0149	0.1739	0.0209	0.0257	0.9241
T36	0.0002	0.0001	0.0004	0.0013	0.0014
T48	0.0005	0.0001	0.0011	0.0125	0.0002

B

CAT1	*PER50*	*GST-F8*	*GST-U5*	*GST-F6*
0.8021	0.0536	0.6069	0.0012	0.6546
0.0034	0.0271	0.8001	0.0426	0.9845
0.7560	0.0045	0.0001	0.0031	0.0026
0.0175	0.0009	0.0000	0.0009	0.0023
0.1540	0.0246	0.0594	0.0014	0.1030
0.0835	0.0100	0.0026	0.0008	0.0012

Eine signifikante, vorübergehende Verringerung der Expression aller Gene wurde bei T6 in Wurzeln von Diclofenac-behandelten Pflanzen beobachtet (Abbildung 7). Darüber hinaus war die Expression von *CAT1*, *PER50*, *GST-F6* und *GST-F8* bei T12 ebenfalls signifikant reduziert. Mit abnehmenden Diclofenac-Konzentrationen konnten wir einen geringeren Einfluss auf die Stressgen-Expression im Vergleich zu Kontrollpflanzen in Salatwurzeln feststellen.

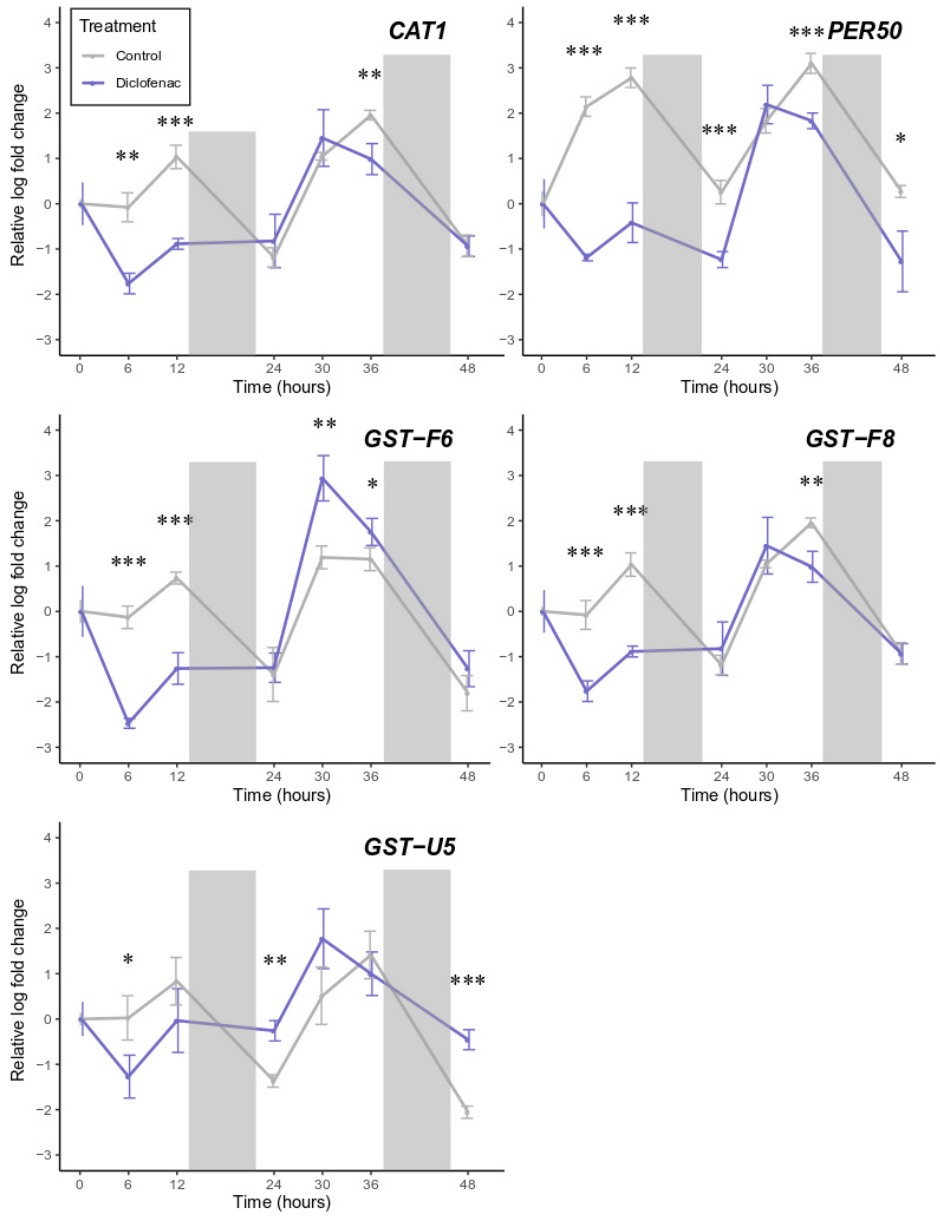

Abbildung 7: Relative Expression (log Fold Change) in Salatwurzeln von drei Glutathion-*S*-Transferasen (*GST-F6*, *GST-F8* und *GST-U5*), einem Katalase- (*CAT1*) und einem Peroxidase- (*PER50*) Gen in Kontrollen und in mit Diclofenac behandelten Pflanzen. Fehlerbalken zeigen ein 95% -Konfidenzintervall an. Signifikante Unterschiede zwischen behandelten Gruppen und Kontrollpflanzen werden gemäß Tukeys HSD-Paartest als "*" für 0,01 ≤ p-Wert ≤ 0,05, "**" für 0,001 ≤ p-Wert ≤ 0,01 und "***" angegeben. für p -Wert ≤ 0,001. Graue Balken: subjektive Nacht.

In Blättern, in denen Diclofenac oder der Metabolit 4'-Hydroxydiclofenac nicht nachgewiesen werden konnte, war der Einfluss auf die Expression von Stressgenen im Allgemeinen gering (Abbildung 8).

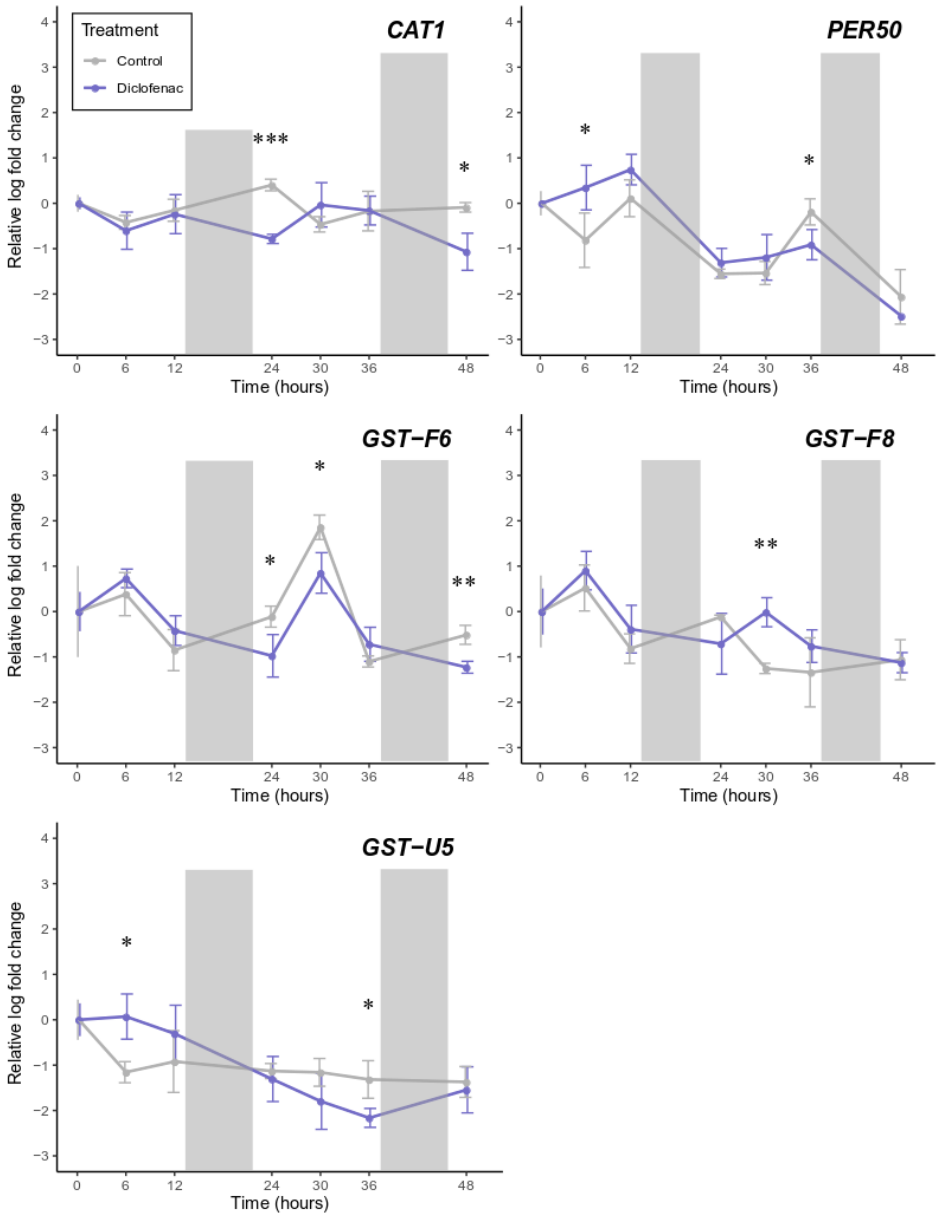

Abbildung 8: Relative Expression (log Fold Change) in Salatblättern von drei Glutathion-*S*-Transferasen (*GST-F6*, *GST-F8* und *GST-U5*), einem Katalase- (*CAT1*) und einem Peroxidase- (*PER50*) Gen in Kontrollen und in mit Diclofenac behandelten Pflanzen. Fehlerbalken zeigen ein 95% -Konfidenzintervall an. Signifikante Unterschiede zwischen behandelten Gruppen und Kontrollpflanzen werden gemäß Tukeys HSD-Paartest als "*" für 0,01 ≤ p-Wert ≤ 0,05, "**" für 0,001 ≤ p-Wert ≤ 0,01 und "***" angegeben. für p -Wert ≤ 0,001. Graue Balken: subjektive Nacht.

Alle oben beschriebenen Ergebnisse wurden in einem Manuskript mit dem Titel *„Effect of the pharmaceuticals diclofenac and lamotrigine on stress responses and stress gene expression in lettuce (Lactuca sativa) at environmentally relevant concentrations“* zusammengefasst und am 30.03.2020 an das „Journal of Hazardous Materials“ für eine Veröffentlichung eingereicht. Mittlerweile ist dieses Manuskript positiv begutachtet und wird zur Veröffentlichung vorbereitet.

Experimente zum Einfluss von Pharmazeutika auf die endophytische Gemeinschaft in Salatwurzeln

Die Verwendung von behandelten Abwässern in der Landwirtschaft kann nicht nur Einfluss auf die Physiologie und Biochemie essbarer Pflanzen selbst haben, sondern auch auf die pflanzen-assoziierten und die im Boden lebenden Mikroorganismen. Mikroorganismen spielen eine zentrale Rolle für die Bodengesundheit und in biogeochemischen Kreisläufen, weshalb Pharmazeutika als Kontamination behandelter Abwässer direkte Auswirkungen auf das Bodenökosystem haben können. Auf der einen Seite können Antibiotika und Antimykotika dabei das Wachstum spezifischer Mikroorganismen, die wichtige Funktionen im Ökosystem haben inhibieren, während auf der anderen Seite die freiwerdende Nische von anderen Mikroorganismen genutzt werden kann, die weniger anfällig oder resistent gegenüber Antibiotika sind. Auch Pharmazeutika anderer Klassen, wie das Antiepileptikum Carbamazepin, zeigten ökotoxikologische Effekte auf die bakterielle Gemeinschaft von Biofilmen in Flussauen, mit einer geringeren bakteriellen Biomasse und einer reduzierten Häufigkeit von Gamma-Proteobakterien und Cyanobakterien (Lawrence et al. 2005). Untersuchungen des Pflanzen-assoziierten Mikrobioms zeigten, dass Bewässerung mit behandelten Abwasser die mikrobielle Gemeinschaft verändert (Zolti et al. 2018).

Gemeinsam mit den Projektpartnern vom Institut National de la Recherche Agronomique (INRAE) in Dijon wurden Versuche durchgeführt, um den Einfluss von Pharmazeutika aus behandeltem Abwasser auf die endophytische (Mikroben, die im inneren des Vegetationskörpers einer Pflanze leben) und mykorrhizale Gemeinschaft zu untersuchen. Dafür wurden Salatpflanzen (*Lactuca sativa*) für 60 Tage in einem Gewächshaus in Dijon kultiviert und entweder mit behandeltem Abwasser oder mit Frischwasser, ohne und mit zusätzlich zugegebenen Pharmazeutika (Konzentrationen: 10 ppb und 100 ppb) gegossen. Danach wurde die Kampagne im gleichen belasteten Boden wiederholt. Die Proben wurden für die chemische Analyse der Pharmazeutika, zur Messung verschiedener mikrobieller Funktionen (Gene und Produkte des N-Zyklus, Antibiotikaresistenzen) (INRAE), und zur Untersuchung der endophytischen und mykorrhizalen Gemeinschaft (HMGU) herangezogen. Anfang Februar erhielten wir die entsprechenden Proben aus Frankreich, um die Sequenzierung des 16S-Gens und eines Mykorrhiza-spezifischen Gens

(AMF-Amplikon basierte Sequenzierung) durchzuführen. Dazu mussten verschiedene PCR- und Aufreinigungsbedingungen getestet werden. Unter anderem wurden für die AMF-Amplikon basierten Sequenzierungen mehrere Primerpaare notwendig, um für beide Gene die optimalen Bedingungen zu bestimmen.

Am 18.03.2020 ging das Helmholtz Zentrum München wegen der Covid-19 Pandemie in den Minimalbetrieb über, und erst seit wenigen Wochen ist es uns wieder möglich, die Arbeiten fortzuführen. Alle notwendigen Laborarbeiten wurden am 24.07.2020 fertiggestellt und die Ergebnisse werden nun bioinformatisch aufgearbeitet. Sie werden somit erst nach dem offiziellen Projektende mit Verspätung vorliegen. Wir erwarten aber wichtige Aussagen über die Zusammensetzung der endophytischen und mykorrhizalen Gemeinschaft und ökotoxikologische Effekte unter Einfluss von Pharmazeutika und behandeltem Abwasser, die gemeinsam publiziert werden.

Buchkapitel „Uptake and translocation of pharmaceuticals in plants – Principles and data analysis“

Pharmazeutika als Kontamination von teilgeklärten Abwässern die zur Bewässerung von landwirtschaftlichen Flächen genutzt werden, können von Kulturpflanzen aufgenommen werden. Im Allgemeinen erfolgt die potentielle Aufnahme über die Wurzeln der Pflanzen und kann zur Bioakkumulation in verschiedenen Pflanzenteilen führen. Die Aufnahme und Translokation ist abhängig von mehreren Parametern, nämlich den physikochemischen Eigenschaften der Verbindungen, der Pflanzenphysiologie und verschiedenen Umweltfaktoren. Ein Buchkapitel unserer Gruppe kombiniert dazu theoretische Hintergrundinformationen zu den Hauptprinzipien der Aufnahme und Translokation von Pharmazeutika in Pflanzen und stellt eine kritische Bewertung der aktuell verfügbaren Literatur durch die Analyse experimenteller Studien zu den Biokonzentrations- und Translokationsfaktoren verschiedener Arzneimittelgruppen in unterschiedlichen Pflanzenarten dar. Dadurch werden interessante Ergebnisse bei der Translokation verschiedener Arzneimittel und von kationischen Verbindungen in Pflanzen gewonnen. Durch den Vergleich verschiedener Studien konnte außerdem gezeigt werden, dass es notwendig ist, neben hohen auch reale Umweltkonzentrationen zu testen, da nach der Exposition mit niedrigeren Konzentrationen zum Teil eine höhere Aufnahme und Translokation einiger Pharmazeutika beobachtet werden konnte. Die Anwendung grundlegender Richtlinien bei den Versuchen könnten eine Möglichkeit bieten, wissenschaftliche Daten vergleichbarer und zuverlässiger zu machen, um zu verhindern, dass die unter bestimmten Bedingungen beobachtete ausbleibende Aufnahme oder Translokation von Arzneimitteln in Pflanzen zu falschen Schlüssen über ihr Umweltverhalten führt. Das Buchkapitel soll Empfehlungen für die zukünftige Forschung geben, um innerhalb der wissenschaftlichen Gemeinschaft valide Schlussfolgerungen zu generieren. Es ist ein wichtiges Ergebnis des Projekts AWARE.

c. die wissenschaftlichen und/oder technischen Erfolgsaussichten nach Projektende

Die Kooperation in AWARE, persönliche Kontakte während der Seminare und die intensive Beschäftigung mit der Fragestellung wurden genutzt, um im Rahmen des Water-JPI einen weiteren Antrag zu stellen, der sich mit weiterführenden Fragestellungen befasst. Es gelang einem Teil des Konsortiums (UM Montpellier, HMGU), mit Partnern aus Südafrika und Brasilien, ein neues Projekt zur dezentralen Abwasserbehandlung zu initiieren. Die Arbeiten am Projekt IDOUM ("Water JPI - Joint Call 2017 - Transnationales Verbundvorhaben: IDOUM Innovative dezentralisierte und kostengünstige Behandlungssysteme für ein optimales kommunales Abwassermanagement“) begannen mit viel Enthusiasmus, sind aber momentan auch aufgrund der misslichen Infektions-Lage in Brasilien und Südafrika annähernd zum Erliegen gekommen. Eine kostenneutrale Verlängerung wurde beantragt, und die Partner sind zuversichtlich, dass die Versuche bald wieder aufgenommen werden können.

d. **Angemessenheit von Aufwand und Zeit**

Der Verbleib und die Wirkung von Pharmazeutika in Pflanzen, die roh dem menschlichen Verzehr dienen, wie Salat und Rettich, sind bisher nicht im Detail untersucht worden, vor allem nicht in solch geringen Konzentrationen, wie sie in Abwässern und Brauchwasser vorkommen. Auf die Erfassung der Wirkstoffe in diesen Konzentrationsbereichen zielten die Arbeiten von AWARE, denn bei der in den vergangenen Jahren beobachtbaren Wasserverknappung wurden Forderungen nach der direkten Verwendung von teilgeklärten Wässern in der Landwirtschaft laut. Um solch niedrige Konzentrationen und die zu erwartenden geringeren Effekte nachweisen zu können, ist der Einsatz hochempfindlicher Methoden und Analytik unabdingbar. Im IDAEA-CSIC Barcelona und im HMGU München stehen entsprechend hochempfindliche MS-Geräte und UPLCs zur Verfügung, und das INRAE Dijon verfügt genau wie das HMGU über empfindliche Next-Generation Sequenzer für die Untersuchung mikrobieller Gene.

Die Extraktionsmethode (QuEChERS) wurde unter der Federführung eines Projektpartners gemeinsam verbessert und an die Gegebenheiten des AWARE-Materials angepasst. Die Ergebnisse werden in mehreren gemeinsamen Publikationen veröffentlicht. Sie werden neue Standards für die Risikobewertung von Brauchwässern auf EU-Ebene setzen. Damit befindet sich das AWARE Projekt an der Spitze der europäischen Forscher-Gemeinschaft, die sich mit diesem Thema befasst. Der Aufwand an Zeit und Ressourcen ist daher mehr als berechtigt.

e. Arbeiten, die zu keiner Lösung geführt haben,

Keine

f. wissenschaftliche, technische und wirtschaftliche Anschlussfähigkeit

Die Kooperation in AWARE, persönliche Kontakte während der Seminare und die intensive Beschäftigung mit der Fragestellung wurden genutzt, um im Rahmen des Water-JPI einen weiteren Antrag zu stellen, der sich mit weiterführenden Fragestellungen befasst. Es gelang einem Teil des Konsortiums (UM Montpellier, HMGU), mit Partnern aus Südafrika und Brasilien, ein neues Projekt zur dezentralen Abwasserbehandlung zu initiieren. Die Arbeiten am Projekt IDOUM ("Water-JPI - Joint Call 2017 - Transnationales Verbundvorhaben: IDOUM Innovative dezentralisierte und kostengünstige Behandlungssysteme für ein optimales kommunales Abwassermanagement“) begannen mit viel Enthusiasmus, sind aber momentan auch aufgrund der misslichen Lage in Brasilien und Südafrika annähernd zum Erliegen gekommen.

g. voraussichtliche Ergebnisnutzung

Die Ergebnisse des Projekts und der am HMGU initiierten Doktorarbeit zum Thema werden verwendet, um weitere Forschungsanträge zu stellen. Sie fließen zudem direkt in die laufende Diskussion zur Wiederverwertung von Wasser und der Verbesserung von Klärprozessen und der Analytik ein. Es war ein großer Erfolg des Projekts, dass im Herbst 2019 alle Partner als Sprecher auf den MUSE-Workshop in Montpellier zur Brauchwassernutzung eingeladen waren, an dem neben Wissenschaftlern auch zahlreiche Stakeholder aus Industrie und Behörden teilnahmen. Weiterhin waren nach der Präsentation unserer Daten auf einer Tagung in Thessaloniki Wissenschaftler des Berliner Verbunds zur Wasserforschung auf unsere Arbeiten aufmerksam geworden. Die Doktorandin und ein PostDoc wurden zu einem Workshop ans Leibniz-Institut für Gemüsebau nach Großbeeren eingeladen, auf dem sie unsere Erkenntnisse mit den dortigen Wissenschaftlern diskutierten. Die Disseminierung der AWARE-Ergebnisse führt also nicht nur zu einer Erweiterung des Wissens zum Thema, sondern auch zur weiteren Umsetzung in der angewandten Forschung.

h. Einhaltung der Kosten- und Zeitplanung

Der Kostenplan wurde eingehalten (siehe Finanzbericht). Das Projektende wurde aufgrund der Covid-19 Pandemie verschoben, denn die Laborarbeiten können erst jetzt erfolgreich abgeschlossen werden. Die Auswertung der Experimente und die Verfassung von Manuskripten wird jedoch noch einige Monate in Anspruch nehmen.

3. (FE-)Ergebnisse, die für eine mögliche Fortführung des Vorhabens relevant sein können

Wassermangel und Wasserwiederverwertung sind wesentliche Themen der EU-Forschungsförderung, und nach den Dürre-Ereignissen der letzten Jahre sind verschiedene Länder- und Bundesministerien mit der Frage befasst. Dabei ist die in unserem Projekt verfolgte Fragestellung zum Verbleib von Pharmazeutika in Oberflächen- und Grundwässern in zahlreichen nationalen und internationalen Projekten aufgegriffen worden. Die von uns im Detail untersuchten Prozesse in Salatpflanzen und Rettich sind aber bisher nicht Gegenstand konkurrierender Projekte geworden. Wir sind allerdings sicher, dass unsere Ergebnisse dazu beitragen werden, ein Augenmerk auf die Exposition über roh zu verzehrende Pflanzen einerseits und die vielverwendeten Analgetika und Antikonvulsiva andererseits zu richten. Das von uns erworbene Wissen im Bereich der Probenvorbereitung, Analytik und Metabolismusforschung wird dabei auch für andere Projekte von großem Nutzen sein.

4. War der Einsatz der Bundesmittel für die Erreichung des geplanten Vorhabenziels ursächlich oder wäre dieses Ziel auch ohne Bundesmittel erreicht worden (einschließlich Bewertung evtl. Mitnahmeeffekte)?

Ohne die zur Verfügung gestellten Bundesmittel wäre die Kooperation mit den exzellenten Partnern nicht zustande gekommen, obwohl bereits Kontakte bestanden. Nur die Förderung ermöglichte es, intensiven Meinungsaustausch in persönlichem Kontakt zu pflegen und die jungen Wissenschaftler in direkter Kooperation an denselben Proben forschen zu lassen. Da die Fragestellung – Wirkung und Verbleib von brauchwassergetragenen Pharmazeutika in essbaren Pflanzen – auch nicht zum Kerngebiet der beteiligten Institute gehört, wäre ohne die Bundesmittel und die Water-JPI-Ausschreibung weder vom deutschen noch vom französischen Partner an diesem Thema geforscht worden.

5. Auflistung der erfolgten und geplanten Veröffentlichungen des Ergebnisses (Belegexemplare befinden sich im Anhang zum Bericht).

- Posterpräsentation: Bigott Y, Chowdhury SP, Montemurro N, Perez S and Schröder P, “Uptake and Metabolism of Pharmaceuticals by *Lactuca sativa*” am 19.06.2019 während der “17th International Conference on Chemistry and the Environment (ICCE)” in Thessaloniki.

- Originalartikel: Bigott Y, Chowdhury SP, Pérez S, Montemurro N, Manasfi R, Schröder P (2020) 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, accepted, in press.

- Originalartikel: Gallego S*, Bigott Y*, Breuil MC, Barceló D, Schröder P, and Martin-Laurent F (2021) Ecotoxicological impact of wastewater-borne pharmaceuticals on mycorrhizal and endophytic communities and functions in lettuce roots. (*Beide sind Erstautoren, bioinformatische Auswertung in Bearbeitung (Laborarbeiten mussten wegen Covid-19 unterbrochen werden))

- Buchkapitel: Bigott Y, Kamel D, Schröder PM, Schröder P, Cruzeiro C (2020) „ Uptake and Translocation of Pharmaceuticals in Plants: Principles and Data Analysis" In: Barcelo D, Perez s et al. (eds): „Interaction and Fate of Pharmaceuticals in Soil-Crop Systems - The Impact of Reclaimed Wastewater" in der Reihe „The Handbook of Environmental Chemistry (698)" von Springer.

6**. Präsentationsmöglichkeiten für mögliche Nutzer – z. B. Anwenderkonferenzen (Angaben, soweit die Art des Vorhabens dies zulässt),**

Die Präsentationen zum Projekt stehen als Powerpoint-Dateien zur Verfügung. Die Protokolle der Projektreffen sind auf der Webseite des Projektleiters abgelegt (https://www.idaea.csic.es/project/aware/). Das IDAEA-CSIC Barcelona ist verantwortlich für Pflege und Updates der Seite.

Die Originalpublikationen werden in open-access veröffentlicht, Vorabversionen sind auf dem epub-server des HMGU erhältlich, und das Buch mit unserem Kapitel ist über Springer oder den Buchhandel kommerziell zu erwerben.

7. Aufführung durchgeführter Maßnahmen des Wissenstransfers bzw. Bildung/ Weiterbildung im Kontext des EU-Gesamtvorhabens

- Mid-term Projektmeeting in Dijon vom 11.-13. März 2019. Dort wurde der Fortschritt sämtlicher Arbeitspakete durch die involvierten Gruppen vorgestellt sowie die Planung gemeinsamer Projekte vorangetrieben.
- Posterpräsentation am 19.06.2019 während der "17th International Conference on Chemistry and the Environment (ICCE)" in Thessaloniki (Poster siehe Anhang).
- Vortrag am 06.10.2019 während des Symposiums „Pharmaceuticals in the Food System" am Leibniz-Institut für Gemüse- und Zierpflanzenbau (Großbeeren).
- 01.-02. Oktober 2019, MUSE Workshop Montpellier https://sites.google.com/view/workshop-reuse/
- 02.-04. Oktober 2019 in Montpellier, France International REUSE MUSE Stakeholder-Workshop: Agricultural Water Reuse - how to address health and environmental challenges? Präsentationen aus dem Projekt:
    - Sandra PEREZ SOLSONA (IDAEA – CSIC, Spain), Fate and uptake of pharmaceuticals and their metabolites in crops irrigated with wastewater-laboratory and field studies
    - Peter SCHRÖDER, Yvonne Bigott, Andres Sauvetre (COMI, Germany), Plant microbe interaction: the role of endophytes in phytoremediation of pharmaceuticals
- Projektmeeting in Barcelona am 27.11.2019. Dort wurde der Fortschritt sämtlicher Arbeitspakete durch die involvierten Gruppen vorgestellt sowie die Planung gemeinsamer Projekte vorangetrieben.
- Zwei Vorträge am 29.11.2019 während der "2nd International Conference on Risk Assessment of Pharmaceuticals in the Environment" in Barcelona.
    - Peter SCHRÖDER, Yvonne Bigott, Andres Sauvetre (COMI, Germany), Plant microbe interaction: the role of endophytes in phytoremediation of pharmaceuticals
    - Yvonne BIGOTT, Peter Schröder (COMI, Germany), Influence of Diclofenac (and Lamotrigine) on the diurnal expression pattern of stress genes in *Lactuca sativa*
- Planung und Beginn der Vorbereitungen für das finale AWARE Projektmeeting, das am 07.-08.04.2020 mit Stakeholder-Beteiligung am Helmholtz Zentrum München hätte stattfinden sollen. Wegen der Covid-19 Pandemie wird es nun jedoch in Zukunft mit Vorträgen zu den Endergebnissen online nachgeholt.

8. Literaturverzeichnis

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9. Anhang

Poster auf der Tagung “17th International Conference on Chemistry and the Environment (ICCE)” in Thessaloniki, GR, September 2019.

Water JPI — Water challenges for a changing world — AWARE

AWARE – Uptake and metabolization of lamotrigine and diclofenac by *Lactuca sativa*

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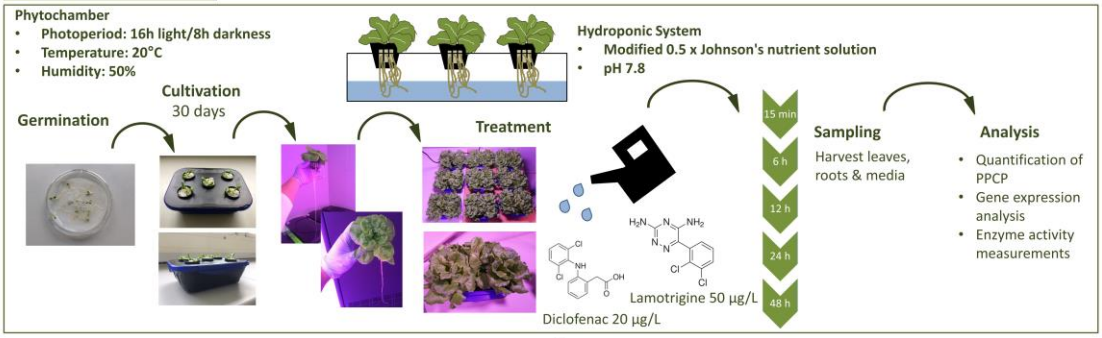
AWARE

Main aims of AWARE:

- Investigate detoxification of xenobiotics by plants and changes in plant physiology with focus on crop quality
- Scrutinize pharmaceuticals & personal care products (PPCP) fate and attenuation processes in soil
- Determine impact of PPCPs on soil microorganisms & earthworms

→ **A**ssess the fate of pesticides and **W**aterborne contaminants in **A**gricultural crops and their **E**nvironmental **R**isks

Experimental setup



Stress gene expression during exposure

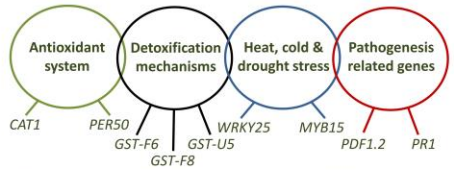


Figure 1: Genes analyzed by RT-qPCR (real-time quantitative PCR) to determine the gene expression during exposure to two chemically different pharmaceuticals (diclofenac and lamotrigine). CAT: Catalase; PER: Peroxidase; GST: Glutathione S-transferase; WRKY & MYB: Transcription factors; PDF: Plant defensin; PR: Pathogenesis related gene.

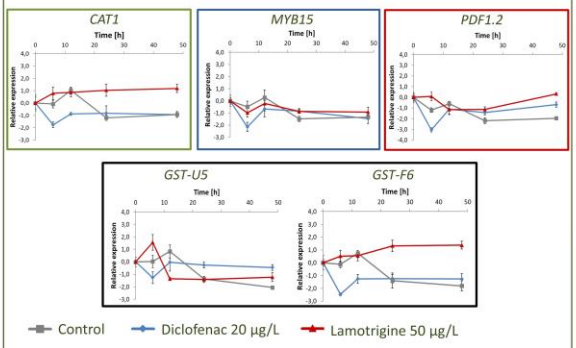


Figure 2: Relative Expression [fold change to T0] of selected genes in lettuce roots grown in hydroponic system treated with either diclofenac or lamotrigine. Only the glutathione S-transferase genes of the Phi class, which are known to be induced by H_2O_2 as well as genes coding for antioxidant enzymes (known to detoxify H_2O_2) were upregulated in lamotrigine treated plants. Other genes which are heat, cold & drought stress induced were not influenced by the treatment. A transient decrease of gene expression of several stress genes could be detected after 6 hours in diclofenac treated plants.

Diurnal expression pattern in controls

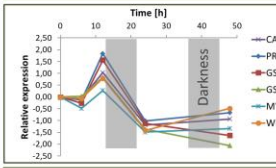


Figure 3: Relative gene expression [fold change to T0] in roots of untreated plants under controlled conditions in a phytochamber with 16h light/8h darkness. photoperiod Most of the studied genes showed a **similar expression pattern**.

Antioxidant defense and detoxification activity

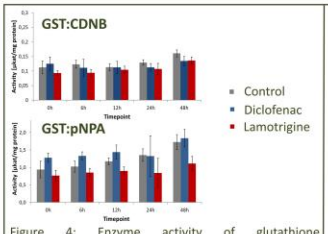


Figure 4: Enzyme activity of glutathione S-transferase measured with two substrates. No differences could be detected with 1-Chlor-2,4-dinitrobenzol (CDNB), and a **decrease of activity in leaves of lamotrigine treated plants** was observed with P-Nitrophenylacetat (pNPA).

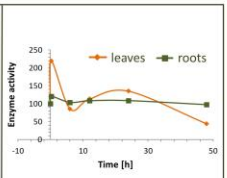


Figure 5: Peroxidase activity in roots and leaves of diclofenac treated lettuce. After 15 min **the activity of the enzyme increased transiently** especially in the leaf samples. This effect was not detectable in lamotrigine treated samples.

Conclusion

→ Relatively low concentrations of lamotrigine and diclofenac triggered different changes in the expression of stress genes and enzyme activities

- Diclofenac caused an oxidative burst demonstrated by a high peroxidase activity after 15 min
- Lamotrigine triggered an increased expression of H_2O_2-dependent genes in lettuce

➢ In the future these results will be supported by H_2O_2- & lipid peroxidation measurements as well as by quantification of the pharmaceuticals and metabolites in plant tissues

10. Anhang: Originalartikel

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Manuscript Draft

Manuscript Number:

Title: Elucidating stress responses in lettuce exposed to the pharmaceuticals diclofenac and lamotrigine using a multidisciplinary approach

Article Type: VSI:Micropollutants in water

Keywords: Treated wastewater; Accumulation in plants; Lactuca sativa; Stress gene expression; Diurnal rhythm

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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 occurred 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.

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*Novelty Statement (maximum limit:100 words)

Statement of Novelty

This study showed for the first time that environmentally relevant concentrations of pharmaceuticals can significantly influence the expression of genes involved in the metabolization of xenobiotics in lettuce, even though concentrations were probably too low to induce measurable oxidative stress reactions. Moreover, these compounds possibly act as zeitgebers affecting the circadian expression of these genes. We also detected that the pharmaceuticals triggered different signal transductions. In the case of diclofenac alterations in gene expression were predominantly pronounced in the roots where the compound was localized, while lamotrigine caused a putative systemic response after its translocation to the leaves.

Elucidating stress responses in lettuce exposed to the pharmaceuticals diclofenac and lamotrigine using a multidisciplinary approach

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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 occurred 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.

Keywords

Treated wastewater, Accumulation in plants, *Lactuca sativa*, Stress gene expression, Diurnal rhythm.

Highlights

- Translocation of lamotrigine, but not of diclofenac to lettuce leaves
- No direct triggering of oxidative stress but significant changes of gene expression

- Altered gene expression localized in root tissue where diclofenac was present
- Translocated lamotrigine to leaves triggered putative systemic response to roots
- Pharmaceuticals possibly act as zeitgebers affecting the expression of stress genes

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 been shown in tomato plants (Christou et al. 2019).

Diclofenac ([2-(2,6-dichloroanilino) phenyl] acetic acid; DCF), is one of the most abundant pharmaceuticals in water derived from wastewater treatment plants and effluents (Pérez & Barceló 2008, Vieno & 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 DCF for 10 days. Moreover, other stress parameters like the ratio of oxidized/reduced thiols, and the peroxidation of lipids were significantly enhanced.

Apart from the oxidative stress induced by this compound, DCF can be rapidly metabolized in plants. This metabolization follows a pattern of three consecutive phases, first described in the “Green Liver” concept by Sandermann Jr (1992). During phase I, compounds are activated by oxidation, reduction or hydroxylation for the conjugation to

71 reactive groups such as amino acids or sugars during phase II. Enzymes like
72 glutathione *S*-transferases or glycosyltransferases catalyze these reactions. Conjugated
73 phase II metabolites are sequestered in vacuoles or cell walls during phase III. In
74 general, metabolization will reduce the toxicity of foreign compounds for the plant,
75 although during phase I activation ROS may be produced that need to be controlled by
76 scavenging enzymes. Huber et al. (2012) observed phase I and phase II metabolization
77 products of DCF in *Hordeum vulgare* (barley) and in hairy root cell cultures of
78 *Armoracia rusticana* (horseradish). The activated hydroxylated metabolite 4′OH-
79 diclofenac as well as the subsequently conjugated glucopyranoside were detected
80 already after three hours of exposure.
81 Similar to DCF, the anti-epileptic drug lamotrigine (LMG) is highly persistent in the
82 environment and can be detected in crops (Paz et al. 2016) even if the concentration
83 found in plant tissue is low and the specific translocation mechanism still unknown.
84 Therefore, Goldstein et al. (2018) hypothesized an adsorption of lamotrigine to the roots
85 or a trapping in root vacuoles with only limited transport to the shoots. Information
86 about lamotrigine-triggered stress responses in plants is lacking, but could provide
87 useful hints for the translocation and perception of this pharmaceutical.
88 Genes involved in abiotic stress responses are often expressed in diurnal rhythms.
89 Mutations in key circadian clock genes caused a greater sensitivity to salt, osmotic, and
90 heat stress in *Arabidopsis thaliana*, which demonstrates the importance of the diurnal
91 rhythms in the modulation of multiple stress responses (Kant et al. 2008). Many cold-
92 and drought-responsive stress genes are rhythmically expressed in *A. thaliana*
93 (Covington et al. 2008, Wilkins et al. 2010). Furthermore, Lai et al. (2012)
94 demonstrated a circadian-regulation of reactive oxygen species (ROS) response. ROS
95 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. In this context, (1) we quantified the concentrations of both pharmaceuticals and 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 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* 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%. Every 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 hours 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 into a fine powder for either RNA, enzyme or H_2O_2 extraction. For the analytical procedure, the plant cultivation and treatments were repeated and samples of time points 0, 6, 12, 24 and 48 hours 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 Montemurro and coworkers (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. The tubes were then vortexed for 2 minutes at 2500 rpm using a BenchMixer XLQ QuEChERS Vortexer (Benchmark Scientific, Sayreville NJ, US). After 1 hour 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 rested for another 30 minutes. Then 10 mL of acetonitrile and 50 µL of concentrated formic acid were added and the tubes were vortexed again. The Original QuEChERS extraction kit was added directly into the tubes and instantly hand shaken for 30 seconds. All tubes were vortexed again and centrifuged (4000 rpm, 10 min, 4 ° C). 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 (150mg PSA, 150mg C18, 900mg $MgSO_4$), 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 to total dryness under a nitrogen stream and then reconstituted with 1 mL of water/MeOH (90:10) solution and 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 an established protocol (Manasfi et al., in preparation). Briefly, 1 g of homogenized freeze-dried root tissue was transferred to a 50-mL falcon tube and hydrated with 8 mL of EDTA-McIlvaine buffer (pH=4), vortexed, and allowed to rest for 30 minutes. After adding 50 µL of IS mix, the tubes were vortexed (2500 rpm, 2.5 min) and rested for another 30 minutes. Then, 10 mL of acetonitrile was added to the samples and they were vortexed for 2 minutes 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 ° C) 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 and 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 the Supplementary Methods (SM).

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). The 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). Primer/gene-specificities were checked by PCR on cDNAs. A housekeeping gene coding for 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 prior to relative quantification by the $2^{-\Delta\Delta C_T}$ method (Livak & 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 DCF and LMG treatments compared to control, 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 and coworkers (2005), and protein content was quantified (Bradford 1976) before measuring enzyme activities in a 96-well spectrophotometer (Spectra MAX 190, Molecular Devices, Germany). GST activity was determined at 400 nm (ε = 17.2 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.

220

221 **3. Results and Discussion**

222 **3.1. Uptake and translocation of pharmaceuticals in lettuce**

223 The highest concentration of DCF was detected in root tissue 6 h after treatment
224 (6.02 µg g^{-1} DW) and a significant reduction of this concentration occurred during the
225 experiment. Simultaneously the analysis of DCF treated root samples revealed the
226 formation of the metabolite 4'-hydroxydiclofenac at the same time point and onwards
227 (Figure 1). Corroborating our results, hydroxylated metabolites had been already
228 detected after 3 h of exposure in a hairy root cell culture of *Armoracia rusticana*
229 (horseradish) (Huber et al. 2012). We observed a rapid metabolization of DCF and a
230 higher concentration of the phase I metabolite than the initial compound after 24 h,
231 similar to results published for *Typha latifolia* by Bartha and coworkers (2014).
232 However, we were not able to detect DCF and the phase I metabolite
233 4'-hydroxydiclofenac in leaves of the treated lettuce plants at any time point. Similar to
234 our observation, in *Typha latifolia* exposed to high concentrations of DCF (1 mg L^{-1})
235 under hydroponic conditions, barely small amounts of the pharmaceutical (4% of
236 amount in roots after 24 h) were quantified in shoots (Bartha et al. 2014). Additionally,
237 it has been reported that only when plants were treated with DCF for a prolonged period,
238 this compound was translocated to tomato fruits (Christou et al. 2017) or to the leaves
239 of *Scirpus validus* (Zhang et al. 2012) at higher rates.
240 Unlike DCF, the concentration of LMG in lettuce roots increased during the first 6 h but
241 stayed constant at a similar concentration (2.14 ± 0.22 µg L^{-1}) afterwards until the end
242 of the experiment (Figure 2). Moreover, a translocation of LMG to the leaves in low but
243 increasing concentrations was detected. It has been proposed that LMG is restricted
244 from passing through plant cell walls or membranes because of its ionic character and

245 therefore rather accumulates in roots than in shoots (Chuang et al. 2019). At the initial
246 pH of the liquid media at pH 5.4, ~ 50% of LMG (pKa 5.7) is charged to form a cation.
247 Charged LMG putatively remains in the apoplastic space and is adsorbed to the root
248 surface, whereas uncharged LMG might be transported by passive diffusion into root
249 cells (pH 7 – 7.4) or to the leaves. After entering root vacuoles (pH 4 – 5.5) the
250 molecule is again charged and cannot pass the tonoplast (Nason et al. 2018).
251 Consequently, the highest accumulation of LMG was detected in roots and only low
252 concentrations were translocated to leaves (Figure 2).
253 In general, our findings highlight a putative passive transport of LMG to leaves, which
254 occurs in low concentrations and at slow rates. This reduced mobility might be caused
255 by the cationic charge of the molecule depending on the pH. The high hydrophobicity of
256 DCF is hypothesized to be the main reason for the missing translocation for this
257 compound to aboveground tissues. As already reported in previous studies, the octanol-
258 water partitioning coefficient (log *Kow*) plays a crucial role to predict the uptake of
259 xenobiotics by plants (Briggs et al. 1982). Highly hydrophobic substances (like DCF;
260 log *Kow* = 4.51) have a large potential for bioconcentration in roots but a low possibility
261 for translocation to shoots and leaves. Moreover, when DCF had entered plant tissue,
262 the molecule underwent rapid metabolization, as was observed by a decrease of the
263 parent compound and a simultaneous increase of the phase I metabolite (Figure 1). Such
264 decrease was not verified for LMG in the present study, but there is also no information
265 about possible metabolisms in plants available in the literature.
266 None of the pharmaceuticals was present in control plants growing in liquid media only.
267 Moreover, for the tested concentrations and exposure time of LMG or DCF neither
268 visual signs of toxicity nor changes in growth were observed in lettuce (Figure S1).

However, the time of exposure was only 48 h and at low concentrations (DCF: 20 µg L^{-1}; LMG: 60 µg L^{-1}).

Concentrations of LMG were also analyzed in liquid media of treated plants and plant-free control groups. During the 48h-experiment, we detected a relatively stable relative concentration of LMG in the plant-free control groups (Figure S2), showing there was negligible loss of the pharmaceutical by sorption to perlite or non-plant related photo- or biodegradation. When plants were present, the initial concentration of 58.32 ± 6.74 µg L^{-1} of LMG in the nutrient media was reduced to 45.48 ± 2.96 µg L^{-1} after 48 h (Figure 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 at high concentrations. The concentration of H_2O_2 in LMG-treated roots and leaves was significantly higher (p–values ≤ 0.001) after 12 h compared to control plants (Figure 3). For the other time points no difference was detected, indicating that LMG is not triggering cellular ROS production but rather a transient oxidative burst, as has been shown for *Salvia officinalis* leaves after they had been exposed to ozone for 5 hours (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 LMG for the first time in the lettuce leaves. We postulate that this oxidative burst might have appeared due to systemic signaling activities from leaves to roots triggered by the presence of LMG or its metabolites in the leaves. Whether LMG 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 LMG published to date, we were not able to test this hypothesis.

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

296 **3.3. Gene expression analysis**

297 Our earlier work showed that the two genes *GST-F6* and *GST-U5* were induced in roots
298 in *Brassica* upon Paracetamol treatment (Bartha 2012). However, the influence of
299 residual pharmaceuticals in water on the circadian rhythm/control of stress signaling
300 genes in plants has not been investigated so far. We determined the expression of these
301 two genes as well as of an additional GST (*GST- F8*) and two other genes involved in
302 the detoxification of ROS (*PER50 and CAT1*) in lettuce after the exposure to DCF or
303 LMG over a time period of 48 h. The expression of all tested genes in the control plants,
304 without exposure to any pharmaceuticals, followed a diurnal pattern over the duration of
305 the experiment (Figure 4; A and B). In lettuce roots, all five tested genes showed
306 maximal expression in the last hour before subjective dusk (T12 and T36), whereas in
307 the leaves the peaks of the expression were observed at different time points for
308 different genes. We detected highest expression of the genes coding for the two GSTs
309 belonging to the plant specific phi class (*GST-F6* and *GST-F8*) during the first 8 h after
310 subjective dawn (T6 and T30), the one coding for the peroxidase (*PER50*) in the last
311 hour before subjective dusk (T12 and T36) (Figure 4; B and D). The diurnal cycles of
312 gene expression in shoots and roots of plants are not usually in-sync. This had been
313 demonstrated in a previous study comparing the circadian clock in roots and shoots in
314 *Arabidopsis*. The rhythmic behavior of gene expression markedly differed between
315 tissues. In this context, a photosynthesis-related signal from the shoots had been
316 identified, affecting the setting of the clock in the roots (James et al. 2008). However,

317 the rhythmic diurnal expression of these genes in lettuce has not been described so far,
318 which makes this an interesting and novel observation.
319 As an exception to the obvious diurnal expression pattern, the gene coding for the tau-
320 class GST (*GST-U5*) was expressed at constant levels in lettuce leaves in control plants.
321 A constitutive expression of the gene *GST-U5* in leaves had been reported previously,
322 suggesting its housekeeping functions (Wagner et al. 2002) although it had also been
323 found to be induced by auxin in roots by another study (van der Kop et al. 1996).
324 Interestingly, the expression of *GST-U5* was significantly increased over all analyzed
325 time points in LMG treated lettuce leaves compared to control plants, indicating a
326 LMG-triggered effect on *GST-U5* (Figure 4; D).
327 All other tested genes (*PER50*, *CAT*, *GST-F6* and *GST-F8*) measured in LMG treated
328 plant roots, which were previously shown to be induced by H_2O_2 (Chen et al. 1996,
329 Guan et al. 2000, Wagner et al. 2002) had a similar expression pattern, differing from
330 control plants (Figure 4; C). In general, we observed a phase shift in the diurnal
331 expression of the genes. There was a trend for an earlier increased expression after 6 h
332 and an enhanced expression over time for *PER50*, *CAT1* and *GST-F6* in roots. We
333 observed that the expression high and low peaks in the circadian rhythm were shifted
334 for most of the genes and their expression at T24, T36 and T48 was significantly
335 different to that in the control plants in roots and leaves (Figure 4 A-D; Table S5).
336 Shortly before this significant change in gene expression, we observed a significant
337 increase of the H_2O_2 concentration in both tissues at T12 in LMG treated plants,
338 highlighting the role of H_2O_2 in intracellular communication and its connection to
339 subsequent downstream signaling like changes in gene expression (Choudhury et al.
340 2017).

341 It has been shown that amongst several other signals, ROS, metabolism and nutrients
342 can act as zeitgebers (external or internal signals acting as time cues) which can affect
343 the functioning of circadian clock of the plants. They can affect a shift in the phase,
344 period or the amplitude of the circadian clock (Lai et al. 2012). The circadian clock has
345 been shown to influence several biological processes in plants, within a complex
346 network of pathways which has been studied in detail for Arabidopsis (Harmer et al.
347 2000, Lai et al. 2012). However, since such information is lacking for lettuce, we may
348 only postulate that LMG or its metabolites could either directly or indirectly act as a
349 stimulus (zeitgeber) or cause a disruption of the circadian clock in lettuce plants.
350 A significant transient reduction of the expression of all genes was observed at T6 in
351 roots of DCF treated plants (Figure 5). Moreover, the expression of *CAT1*, *PER50*,
352 *GST-F6* and *GST-F8* was also significantly reduced at T12. With decreasing
353 concentrations of DCF we detected a reduced influence on stress gene expression
354 compared to control plants in lettuce roots. In leaves, where we were not able to detect
355 DCF or its metabolite 4'-hydroxydiclofenac, the influence on stress gene expression
356 was generally low (Figure 6). Nevertheless, a reduced expression of stress genes might
357 lead to a decreased defense status against biotic and abiotic stressors and therefore to
358 higher susceptibility of the plant when the compound was present.

359 **3.4. Stress enzyme activity**

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

In *Typha latifolia*, POX activity was inhibited during the first 14 days of 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, the translocation of LMG to lettuce leaves is relatively low; hence we measured no significant change of POX activity in the leaves compared to control plants. Plant peroxidases were reported to oxidize DCF to activate the molecule for further conjugation (Huber et al. 2016). When *Typha latifolia* was incubated with 1 mg L^{-1} of DCF, 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 (Figure 7).

The activity of enzymes involved in the conjugation of activated xenobiotics to glutathione during detoxification processes was comparable between DCF (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 DCF concentrations (100 µg L^{-1}) caused significantly increased *Lemna* GST activities. Moreover, no change of GST activities was caused by the exposure to LMG, 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 LMG by lettuce roots and the low translocation to the leaves. In contrast, the concentration of DCF in the tissue seemed too low to induce a change of enzyme activities.

4. Conclusions

Our results indicate that low concentrations of DCF and LMG 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 DCF were predominantly pronounced in the roots where the pharmaceutical was localized whereas LMG triggered a putative systemic response after the pharmaceutical was translocated to the leaves. We show for the first time that pharmaceuticals like LMG and DCF can possibly act as signals or zeitgebers, which can 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 constant presence of various pharmaceuticals in wastewater and their uptake by crops may influence the expression of plant stress genes in different ways. Especially the circadian dysfunction of 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.

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418

419 **Conflict of Interest Statement**

420 The authors declare that this research was conducted in the absence of any commercial
421 or financial relationships that could be construed as a potential conflict of interest.
422

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427

428 **5. Sources**

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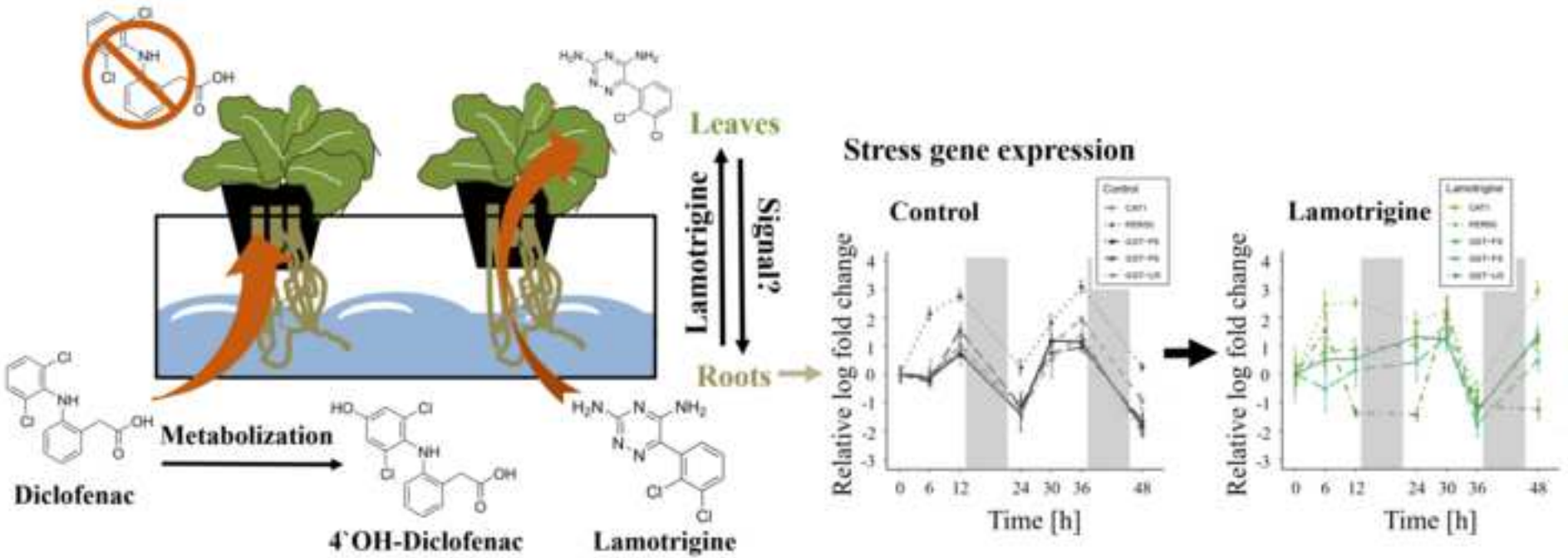
Graphical Abstract



Figures

Elucidating stress responses in lettuce exposed to the pharmaceuticals diclofenac and lamotrigine using a multidisciplinary approach

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Contents

47 0.05, “**” for 0.001 ≤ p–value ≤ 0.01, and “***” for p–value ≤ 0.001. Grey bars:
48 subjective night.
49 **Figure 6:** Relative expression (log fold change) in lettuce leaves of three glutathione
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53 are indicated according to Tukey`s HSD pairwise testing as “*” for 0.01 ≤ p–value ≤
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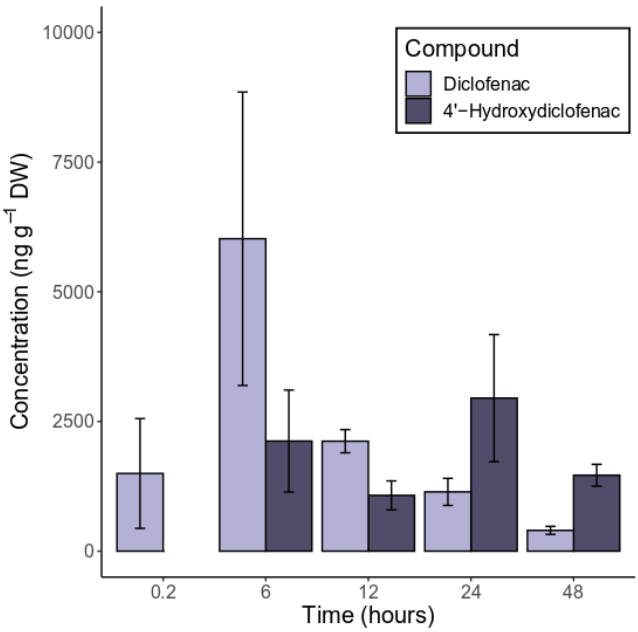


Figure 1: Concentration of diclofenac and its metabolite 4'-hydroxydiclofenac (ng g^{-1}) in lettuce roots of diclofenac treated groups. Data are mean concentrations (dry weight, DW) ± standard error (n = 3).

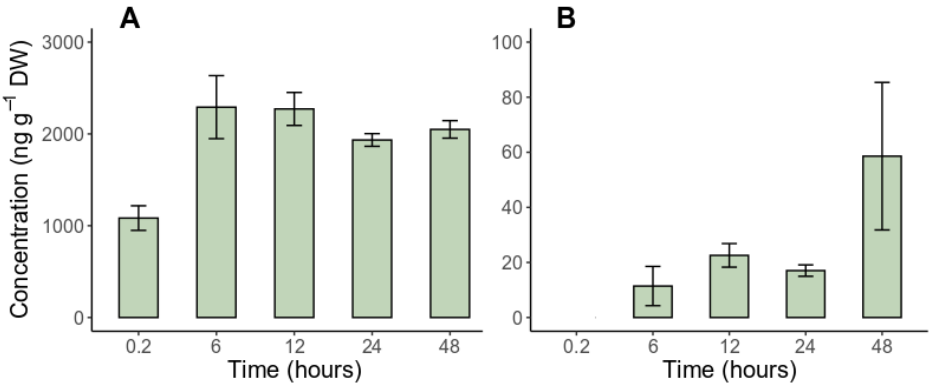


Figure 2: Concentration of lamotrigine (ng g^{-1}) in lettuce tissue ((A) roots, (B) leaves) of lamotrigine treated groups. Data are mean concentrations (dry weight, DW) ± standard error (n = 3).

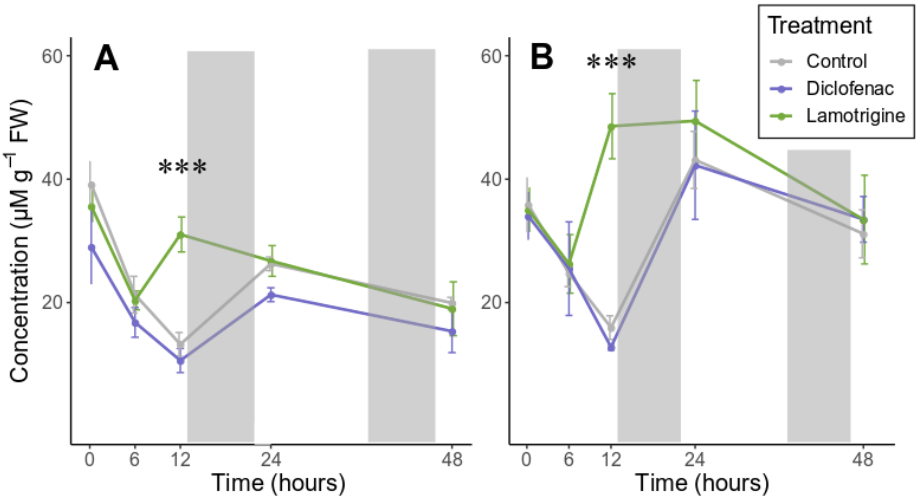
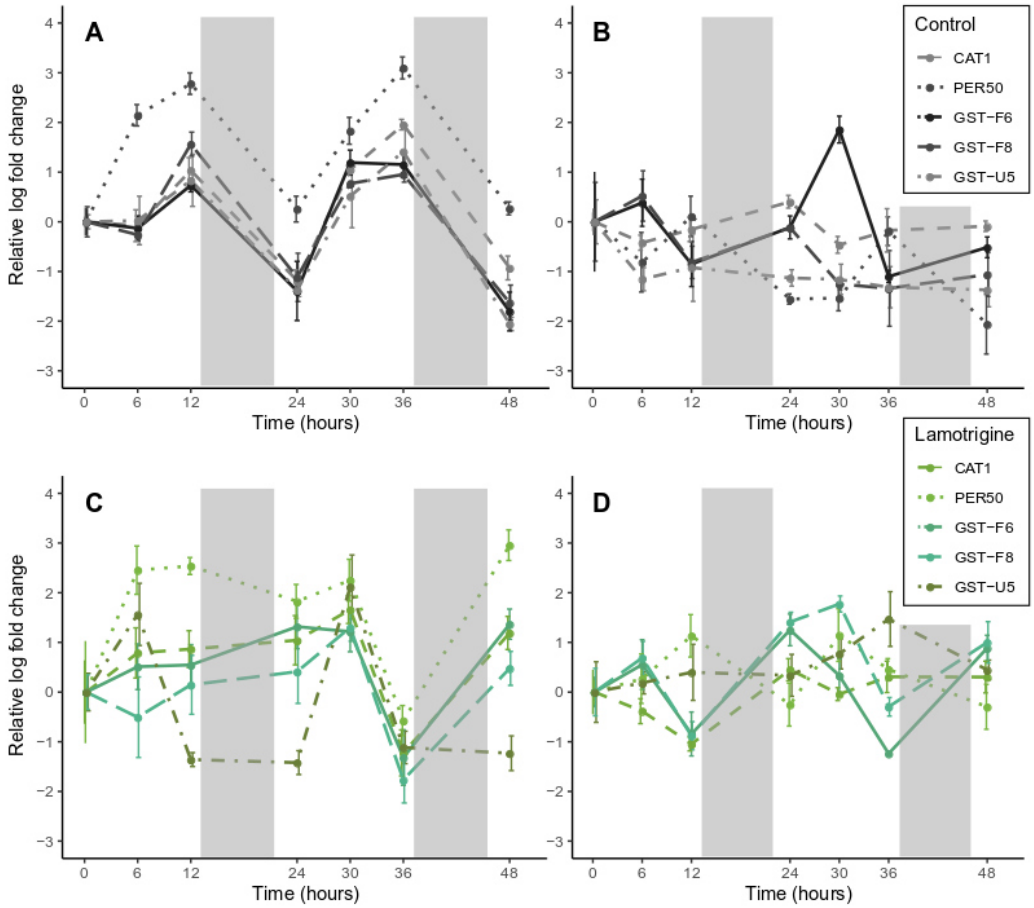


Figure 3: Concentration of hydrogen peroxide (µM g^{-1}) in lettuce tissue ((A) roots, (B) leaves) in control plants and diclofenac or lamotrigine treated groups. Data are mean concentrations (fresh weight, FW) ± standard error (n = 3). Significant differences between treated groups and control plants are indicated according to ANOVA as "***" for p–value ≤ 0.001. Grey bars: subjective night.



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79 **Figure 4:** Relative expression (log fold change) of three glutathione *S*-transferases
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81 in (A+B) control and (C+D) lamotrigine treated lettuce in different plant tissues ((A+C)
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83 differences were observed in the expression pattern of all genes when compared to
84 control plants at different time points revealed by Tukey`s HSD pairwise testing (Table
85 S 1). Grey bars: subjective night.

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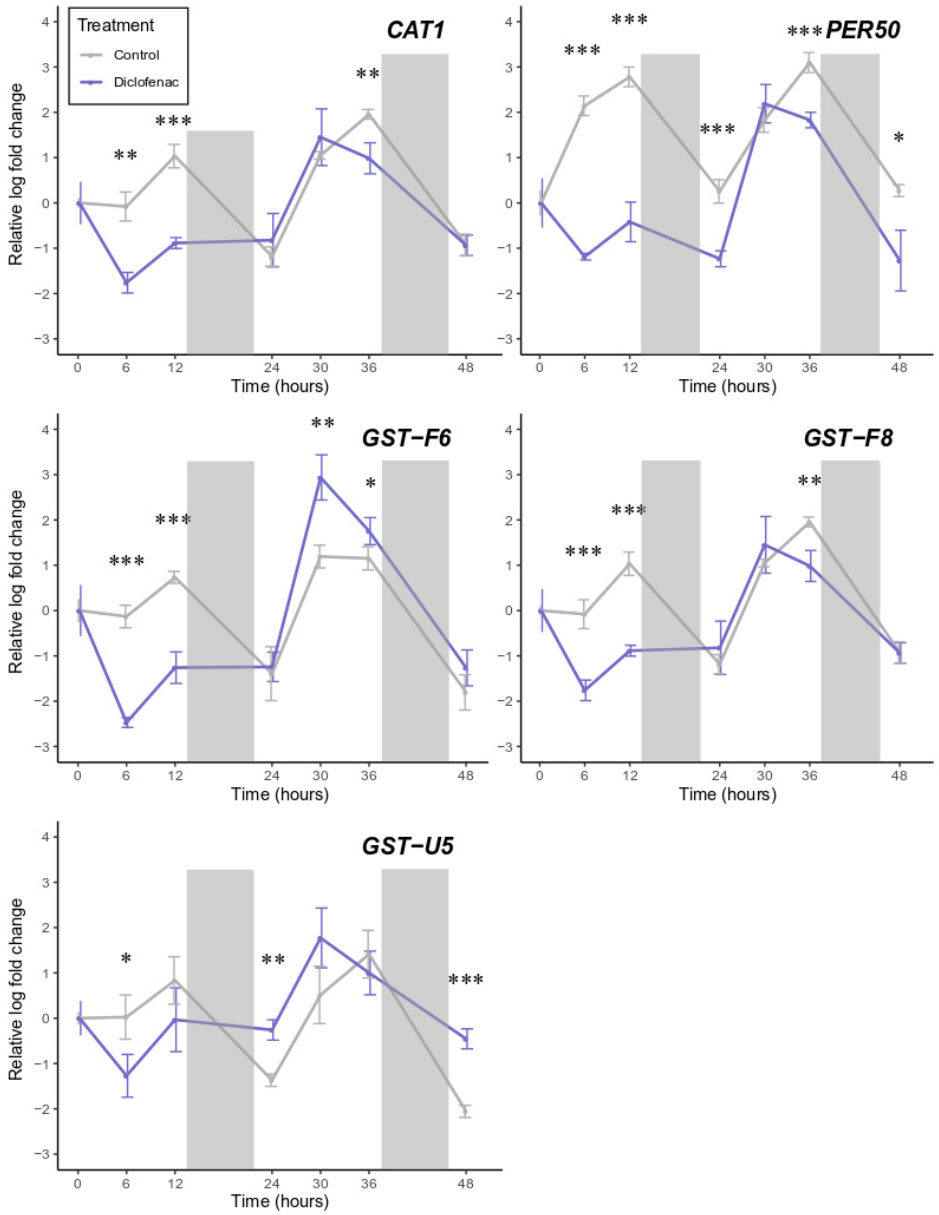

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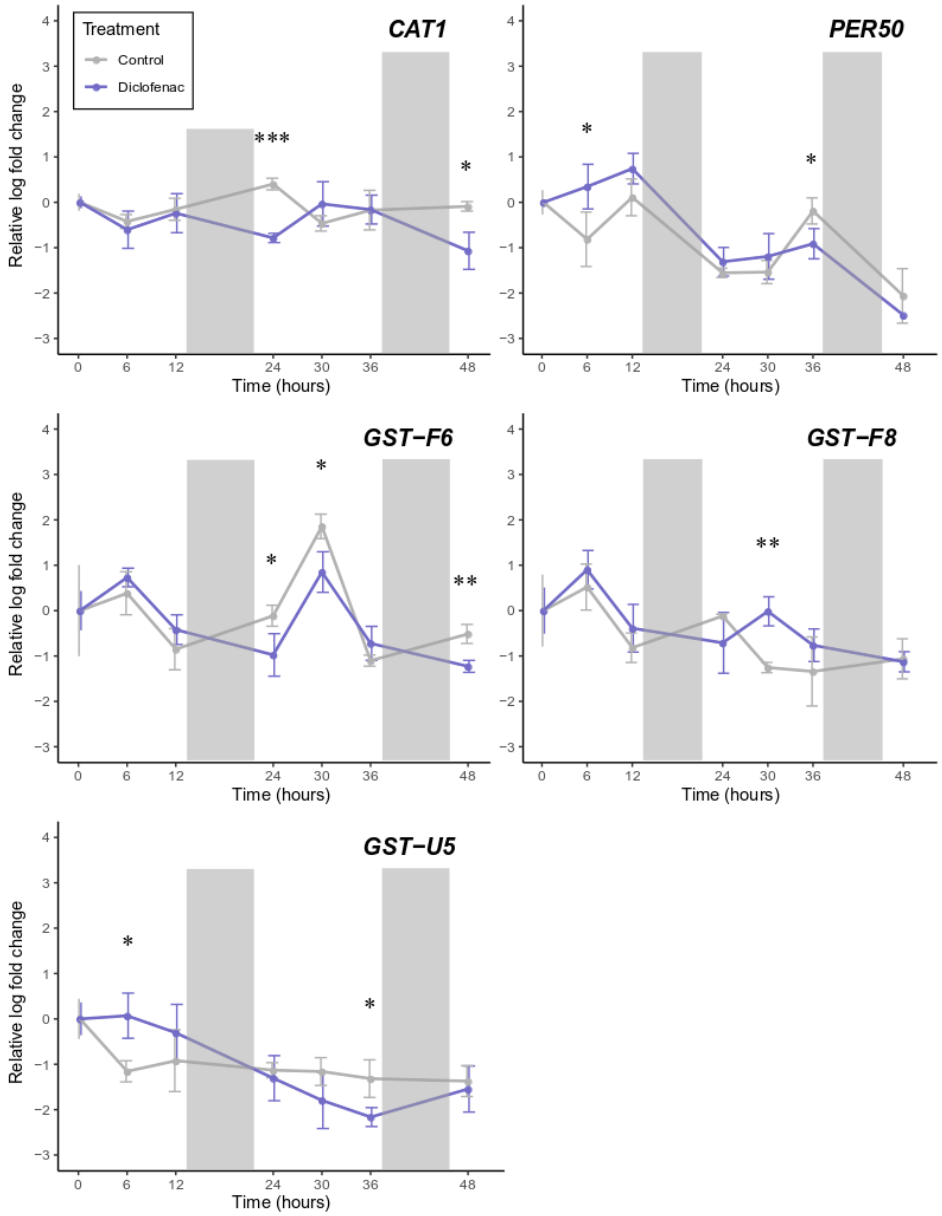

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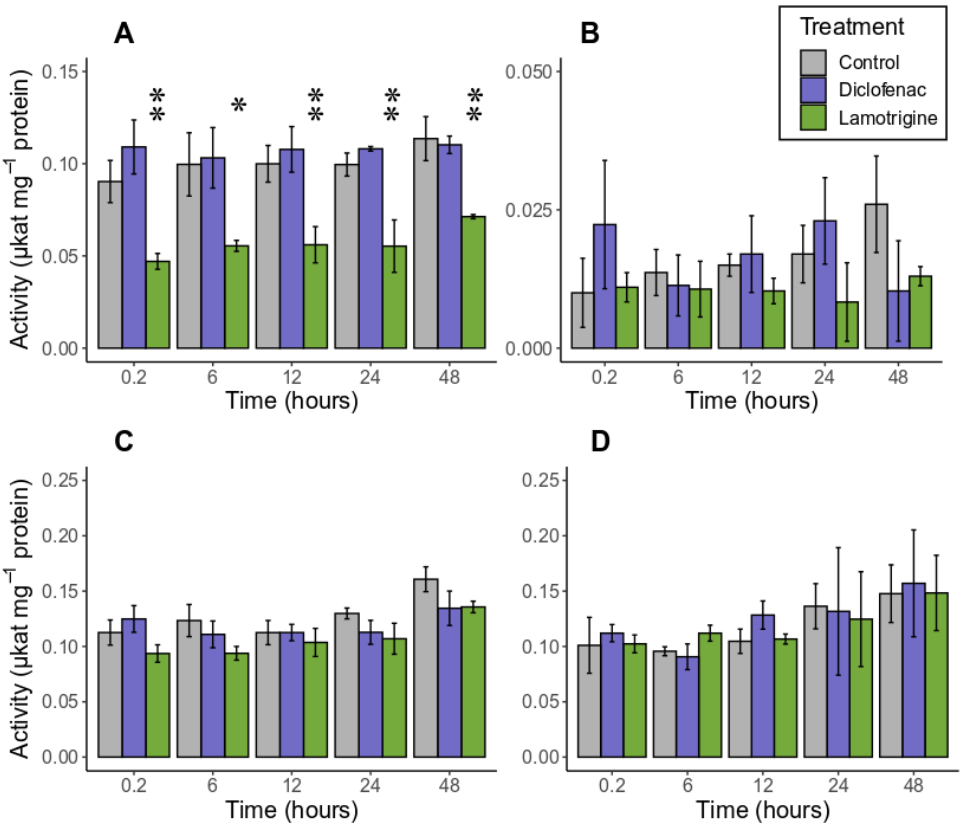

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Anhang: Buchkapitel

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

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

Contents

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 used to perform a meta-analysis approach 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 transformation like hydrolysis, which can occur during wastewater treatment or in the soil environment, redox reactions may occur in the clay fraction through reactive mineral phases, influencing 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. 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

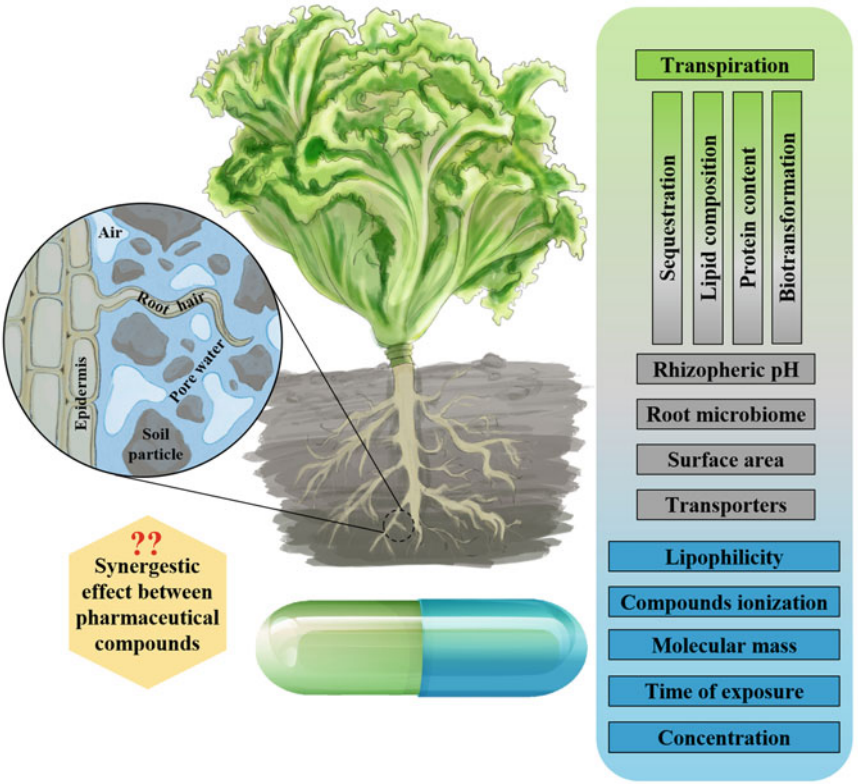

Fig. 1 Multiple parameters, which play a critical role on plants' uptake of pharmaceuticals along with their distribution among different plant organs

carbamazepine was not affected in presence of lamotrigine in cucumber plants (*Cucumis sativus*) grown under hydroponic conditions [10]. Moreover, the 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 versus 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 a 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 plant growth promoters and 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 Chap. 12).

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, 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. pKa) of a molecule in aqueous solution [24]. AU5

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 point 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 (available 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 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 solids 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

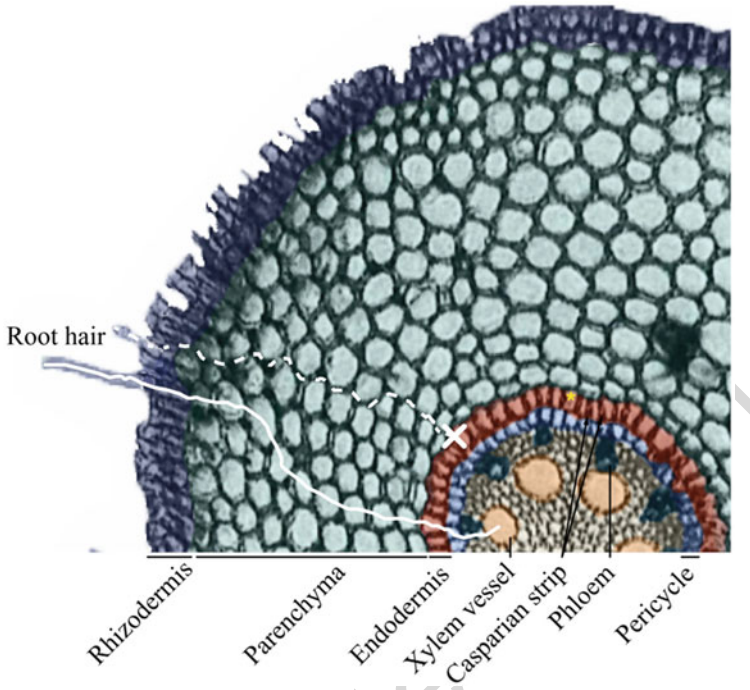

Fig. 2 Cross section of an iris (*Iris pseudacorus*) root. Diffusive uptake of chemicals can occur via the apoplast, i.e. through the cell wall continuum (dotted line). However, at the endodermis with its thickened suberized cell walls (Casparian strip; red), diffusive apoplastic transfer is stopped. 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

compounds is transported via the symplastic movement into cellular vacuoles [39]. Once in the symplast, these compounds tend to move from xylem to phloem and vice versa and thus are being transported predominantly 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* all enhance transpiration rates. Transpiration rates determine 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), sorb to lipids in plant cells and membranes or to hydrophobic xylem vessels, hindering their translocation [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 in [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*-

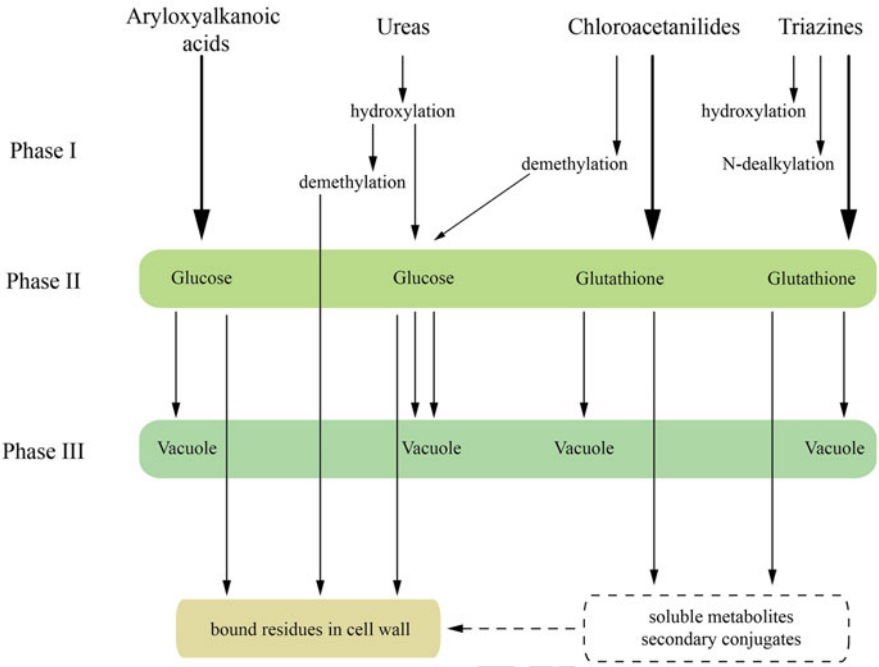

Fig. 3 The metabolic cascade of the green liver concept implies three phases for the fate of herbicides and foreign compounds in plants. It can be assumed that pharmaceuticals follow the same routes. While many compounds are finally bound to cell wall material to form insoluble residues, other xenobiotics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

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 insoluble residues in the cell wall (*bound residues*) [80] (see Chap. 9 for more detailed information). 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 transported in plants via the vascular tissue [87, 88] (more details in Chap. 10). 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 analyses 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, there 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, TF > 1 means that the target compound was effectively translocated from roots to shoots. In contrast, 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 meta-analysis 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 analyses, 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

t1.1 **Table 1** Chemical properties of neutral pharmaceuticals, as well as the average BCFs and TFs calculated per compound in hydroponic studies

t1.2 Compounds	log K_{OW}	pH	p*Ka*	log D_{OW}	BCF	TF	Authors
		Average values					
t1.4 *Analgesic*							
t1.5 Acetaminophen	0.46	5.60	0.00	0.09	1.43	0.49	[20, 58, 107–111]
t1.6 *Antibacterial*							
t1.7 Triclocarban	4.34	na	0.00	5.23	31.39	0.01	[109, 110]
t1.8 *Antibiotic*							
t1.9 Sulfamethoxazole	0.89	5.59	0.00	−0.06	0.55	0.13	[11, 109, 110, 112–114]
t1.10 Sulfapyridine	0.35	6.53	0.00	4.21	3.29	0.03	[105]
t1.11 *Anticonvulsant*							
t1.12 Carbamazepine	2.45	6.23	0.00	3.64	0.93	2.05	[10, 11, 20, 110, 114–119]
t1.13 Primidone	0.91	na	0.00	−1.23	1.61	0.17	[109]
t1.14 *Hormone*							
t1.15 17β-estradiol	0.20	na	0.00	4.33	2.01	1.11	[106]
t1.16 17α-ethinylestradiol	3.67	5.30	0.00	4.94	0.98	1.04	[106, 112, 114]
t1.17 Beta-estradiol	3.67	5.55	0.00	4.33	0.01	nd in leaf	[20, 114]
t1.18 Estrone	3.13	5.55	0.00	4.23	0.13	0.07	[20, 114]
t1.19 Levonorgestrel	3.48	na	0.00	4.27	17.26	nd in leaf	[101]
t1.20 *Lipid regulator*							
t1.21 Atorvastatin	6.36	na	0.00	2.38	0.48	0.26	[109]
t1.22 *Psychotropic drug*							
t1.23 Meprobamate	0.70	na	0.00	1.16	0.37	6.11	[109, 110]
t1.24 *Stimulant*							
t1.25 Caffeine	−0.07	5.68	0.00	0.95	0.32	12.06	[20, 109, 114, 116, 119, 120]

t1.26 *Symbols: na*, means not available; *nd*, means not detected

as hypothesized for pharmaceuticals with molar mass ≥400 g/mol [20]. None of the studied pharmaceuticals was ≥1,000 g/mol, and only eight of them can be 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 t2.1

Compounds	log K_{OW}	pH	p*Ka*	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

t3.1 **Table 3** Chemical properties of anionic pharmaceuticals, as well as the average BCFs and TFs calculated per compound in hydroponic studies

t3.2 Compounds	log K_{OW}	pH	p*K*a	log D_{OW}	BCF	TF	Authors
	Average values						
t3.4 *Antibacterial*							
t3.5 Triclosan	4.76	5.47	−0.01	5.42	1.50	0.20	[9, 19, 41, 55, 117]
t3.6 *Antibiotic*							
t3.7 Ofloxacin	−0.39	7.80	−0.16	0.74	0.01	nm in leaves	[131]
t3.8 Oxytetracycline	−0.90	5.97	−0.15	−6.49	0.59	0.02	[25, 55]
t3.9 Sulfadiazine	−0.09	6.81	−0.88	1.04	14.87	0.15	[46, 87]
t3.10 Sulfamerazine	0.14	6.81	−0.58	3.54	25.98	0.03	[105]
t3.11 Sulfamethazine	0.89	6.85	−0.23	4.39	9.13	0.02	[105, 116]
t3.12 Sulfamethoxazole	0.89	6.85	−0.002	−0.06	16.61	0.02	[105]
t3.13 Sulfapyridine	0.35	6.91	−0.01	4.21	11.78	0.03	[105]
t3.14 Tetracycline	−1.30	na	−0.08	−5.44	0.15	3.18	[103]
t3.15 *Anticonvulsant*							
t3.16 Dilantin	2.47	na	−0.003	1.71	0.95	2.98	[41, 110]
t3.17 *Anti-inflammatory*							
t3.18 Diclofenac	4.51	6.61	−0.98	1.85	2.82	0.24	[43, 55, 56, 65, 101, 132]
t3.19 Ibuprofen	3.97	5.48	−0.90	2.25	0.21	1.52	[109, 110, 116, 133–135]
t3.20 Naproxen	3.18	5.65	−0.89	2.09	0.61	0.90	[109, 110, 114, 119]
t3.21 *Lipid regulator*							
t3.22 Clofibric acid	3.32	6.00	−0.99	1.20	1.37	1.59	[119]
t3.23 Gemfibrozil	4.77	na	−1.00	2.92	4.03	0.04	[109]

t3.24 *Symbols*: *na*, means not available; *nm,* means not measured

629 dynamic between BCF and TF reported in the two studies was the opposite of what
630 was expected (i.e. BCF > TF) but might be explained due to the high lipophilicity of
631 triclocarban (log K_{OW} = 4.34). Indeed, several models purposed different ranges
632 during which translocation is favoured or not. All of these models predicted a low
633 transfer for compounds with around log K_{OW} > 4 [25, 61, 140, 141].
634 Another neutral compound that stands out from the proposed observation was the
635 levonorgestrel (hormone). Li and co-workers [101] described a BCF average of 17.3,
636 where no compound was detected in stems and leaves. Therefore, further investiga-
637 tion is needed to scrutinize these results.
638 Sulfamethoxazole was another pharmaceutical, which was studied intensively in
639 hydroponic and soil experiments. This antibiotic and the analgesic acetaminophen,
640 which is also studied on several hydroponic experiments, showed a slightly higher
641 average BCF > TF. Moreover, as many observations were showing similar results
642 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	p*K*a	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

t5.1 **Table 5** Chemical properties of cationic pharmaceuticals, as well as the average BCFs and TFs calculated per compound in hydroponic studies

t5.2 Compounds	log K_{OW}	pH	p*K*a	log D_{OW}	BCF	TF	Authors
		Average values					
t5.4 *Antibiotic*							
t5.5 Clarithromycin	3.16	na	1.00	−1.98	9.91	0.04	[137]
t5.6 Lincomycin	0.20	5.80	1.00	−4.84	0.33	0.08	[20]
t5.7 Trimethoprim	0.91	5.80	0.88	0.09	2.45	0.23	[20, 97, 110, 112]
t5.8 *Anticonvulsant*							
t5.9 Lamotrigine	2.57	6.05	0.65	2.49	7.86	0.12	[138]
t5.10 *Antidiabetic*							
t5.11 Metformin	−2.64	6.00	1.01	−2.56	32.14	0.02	[65]
t5.12 *Beta-blocker*							
t5.13 Atenolol	0.16	7.80	0.98	−2.23	0.21	2.79	[11, 109, 131]
t5.14 Propranolol	3.48	na	1.00	0.15	0.92	0.22	[116]
t5.15 *Lipid regulator*							
t5.16 Gemfibrozil	4.77	5.30	0.81	3.56	–	0.06	[114]
t5.17 *Psychotropic drug*							
t5.18 Amitriptyline	4.92	7.00	1.00	3.60	29.85	1.11	[117]
t5.19 Clomipramine	5.19	na	1.00	2.35	0.18	0.62	[139]
t5.20 Diazepam	2.82	na	0.01	4.73	3.21	0.45	[109, 110]
t5.21 Fluoxetine	4.05	7.00	1.00	1.05	13.96	1.03	[110, 117]
t5.22 Sertraline	1.37	na	1.00	2.27	0.43	0.12	[139]
t5.23 Trazodone	3.21	na	0.10	3.97	0.09	2.89	[139]

t5.24 *Symbols: na*, means not available; – not possible to calculate

657 slower, when compared to cationic and neutral compounds due to electrostatic
658 repulsion between root surface and anionic substances. However, looking at data
659 from the hydroponic experiment of Tai and co-workers [105] (Table 3, Fig. 5a), high
660 BCF ratios of SAs, ranging from 9.1 to 26.0, were quantified in two wetland plant
661 species (Indian shot (*Canna indica*) and yellow iris (*Iris pseudacorus*)) in a 7-day
662 trial. In this work, authors suggested that plants take up SAs via active processes.
663 However, the high BCF values might be related to the plant lipid content, since it is
664 considered as the main storage site for hydrophobic organic contaminants, as
665 hypothesized by the same group. To support this hypothesis, a positive correlation
666 between the obtained BCF and the respective log D_{OW}, for several nutrient media
667 and soil articles (cited in Tables 3 and 4), was calculated (0.29 and 0.42, accordingly
668 ($p > 0.05$)). Nonetheless, for a specific antibiotic (tetracycline), the results were the
669 opposite (i.e. TF > BCF), meaning that this compound is rather translocated to the
670 aerial parts than being stored in roots [104], which can be explained by its hydro-
671 philic behaviour (log D_{OW} −5.44).
672 As observed for SAs, high average BCF > TF values for triclosan, diclofenac and
673 gemfibrozil were registered in hydroponic experiments (see Table 3). Several studies
674 focused on the antibacterial pharmaceutical triclosan, but only in some of them, high
675 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 t6.1

Compounds	log K_{OW}	pH	p*Ka*	log D_{OW}	BCF	*TF*	Authors t6.2
		Average values					
Antibiotic							t6.4
Lincomycin	0.20	7.58	0.75	−3.35	0.00	9.96	[102] t6.5
Anticonvulsant							t6.6
Lamotrigine	2.57	8.10	0.18	2.70	0.03	1.93	[102] t6.7
Antidiabetic							t6.8
Metformin	−2.64	na	1.01	−2.56	0.34	0.61	[126] t6.9
Beta-blocker							t6.10
Atenolol	0.16	6.96	0.99	−2.60	0.39	3.51	[124] t6.11
Propranolol	3.48	6.63	0.99	0.59	2.59	1.97	[125] t6.12
Psychotropic drug							t6.13
Chlordiazepoxide	2.44	6.30	0.43	−0.12	0.04	6.58	[130] t6.14
Clonazepam	2.41	6.30	0.01	3.56	0.01	16.82	[130] t6.15
Fluoxetine	4.05	6.25	1.00	1.07	0.04	0.24	[125] t6.16
Flurazepam	3.80	6.30	1.00	3.77	0.01	1.24	[130] t6.17

Symbols: *na*, means not available t6.18

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 and translocation should be favoured, however it is not the case. However, as it was 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*Ka* 0.8 to −0.99) in a very short pH interval (5.3–6).

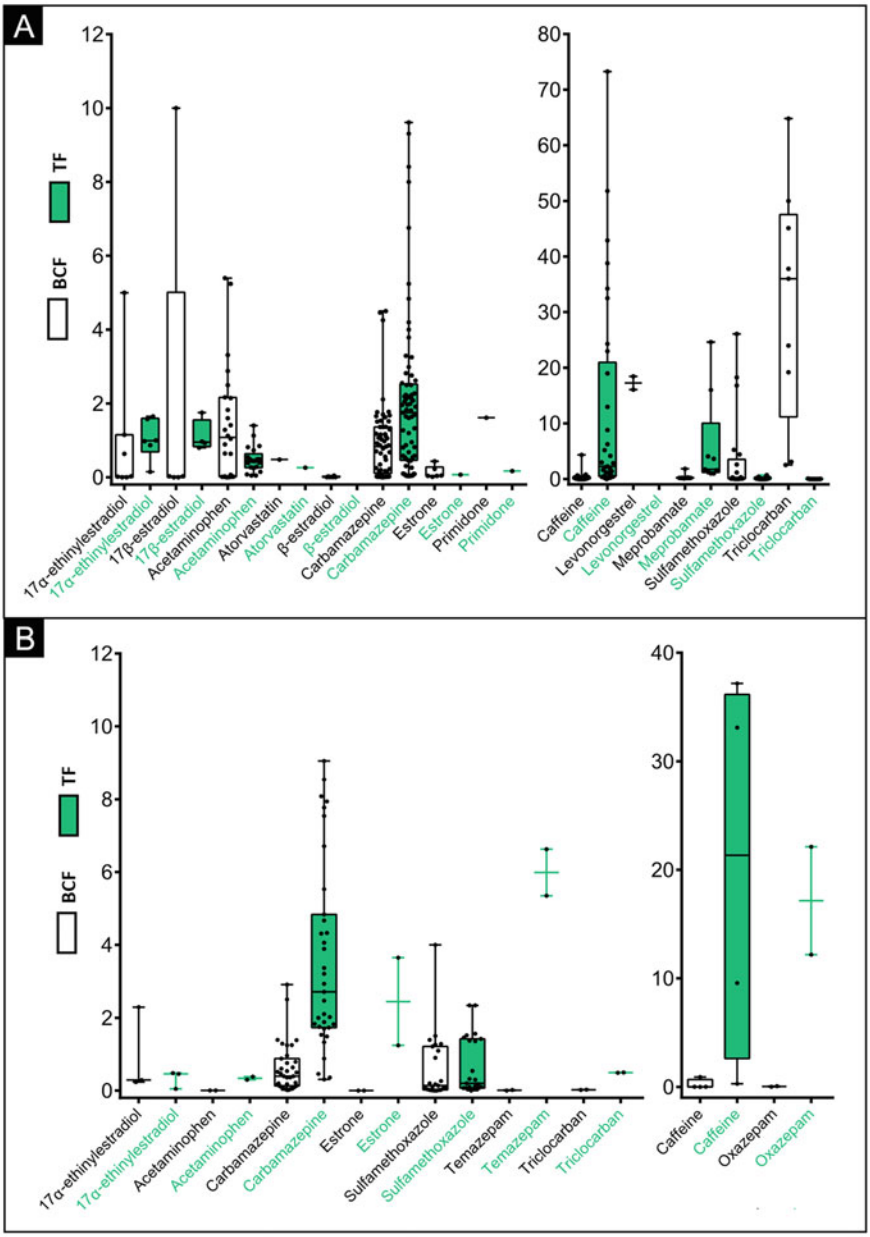

Fig. 4 Boxplot visualization of all BCF (black) and TF (green) values of several neutral compounds (every dot, represents an observation) from hydroponic (**a**) and soil studies (**b**); data references in Tables 1 and 2

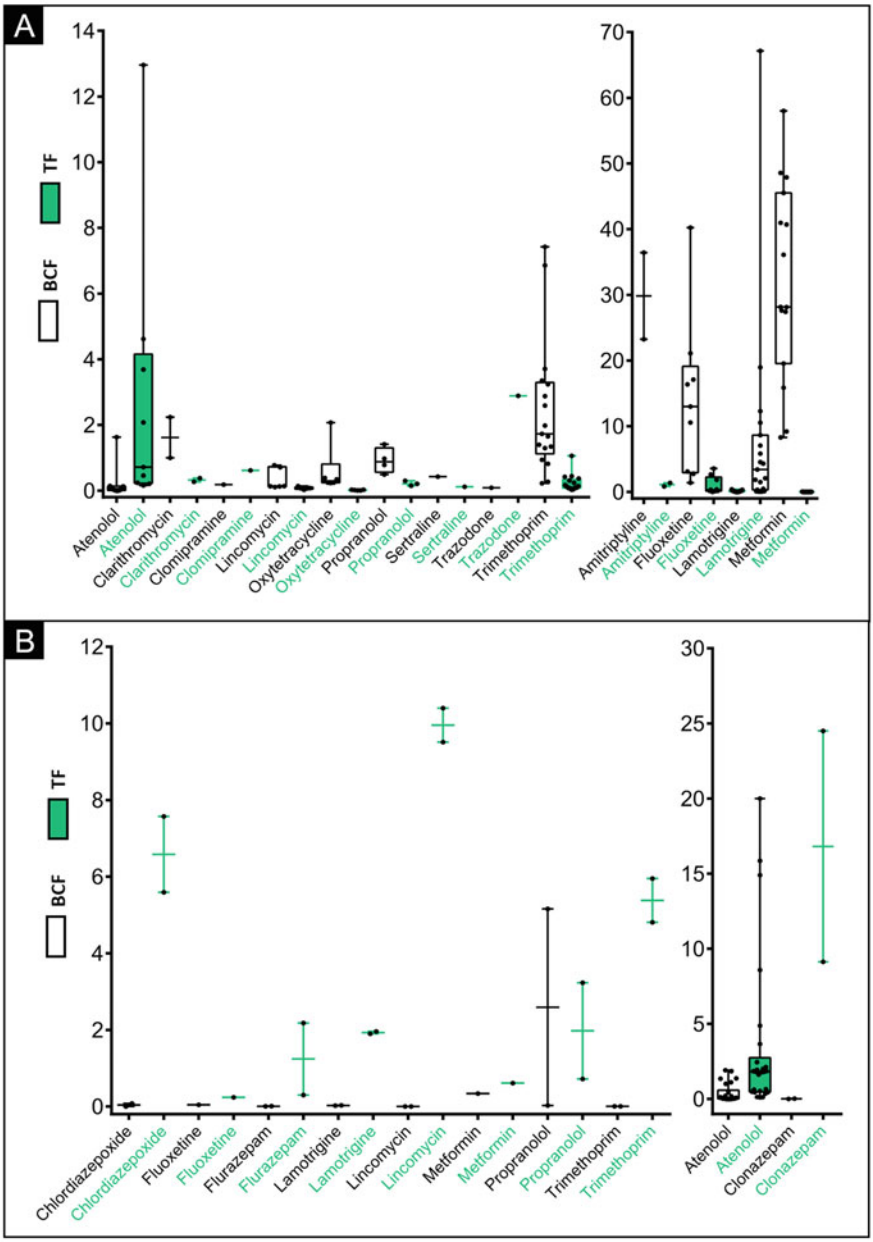

Fig. 5 Boxplot visualization of all BCF (black) and TF (green) values of several anionic compounds (every dot, represents an observation) from hydroponic (**a**) and soil studies (**b**); data references in Tables 3 and 4

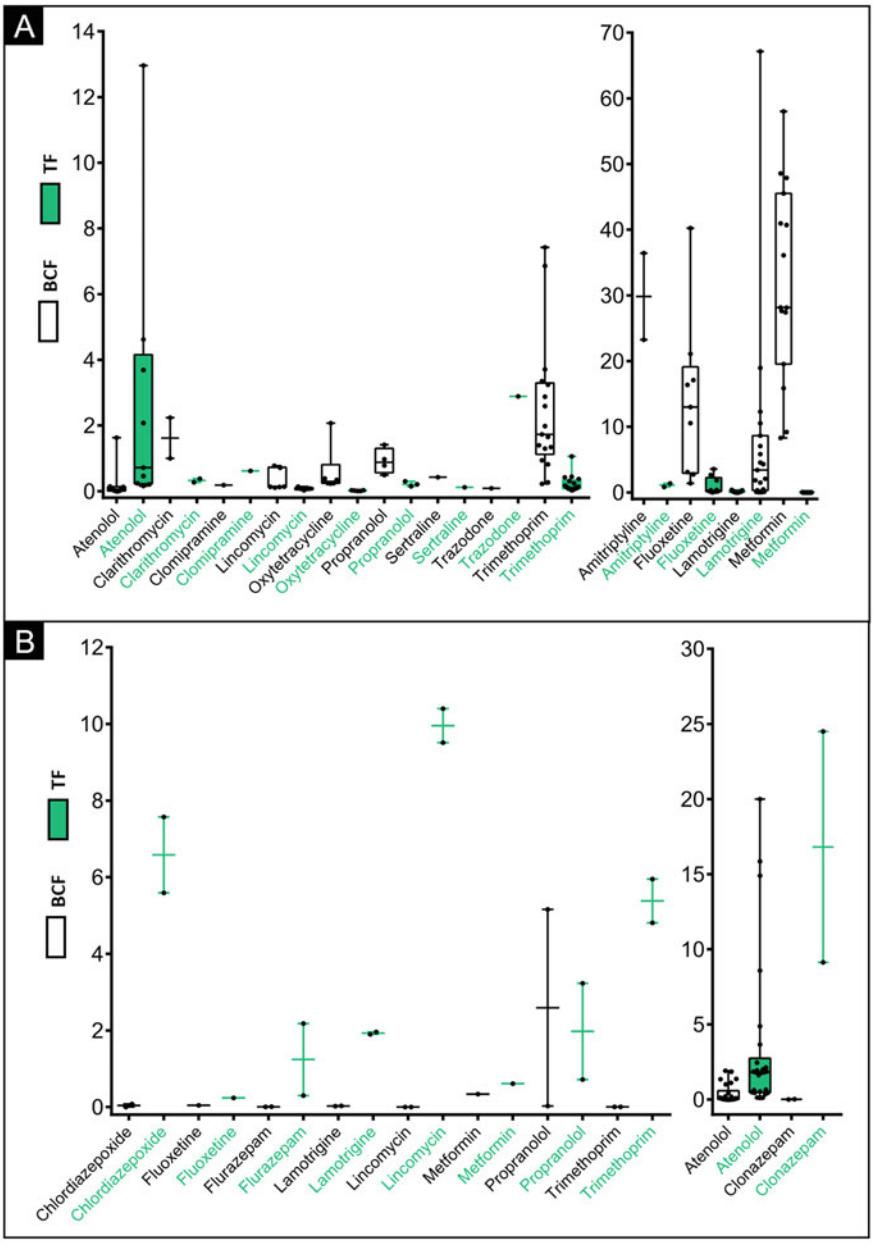

Fig. 6 Boxplot visualization of all BCF (black) and TF (green) values of several cationic compounds (every dot, represents an observation) from hydroponic (**a**) and soil studies (**b**); data references in Tables 5 and 6

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) (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 Conclusions from Data Analyses

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, the 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 sample collection time, in order to relate it to the concentration in the plant at that specific point of time 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 for compounds that their ionic status can easily change 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 as well, 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 both 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 analyses and interpretation.

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AWARE-Kurzfassung zum Abschlussbericht

Der Mangel an Wasserressourcen ist ein Hauptproblem der landwirtschaftlichen Lebensmittelproduktion. Um den enormen Wasserbedarf für die Bewässerung landwirtschaftlicher Flächen zu decken, wird bereits heute in einigen EU-Ländern behandeltes Abwasser verwendet. Solche regionalen Wassermanagementpraktiken machen aber organische Schadstoffe pflanzenverfügbar. AWARE zielte darauf ab, Schicksal und potenzielle Auswirkungen von durch Abwasser übertragene Kontaminanten (Arzneimittel und Pflegeprodukte) in landwirtschaftliche Kulturen und Böden zu untersuchen, um so deren Umweltrisiken zu bewerten. Diese Ziele wurden erreicht durch: i) Bewertung des mikrobiellen Abbaus von Pharmaka in der Rhizosphäre, ii) Untersuchung der Aufnahme und Metabolisierung ausgewählter Kontaminanten in Topfversuchen unter Verwendung von echtem oder versetztem Abwasser; iii) Abschätzung der ökotoxikologischen Auswirkungen von Kontaminanten auf mikrobielle Vielfalt und Bodenfunktionen sowie Untersuchung von Antibiotikaresistenzgenen; iv) Bewertung der Auswirkungen auf Regenwürmer, und v) Bewertung der Risiken der Verwendung von Abwasser zur Bewässerung von Kulturpflanzen in realem Maßstab. Dabei konnte das Helmholtz Zentrum München eine spezifische Beeinflussung der zirkadianen Rhythmik pflanzlicher Stressgene und der Aktivität pflanzlicher Stressenzyme durch Exposition mit unterschiedlichen Pharmazeutika zeigen, Hinweise auf die Metabolisierung von Lamotrigin geben und den Einfluss eines Arzneimittel-Cocktails in echtem Abwasser auf Diversität und Zusammensetzung der endopytischen und der Mykorrhiza- Gemeinschaft zeigen.
Der Mehrwert des AWARE-Projekts (CSIC, HMGU, INRAE, NIBIO, UM) entstand durch Nutzung unterschiedlichen Fachwissens und der perfekten Kombination zwischen Universitäten und Forschungseinrichtungen. Der Wissensaustausch mit Stakeholdern aus Industrie und Behörden trug dazu bei, die Diskussion über neuartige Bewässerungsstrategien mit abgesicherten Daten zu unterstützen.