PLANT ACCUMULATION OF PHARMACEUTICALS

By

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ABSTRACT

Pharmaceuticals are frequent contaminants in reclaimed wastewater throughout the world, and using reclaimed wastewater for irrigation of food crops is increasingly common in arid regions. Therefore, crop plants are regularly exposed to pharmaceuticals, and previous literature has shown that plants can accumulate pharmaceuticals in edible tissues under field conditions. Developing predictive capabilities for estimating pharmaceutical accumulation in plants is important, as monitoring produce for all potential contaminants is impractical, but simple correlations based on compound properties have had limited success. A lack of mechanistic knowledge hinders the creation of more complex models.

Here, we compile previous literature on plant uptake of pharmaceuticals and investigate various factors that are important for building a mechanistic understanding of pharmaceutical uptake. We demonstrate that changing the form of nitrogen available to wheat plants results in alteration of rhizosphere pH, which in turn affects plant accumulation of lamotrigine, an ionizable antiepileptic drug. Additionally, we demonstrate that spinach accumulation of four pharmaceuticals is driven by transpiration (water flow through the plant), though other factors are important for determining differences in accumulation between compounds. Furthermore, we find that exposing spinach to mixtures of pharmaceuticals has effects on *in planta* metabolism of some compounds. We also investigate species differences in plant uptake of the anti-epileptic drugs carbamazepine and lamotrigine, and find significant variation in accumulation, distribution within the plant and compound metabolism between species. Overall, our results show that accounting for plant nutrition, water uptake, and metabolism is important for understanding pharmaceutical accumulation, and that results from one plant species may not be applicable to others.

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INTRODUCTION

Human civilization requires access to fresh water. As population grows and water supplies become more unpredictable due to climate change, there is increasing concern regarding water scarcity. Irrigation accounts for 70% of water withdrawals for human benefit and supports roughly 40% of global food output, with future increases expected.[1] Reuse of treated wastewater (TWW) is an important strategy for decreasing demand from water sources that are not replenished as quickly as water is withdrawn. Use of TWW for irrigation is an increasingly common practice in arid and semi-arid agricultural areas such as Israel and California, USA. [2]

While wastewater reuse is important for addressing water scarcity issues, TWW contains many contaminants that are not effectively removed during conventional treatment processes.[3] Many pharmaceuticals have been detected in wastewater, as a fraction of each dose of medication administered is excreted without metabolism, and most wastewater treatment processes are not designed to remove pharmaceuticals and other polar and ionizable organic contaminants. Thus, use of TWW for irrigation exposes plants (including crops designated for human consumption) to pharmaceuticals and many other contaminants that have not been removed.

Unintended human exposure to pharmaceuticals via contaminated crops is a potential risk to human health. An extensive body of literature already exists that demonstrates that plants can accumulate pharmaceuticals in edible tissues under field conditions.[4,5] While several studies have found that pharmaceutical levels in plants are unlikely to reach levels that affect humans,[6–9] the antiepileptic drug lamotrigine and a metabolite of the antiepileptic drug carbamazepine have been found to accumulate to levels exceeding the threshold for toxicological concern for adults and children under normal consumption levels.[10,11] The threshold for toxicological concern is a conservative estimate used for compounds present at very low concentrations for which minimal

toxicological data are available, and is meant to serve as an indicator of when additional study of toxic effects may be warranted.[12] Additionally, TWW contains complex mixtures of potentially harmful contaminants, albeit at very low levels, and the effects of individual compounds may be additive, synergistic, or antagonistic, making it necessary to consider the whole group of contaminants, rather than each one individually. Currently, not much is known about the health impacts of chronic low-level exposure to pharmaceuticals, singly or in mixtures. While studies on human exposure and health effects are costly and logistically difficult, so far one study has been published where carbamazepine and its metabolites were found at higher levels in the urine of people who consumed produce grown using TWW irrigation than those who consumed produce grown with freshwater irrigation.[13] However, this study only addressed exposure and did not approach the topic of potential health risks.

While experiments testing plant accumulation of pharmaceuticals under various conditions are important for gaining an understanding of plant uptake processes, it is also important to develop methods of prediction. New drugs are constantly entering the market, the contaminants present in TWW are constantly changing, and monitoring all produce for potential contaminants is highly impractical. However, prediction of plant uptake of pharmaceuticals, particularly ionizable ones, has proven to be difficult. Models based solely on properties of the compounds have had limited success, and there is a lack of understanding at the mechanistic level that leads to difficulties when considering models that incorporate plant biology aspects.[4]

My dissertation consists of four main chapters that address the current state of the literature on plant uptake of pharmaceuticals and move beyond it to develop a more mechanistic understanding of how pharmaceuticals are taken up, translocated, and metabolized by plants. Each

chapter is formatted as an independent manuscript that has been published in or is in preparation for submission to a peer reviewed journal. Below I provide a synopsis of each chapter.

Chapter 1, Root Uptake of Pharmaceuticals and Personal Care Product Ingredients, is a critical review that was published in *Environmental Science and Technology* in 2016.[4] This chapter includes an in-depth overview of the literature on plant uptake or pharmaceuticals, attempts to use literature data to create predictive models based on compound properties, and identifies knowledge gaps where additional research is required. The main takeaways from the chapter include that plants are active organisms that have some control over the compounds they take up and how they are distributed and transformed, and that better reporting is necessary in experimental studies to allow data to be compared across studies and used to develop better predictions of plant accumulation. The remaining chapters of my dissertation seek to fill in some of the knowledge gaps identified in Chapter 1, including the effects of rhizosphere processes on plant accumulation of pharmaceuticals, mechanisms of plant uptake, and *in planta* transformation of pharmaceuticals.

Chapter 2, Plant-induced Changes to Rhizosphere pH Influence Uptake of Ionizable Organic Contaminants, is a manuscript in preparation for submission to *Environmental Science and Technology Letters*. This chapter focuses on plant-driven changes in rhizosphere pH in response to different nitrogen sources, and how the pH changes can influence availability and uptake of ionizable compounds. The rhizosphere consists of the soil and water directly surrounding the plant roots, and the chemical composition and properties of the rhizosphere can differ significantly from those of bulk soil. We found that plants provided with nitrate as the sole nitrogen source increased rhizosphere pH and had higher uptake of lamotrigine, an ionizable pharmaceutical, while those provided with both nitrate and ammonium had lower rhizosphere pH

and less uptake of lamotrigine. Plant accumulation of carbamazepine, a neutral molecule across the pH range of this study, was not affected.

Chapter 3, Effects of Transpiration and Binary Mixtures on Accumulation of Pharmaceuticals by Spinach, is a manuscript in preparation for submission to *Environmental Science and Technology*. This chapter focuses on spinach accumulation of the antiepileptic drugs carbamazepine and lamotrigine and the antidepressants amitriptyline and fluoxetine. We found that amitriptyline and fluoxetine affect the accumulation of a carbamazepine metabolite, and that accumulation of each of the compounds correlates strongly with transpiration. We identified amitriptyline as a molecule that accumulates to a larger extent than would be expected from water movement into the plant, and hypothesize that its accumulation may be assisted by a plant transporter protein.

Chapter 4, Plant Accumulation and Metabolism of Carbamazepine and Lamotrigine: An Interspecies Comparison, reflects a joint study between researchers at the University of Wisconsin – Madison and the Hebrew University of Jerusalem. The manuscript is a work in progress and is in preparation for submission to *Environmental Toxicology and Chemistry*. We grew *Arabidopsis thaliana*, spinach, cucumber, and tomato plants and exposed them to carbamazepine and lamotrigine alone and in a mixture, and compared uptake and metabolism among species. We found accumulation, mixture effects, and metabolism to vary among species. In particular, much of the initial compound provided was not detected in cucumber and tomato plants or nutrient solution at the end of the exposure period. We saw less compound loss for spinach and *A. thaliana*. We hypothesize that this may be due to differences in metabolism enzymes found in the different species or due to photodegradation. Some method validation for the data in this manuscript is still under way at the Hebrew University of Jerusalem, so this chapter currently includes a note about

how our results may change pending the method validation results. I expect to have all of the method validation data and updated results included by the time my dissertation is officially submitted to the university after my defense.

In combination, these chapters represent a significant body of research that contributes to the overall understanding of plant accumulation of pharmaceuticals. This is an important topic to address as water scarcity and reuse of TWW continue to increase.

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STATEMENT OF COLLABORATIVE WORK

The four main chapters of this dissertation were each written in collaboration with other graduate students and the professors we work with.

Elizabeth L. Miller, a Ph.D. candidate in Molecular and Environmental Toxicology at the University of Wisconsin – Madison, is the primary author of Chapter 1 and I am the second author. My main contributions to the publication include helping to determine the initial outline of the paper and compiling sources to include, assisting with the compilation of data to use in the literature-based predictive modeling attempts, writing several sections of the initial manuscript draft, and writing the final sections on rhizosphere processes. I also assisted with responding to reviewer comments and editing the manuscript after we initially submitted the paper.

I am the primary author on Chapter 2. I had the initial idea of focusing a study on rhizosphere processes and designed the experiments. I built the plant growth system and conducted preliminary plant uptake and sand sorption experiments. Second author Elizabeth Miller grew the plants used for the analysis included in the manuscript. I assisted with the exposure set-up and plant harvesting. Elizabeth Miller conducted plant extractions, while I was responsible for all LC-MS/MS analysis. I conducted all data analysis and wrote the manuscript.

I am also the primary author on Chapter 3. I designed the experiments, grew the plants, conducted all of the LC-MS/MS and data analysis, and wrote the manuscript. Second author Elizabeth Miller assisted with the exposure set-up and plant harvest and did all of the plant extractions.

I am the first primary author on Chapter 4. Tomer Malchi, a Ph.D. candidate at the Hebrew University of Jerusalem is a co-first author and Elizabeth Miller is second author. I designed the experiments in collaboration with Professor Benny Chefetz at the Hebrew University of Jerusalem.

I grew spinach and *Arabidopsis thaliana* plants and conducted exposure experiments with those species (assisted by Elizabeth Miller), while Tomer Malchi grew cucumber and tomato plants and conducted equivalent experiments. Tomer Malchi and I analyzed the samples grown in our respective labs, and divided the initial data analysis process. I wrote the abstract, introduction, and materials and methods sections of the current manuscript. Tomer Malchi initially drafted the first half of the results and discussion, which I have since revised. I wrote the remainder of the results and discussion, including the sections on mass balance, comparison of metabolism enzymes, and environmental implications.

Joel Pedersen and K.G. Karthikeyan guided the planning of each of the chapters, offered comments on content and style of the writing, and were generally very helpful.

CHAPTER ONE

ROOT UPTAKE OF PHARMACEUTICAL AND PERSONAL CARE PRODUCT INGREDIENTS

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ABSTRACT

Crops irrigated with reclaimed wastewater or grown in biosolids-amended soils may take up pharmaceutical and personal care product ingredients (PPCPs) through their roots. The uptake pathways followed by PPCPs and the likelihood that these compounds bioaccumulate in food crops are still not well understood. In this critical review we discuss processes expected to influence root uptake of PPCPs, evaluate current literature on uptake of PPCPs, assess models for predicting plant uptake of these compounds, and provide recommendations for future research, highlighting processes warranting study that hold promise for improving mechanistic understanding of plant uptake of PPCPs. We find that many processes that are expected to influence PPCP uptake and accumulation have received little study, particularly rhizosphere interactions, *in planta* transformations, and physicochemical properties beyond lipophilicity (as measured by K_{ow}). Data gaps and discrepancies in methodology and reporting have so far hindered development of models that accurately predict plant uptake of PPCPs. Topics warranting investigation in future research include the influence of rhizosphere processes on uptake, determining mechanisms of uptake and accumulation, *in planta* transformations, the effects of PPCPs on plants, and the development of predictive models.

INTRODUCTION

Worldwide, agriculture accounts for 67% of total water withdrawals and 86% of water consumption.[1] Strategic changes to agricultural water systems therefore have potential to achieve large improvements in global water management. Integration of municipal and agricultural water management systems through use of reclaimed wastewater (RWW) to irrigate crops adds to the portfolio of options available to ameliorate water demand in water-stressed regions. Irrigation with RWW is already widespread, particularly in regions where freshwater is limited or negative environmental effects from wastewater discharge provide incentive for reclamation. However, concerns remain about the safety of irrigation with RWW. The fate of RWW-derived organic micropollutants in agro-ecosystems and the risks of chronic exposure to these compounds through consumption of RWW-irrigated crops warrant further investigation.[2]

Conventional wastewater treatment processes are only moderately effective at removing many wastewater-derived organic contaminants, including pharmaceuticals and personal care product ingredients (PPCPs),[3] many of which are inherently bioactive substances. PPCPs have been detected in wastewater effluents, biosolids, biosolids-amended soils, and surface and groundwater systems receiving RWW.[4–10] Routes for PPCPs to enter agro-ecosystems include RWW irrigation, soil amendment with biosolids, sludge, or animal manure, and irrigation from freshwater bodies receiving wastewater effluent, sewer overflow, or runoff from confined animal feeding operations. Consequently, crop plants in such agricultural systems are exposed to PPCPs. In addition to PPCPs, irrigation with RWW and application of biosolids to agricultural lands can introduce a variety of other organic micropollutants to agro-ecosystems, including disinfection byproducts, flame retardants, steroidal estrogens, and perfluoroalkyl acids.

The majority of PPCPs present in RWW and biosolids are polar compounds with low volatility; many contain ionizable functional groups. Root uptake is therefore expected to be an important route of exposure for these compounds when they are applied in irrigation water and soil amendments. A large number of predominately descriptive studies have investigated root uptake of PPCPs. Despite this, mechanistic understanding of PPCP uptake by plants remains rather limited. Uptake of many PPCPs has been reported, but differences in experimental design and analytical methods complicate comparisons of uptake among studies that are needed to develop a fundamental knowledge of plant uptake of PPCPs. The resources do not exist to experimentally quantify the uptake of each of the thousands of current and future PPCPs found in RWW by multiple crop types, motivating development of models to predict contaminant accumulation in crops.

The purpose of this review is to critically review current knowledge of uptake of PPCPs by plant roots and translocation to above-ground tissues, and to suggest directions for future research. We first discuss processes occurring in the bulk soil and rhizosphere that affect contaminant availability for root uptake. We next discuss how root uptake is driven by plant physiology, summarize current literature on root uptake of PPCPs, and the correlation of root uptake with contaminant physicochemical properties. This is followed by a discussion of PPCP translocation to aerial tissues and how contaminant properties influence translocation. We then discuss *in planta* transformations and how these may affect estimates of uptake. We then discuss models for predicting root uptake of organic contaminants, testing an approach based on multiple physiochemical properties. We conclude by providing recommendations for future research, highlighting topics that hold promise for improving mechanistic understanding of plant uptake of PPCPs. Risk assessment of human exposure to PPCPs via consumption of contaminated crops is

outside the scope of this review; nonetheless, we discuss this topic briefly in the Supporting Information (SI).

PROCESSES IMPACTING AVAILABILITY OF PPCPS TO PLANT ROOTS

Sorption. Only the fraction of an organic compound dissolved in the soil pore water is considered available for uptake by plant roots. The dissolved fraction is also susceptible to leaching through soils, removing contaminants from the root zone and thereby decreasing their availability to plants. Sorption also influences the availability of PPCPs to microorganisms and consequently their microbial transformation. Polar and ionizable PPCPs contain structural moieties that allow interactions with both soil organic matter (SOM) and mineral surfaces.[11–17] Plants induce changes in the rhizosphere, the narrow zone of soil around plant roots (~2-3 mm), that can alter the interaction of organic contaminants with soil constituents.

Soil organic matter is an important sorbent for PPCPs. For nonpolar and moderately polar neutral organic compounds in bulk soil, the amount present in pore water is controlled primarily by sorption to SOM. Partitioning between SOM and water is typically described using an organic carbon-normalized sorption coefficient, K_{oc}.[18] Poly-parameter linear free energy relationships (pp-LFERs) have proven successful for estimating K_{oc} for a wide range of nonpolar and polar neutral organic compounds.[19–21] The pp-LFERs employ solute descriptors to account for relevant intermolecular interactions between organic contaminants and SOM (see review by Endo and Goss[21]). For neutral polar organic compounds, the degree of sorption to SOM tends to decrease as compound polarity increases.[22] Currently, the ability to predict sorption of ionized organic compounds to SOM from solute descriptors is limited, although sorption of organic anions to SOM is generally lower than that of the corresponding neutral species (reductions by a factor of 7 to 60 have been reported).[22] Application of pp-LFERs for some PPCPs may be hindered by the lack of available solute predictors.[21,23] Root uptake of nonpolar and moderately polar PPCPs is inversely related to sorption to SOM. For example, uptake of polycyclic musks by carrot roots from soils

increased as SOM content, and therefore sorption, decreased.[24] Similarly, uptake of the polar, uncharged antiepileptic drug carbamazepine related inversely to SOM content.[25–27] The effect of SOM content on PPCP uptake depends on its importance as a sorbent relative to other phases in the soil.[26,27] Sorption to biochar can also diminish accessibility of PPCPs for uptake by plant roots.[28,29]

Polar and ionizable PPCPs can engage in interactions beyond hydrophobic partitioning including electron donor-acceptor interactions (e.g., hydrogen bonding), cation exchange, anion exchange, protonation, water bridging, cation bridging, and surface complexation.[30–32] For ionizable compounds, solution chemistry (i.e., pH, ionic strength, concentration of competing ions) strongly influences the degree of association with soil particles.[12,15,33–38] Anionic organic species can exchange with inorganic anions at positively charged sites on metal oxides (e.g., iron and aluminum oxides) and on the edges of phyllosilicate clay minerals, as well as engage in cation bridging and surface complexation.[32] The anticonvulsant phenytoin ($pK_a = 8.3$) appears to interact with iron oxide minerals via weak electrostatic attraction.[41] Zwitterionic tetracycline and fluoroquinolone antibiotics form surface complexes with hydrous aluminum and iron oxides.[39,40] Uptake of fluoroquinolones by carrots was higher in sandy than loamy soils.[42]

Sorption of organic cations is strongly influenced by the density of exchange sites on SOM, phyllosilicate clay, and other mineral surfaces.[14,15,43] Sorption of the protonated base to these sorbents is generally stronger than that of the neutral species.[35,37,43,44] Organic cations compete with inorganic cations for exchange sites.[38,45] Approaches to predict organic cation sorption to soil constituents is currently a topic of investigation by several groups.[36,38,46,47] For organic cations bearing alkyl chains, sorption to SOM increases with alkyl chain length and is larger for primary relative to equally sized quaternary amines.[46] A linear interaction method in molecular dynamics

simulations has been applied to predict free energies of association of organic cations with phyllosilicate clay minerals.[47]

Extensive research has been conducted to evaluate the sorption of PPCPs, particularly antibiotics, to soil constituents; however, with the exception of the studies mentioned above on the impact of SOM on uptake of non-ionic compounds, few have examined the impact of soil properties on PPCP uptake by plants.

Effects of Rhizosphere Processes on Sorption. In the rhizosphere, root exudates can alter the bioavailability of organic contaminants to plants.[48,49] This topic has been studied extensively in the context of phytoremediation.[48–50] For example, citric, oxalic, and malonic acids (commonly found in root exudates) can promote desorption of polycyclic aromatic hydrocarbons (PAHs) from soil,[51] and sterilized root exudates can decrease naphthalene partitioning to soil.[50] Compounds found in root exudates can also increase mineralization of SOM,[52] which may also impact contaminant sorption.

Root exudates can strongly impact soil properties that influence sorption of ionizable organic contaminants. For example, plants can modulate rhizosphere pH in response to nutrient availability by secreting H^+, OH^-, and organic acids and can alter pH by up to 2 units as far as 2-3 mm from the root surface.[53,54] The speciation of ionizable PPCPs and soil constituents in the rhizosphere may therefore differ from that in bulk soil, with concomitant effects on sorption. The importance of nutrient availability and rhizosphere pH on the bioavailability of metals has been demonstrated. For example, rhizosphere alkalinization (when nitrate is the sole nitrogen source) strongly influences plant uptake of copper due to pH-driven changes in solubility and solution speciation.[55] The effect of nutrient-driven pH modulation in the rhizosphere on PPCP uptake by plants has not been studied, but warrants investigation. Additionally, microorganisms in the

rhizosphere, including plant symbiont bacteria and mycorrhizal fungi, can affect contaminant availability by altering the rhizosphere solution chemistry and mineralogy.[56] The effects of rhizosphere biota on PPCP sorption has not yet been explored.

Sorption to Dissolved Organic Matter (DOM). Sorption of PPCPs to effluent-derived DOM may also influence plant uptake. Dissolved OM can facilitate the movement of pesticides and PPCPs within soils by forming soluble complexes with DOM or by competing for sorption sites on soil particles.[57,58] In some cases, soil particles may sorb DOM and promote association of PPCPs.[58] The sorption of a variety of PPCPs to DOM has been investigated.[36–38,59–62] Non-steroidal anti-inflammatory drugs (NSAIDs) exhibit lower retardation factors in soils irrigated with RWW, and uptake of thee NSAIDS and several other weakly acidic compounds into cucumber leaves was lower when the plants were irrigated with RWW than with spiked freshwater.[7,27] The increased mobility of the NSAIDs in RWW-irrigated soils appears to be due to changes in pH rather than sorption to DOM.[63] In contrast, biosolids-derived DOM was shown to reduce the leaching of weakly acidic PPCPs.[63]

Transformations in Soil. Concentrations of PPCPs available for uptake by plant roots may be altered by abiotic and microbial transformation processes. In addition, such processes may result in the formation of biologically active transformation products that accumulate in plants.[64–67] Within the rhizosphere, root exudates may impact transformations of PPCPs as may the bacteria and mycorrhizal fungi that depend on carbohydrates excreted by plant roots.

Abiotic Transformations. Abiotic transformation processes occurring in soil include photolysis, hydrolysis and redox reactions. Direct photolysis of organic contaminants in soils is considered relatively unimportant due to light attenuation (the soil photic zone is limited to the top ~0.5 mm).[68,69] For compounds susceptible to indirect photolysis, degradation rates may be

enhanced immediately at the soil surface due to light-induced production of transient photoxoxidants such as singlet oxygen.[69] Antibiotics susceptible to photodegradation in water show lower rates of photodegradation in soils.[70] Compounds susceptible to hydrolysis may be hydrolyzed *in vivo*, during wastewater treatment, or in the soil environment.[65] β-Lactam antibiotics can be rapidly hydrolyzed in soils.[63] Interaction with metal oxide surfaces can inhibit or catalyze hydrolysis depending on compound structure.[71,72] Oxidation of PPCPs by reactive mineral phases in the clay fraction may occur. For example manganese oxides can transform oxytetracycline and sulfamethazine.[73,74]

Microbial Transformations. Many types of PPCPs are susceptible to microbial degradation in soils.[70] In some cases, conjugated metabolites can be deconjugated in soil, increasing the concentration of the parent compound.[75] Antibiotics can change the composition of soil microbial communities[65,70,76] and decrease soil respiration and nitrification rates.[77] In some cases, antibiotics may decrease the rate that other PPCPs are degraded.[78] Biosolids-amended soils exhibited reduced biodegradation of 15 pharmaceuticals compared to rates measured individually in other laboratory studies or predicted by the USEPA's EPISuite software.[79] Pre-exposure to low levels (0.1-4.5 $\mu g \cdot L^{-1}$) of PPCPs in irrigation water did not change biodegradation rates,[80] indicating that higher levels are necessary to induce changes in enzyme expression or community structure.[80] High SOM content often correlates with decreased biodegradation,[81–83] probably due to reduced bioavailability from increased sorption. However, addition of biosolids does not always decrease biodegradation, possibly due to increased microbial activity from increased nutrient availability.[67,84,85] Anoxic conditions generally decrease biodegradation rates.[70,83,84,86–88] Complete mineralization of many PPCPs is low (< 2% of total mass),[64,65,85,89–91] although larger fractions of highly reactive compounds like analgesics and NSAIDs can be mineralized by microbial

processes.[66,92] Compound susceptibility to microbial degradation may be predicted using models, such as UM-PPS, that base predictions on contaminant structure and known microbial degradation pathways.[93,94] Hydroxy, ester, and acid groups promote enzymatically catalyzed transformations, while aromatic rings and halogen substituents diminish biodegradability.[95]

The transformation of organic contaminants in the rhizosphere may be enhanced relative to bulk soil. Plants and rhizosphere-associated microorganisms secrete enzymes such as laccases and peroxidases that can transform contaminants, and secretion of compounds structurally analogous to contaminants may stimulate co-metabolic processes.[48] The carbohydrates in root exudates serve as a carbon source for microorganisms in the rhizosphere, leading to higher microbial activity. Degradation of contaminants such as PAHs and PCBs is increased in the rhizosphere,[96] as is dissipation of the antibiotic sulfadiazine.[97]

Bound Residues. Organic contaminants bearing appropriate functional groups (e.g., aromatic amines, phenols) may covalently bind to SOM for form bound residues. This process is often mediated by soil microbial activity.[98–100] Published studies often fail to distinguish between bound and non-extractable residues (NERs) because of the difficulty in verifying the former. Non-extractable residues are operationally defined and may be due to covalent binding to SOM, intercalation in smectites, or entrapment in SOM domains poorly accessible to extractants.[101] Plant uptake of ^{14}C from pesticide and PAH NERs has been demonstrated.[102,103] Pesticides and their metabolites possessing reactive moieties can form bound residues by covalently binding to carbonyl, quinone, or carboxyl groups via oxidative coupling reactions.[102] Sulfonamide antibiotics can form bound residues via covalent binding to humic substances.[104–108] Sulfonamides can form Michael adducts with humic acids.[105] Phenoloxidases mediate bound residue formation by oxidatively transforming phenolic SOM constituents to create sites for nucleophilic attack by

sulfonamides.[106] Other pharmaceuticals (viz. NSAIDs, paracetamol, diphenhydramine, and carbamazepine) have been hypothesized to covalently bind to soil components; to date only NER formation has been demonstrated.[64,66,85,89,92] We are aware of no studies on plant uptake of bound PPCP residues.

ROOT UPTAKE OF PPCPS

Root Physiology and Processes. From the rhizosphere, PPCPs enter the plant through the roots. Figure 1 shows typical root anatomy for a dicot vascular plant. Water and small solutes ($M_r \leq 500$)[109] can enter the root through the epidermis of growing root tips, including root hairs, which contribute the bulk of root surface area. Mature regions of the root may develop an exodermis, an additional outer layer relatively impermeable to water and solutes. Once in the epidermis, water and solutes cross the cortex, pass into the vascular tissue through the endodermis, and can then be transported via the xylem/phloem to aboveground tissues (Figure 1). Compounds that do not reach the vascular tissue are not translocated out of the plant roots.

Water and solutes can move from soil pore water to the vasculature via three pathways: the transmembrane (between cells through cell walls and membranes), symplastic (between cells through interconnecting plasmodesmata) and apoplastic (along cell walls through the intercellular space) routes (Figure 1). The transport pathway taken depends on the ability of the solute to cross membranes into cells. The Casparian strip, impregnations of the endodermal transverse cell walls composed of lignin and lamellar suberin,[110,111] acts as a hydrophobic barrier between the apoplast (the extracellular space in the epidermis) and the vascular tissue.[112] Compounds taken up solely by the apoplastic route cannot cross the Casparian strip; they must cross at least one lipid bilayer to enter the xylem or phloem (and consequently be transported to above-ground tissues). Casparian strip development and maturation depends on plant species/variety and growth medium (i.e., hydroponics vs. soil).[113]

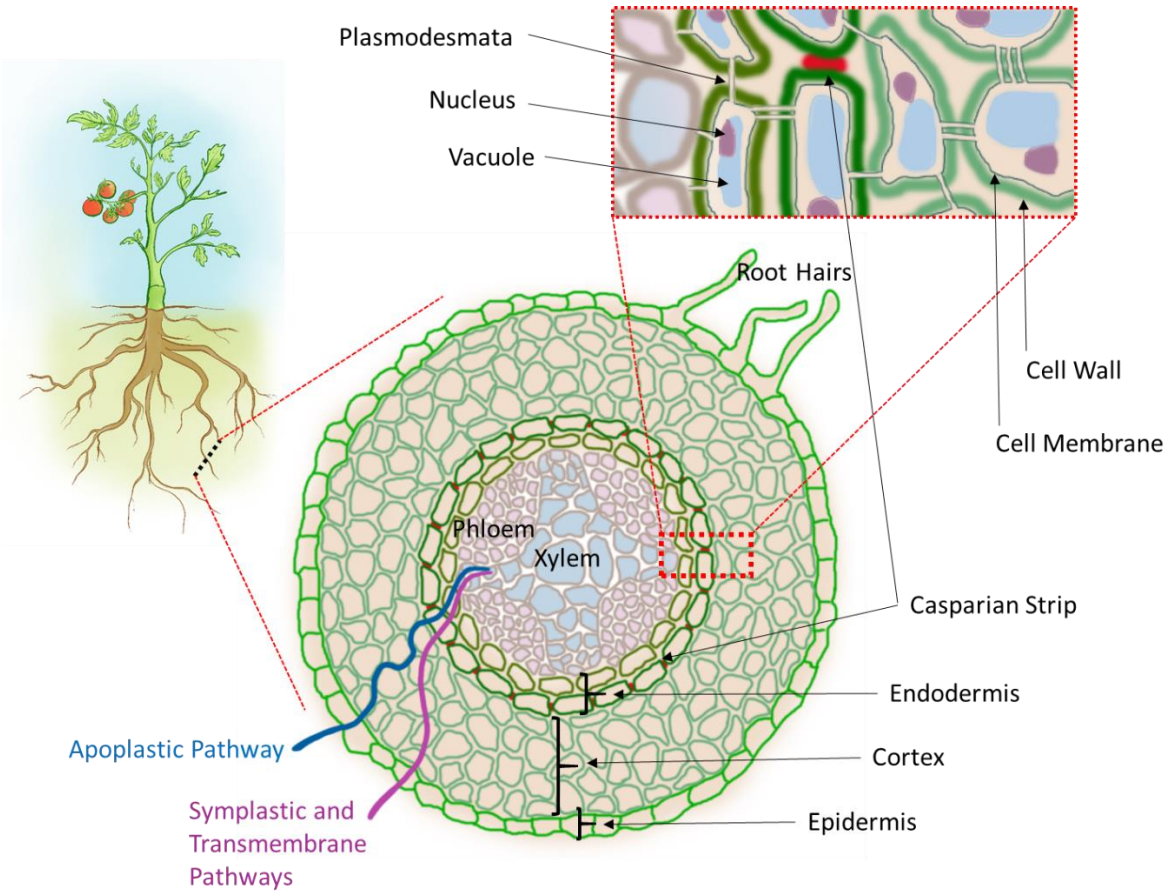

Figure 1. Cross-sectional diagram of a young dicot root. Water and solutes may travel from the soil solution to the vascular tissue via apoplastic (between cells along cell walls), symplastic (through cells via plasmodesmata), or transmembrane pathways (through cells via cell membranes). The Casparian strip blocks apoplastic transport at the endodermis. We note that during lateral root formation, small holes or breaking points in the Casparian strip allow localized primordium penetration,[259] but endodermal disruption seems to be a transient feature of lateral root development, and thus is not expected to affect contaminant uptake.

The lipophilicity and speciation of PPCPs strongly affects their ability to passively cross plant cell membranes. Higher lipophilicity allows more rapid diffusion across lipid bilayers.[114] Introducing charge to a molecule decreases its lipophilicity and leads to interaction with the negative surface potential of the cytoplasmic membrane surface potential.[115] Partitioning to lipids and membrane permeability are often estimated from K_{ow}, an approach that is inaccurate for

organic ions because lipid bilayers can more easily accommodate charged organic species than *n*-octanol can.[18,115] Phospholipid-water partition coefficients more accurately predict association of polar and ionizable compounds with animal membranes.[18,116] The composition of plant cytoplasmic membranes varies among species and tissues.[117] Biologically relevant differences in membrane composition can result in up to order-of-magnitude differences in affinity and permeation of pharmaceuticals into lipid membranes.[118–120] Interaction of ionizable organic compounds with plant cell membranes warrants investigation.

Ion trapping occurs when a compound is neutral in the apoplast (pH 4-6) but ionizes inside the cell (pH 7-7.5), leading to accumulation within cells.[121] Compounds such as sulfonamide and fluoroquinolone antibiotics, the anti-histamine cimetidine, the anticoagulant warfarin, and the anti-convulsant lamotrigine, with pK_a values between these pH values, are expected be subject to ion trapping. We are not aware of any experimental studies explicitly demonstrating ion trapping of PPCPs in plants.

Sorption of cationic PPCPs to plant cell walls is expected to impact their uptake. The composition (cellulose embedded in a noncellulosic polysaccharide matrix)[122] and cation exchange capacity of plant cell walls vary by species and tissue, but all cell walls bear negative charge.[122–124] Ion exchange at negatively charged sites in plant cell walls is expected to impede the diffusion of organic cations through the cell wall matrix. The importance of this process has been demonstrated for the cationic dye methylene blue: apparent diffusion coefficients in roots were comparable with those for isolated cell walls.[125] No information is currently available about the interaction of cationic PPCPs with plant cell walls.

Proteins can mediate the uptake of organic compounds into root cells, but this has not yet been demonstrated for PPCPs. Protein-mediated transport requires energy when transport is

directed against concentration gradients. Energy-dependent uptake processes would lead to accumulation of PPCPs in excess of predictions based on passive uptake. Plants take up some herbicides[126,127] and amino acids via energy-dependent mechanisms.[128] To our knowledge, the herbicide transporters involved have not been reported. Depending on the charge of their side chains, amino acids are transported into root cells by specific transporters.[128] Both mycorrhizal and non-mycorrhizal plants take up and use organic nitrogen (e.g., short peptides, quaternary ammonium compounds),[128–132] although uptake mechanisms for peptides have not been elucidated. These compounds vary in size, from < 100 Da to several thousand Da. Gamma-amino butyric acid transporters are responsible for the energy-dependent uptake of quaternary ammonium compounds in *Arabidopsis*.[133] Many organic nitrogen transporters have low selectivity,[134] suggesting that they could be involved in the uptake of PPCPs with structures similar to the natural organic compounds they transport.[135,136] For example, the corrosion inhibitor benzotriazole is hypothesized to be taken up into *Arabidopsis* by transporters for the structurally similar amino acid tryptophan.[137] Involvement of organic cation transporters has been postulated to explain the high accumulation of metformin in rapeseed.[136,138] Metformin is structurally similar to many endogenous plant compounds (e.g., guanidine), and transport across the cell membrane via non-selective channels would allow bypassing of the Casparian strip. Metformin and other basic compounds may be taken up by protein-mediated processes due to the similarity of their nitrogen-containing functional groups to those in natural nitrogenous compounds taken up by these routes (*vide supra*), but this remains to be investigated.

The PPCPs with the highest propensity to accumulate in roots are those blocked by the Casparian strip from entering the xylem and phloem, those having high affinity for root lipids, and possibly those conjugated and sequestered in root cell vacuoles before they make it to the

vasculature (*vide infra*). Little research has been directed at elucidating PPCP uptake mechanisms and pathways, knowledge that is needed to develop models to predict uptake and accumulation.

Root Uptake Studies. Uptake of more than 100 PPCPs by plants has been studied.[139–141] Some of the studies discussed in this and the following sections were conducted in the absence of soil under hydroponic conditions. Hydroponic studies can be useful to study the influence of PPCP physicochemical properties on root uptake without the complicating factors of sorption to soil constituents and transformations mediated by soil microorganisms and particles. Hydroponic exposure may be considered the condition allowing maximum availability of PPCPs to plant roots,[141] but does not always result in more uptake than for plants grown in soil. Hydroponic studies are more easily compared to one another than are studies using plants grown in soil, and comparison with studies using well-characterized soils may yield insight into how plant-soil interactions affect PPCP uptake. Plants grown hydroponically may exhibit some physiological differences from those grown in soil however.

Nearly all studied PPCPs associate with plant roots, regardless of their physicochemical properties. The large volume of literature showing detectable levels of PPCPs in root extracts indicate that most PPCPs may accumulate in or on roots of most types of plants, including edible root crops. In many cases, however, uptake into the root has not been confirmed; extracting PPCPs from whole roots does not discriminate sorption to the root exterior from uptake into the root. Analyses of the peels and cores of root vegetables grown in soil separately typically show substantially higher concentrations in the peel than in the core.[136,142–146] This indicates the exodermis at least partially blocks their entry into the root cortex. Some reports are not consistent with this trend, however; hydroponically grown potatoes had higher triclosan and triclocarban concentrations in the core than in the peel.[147] This finding may be due to higher availability in

hydroponics compared to soil or differential development of the exodermis. Whether accumulation occurs mostly in the peel or also in the core of the root is plant- and compound-specific.[145,148]

Accumulation in roots is often expressed using the root concentration factor (RCF), the ratio of the concentration in roots to that in the exposure medium (typically bulk soil rather than soil pore water). Root concentration factors for PPCPs depend on exposure time,[149–151] plant species,[147,149,150,152–154] soil properties, humidity, temperature,[153,155] and whether concentrations are calculated as wet or dry weights.[156] Comparison among studies would be facilitated by reporting these factors and calculating RCFs relative to pore water concentrations. Reported RCF values for many types of PPCPs span a range of several orders of magnitude, from ~0.01 to ~1000.[138,141,142,147,149–154,157–160] Although compounds from most classes can associate with or be taken up by roots, no uptake of macrolide antibiotics from soil has been reported.[142,144,154,161,162] The large size of macrolides (M_r > 500) appears to preclude uptake via passive diffusion; no protein-mediated or energy-dependent uptake has been reported for these compounds.

Root uptake mechanisms for PPCPs appear to have been investigated only for tetracycline antibiotics; results published to date seem contradictory. One study relied on inhibition of metabolic activity (thus halting energy-dependent processes) and aquaporin (water transport protein) and found that inhibition of metabolic activity by 2,4-dinitrophenol prevented oxytetracycline uptake by alfalfa, while the aquaporin competitors had no effect.[163] Interpretation of these results is difficult because 2,4-dinitrophenol treatment not only stopped energy-dependent processes, but also inhibited transpiration, which could have decreased passive uptake. Uptake was also decreased when cells were exposed to Hg^{2+}, indicating general cellular stress can inhibit uptake.[163] Correlations between tetracycline antibiotic properties (e.g., permeability through cellophane, K_{ow}) and root uptake kinetics in rice were consistent with non-facilitated passive

uptake.[164] Other classes of PPCPs may have different uptake pathways (e.g., facilitated passive uptake through protein channels, protein-mediated energy-dependent uptake), but to our knowledge, this has not yet been studied.

Correlations between Root Uptake and Compound Properties. The RCF generally correlates with compound lipophilicity for neutral compounds.[165–167] Lipophilic compounds are expected to partition to root lipids (membrane and storage lipids) and thus concentrate in roots. For polar and ionizable PPCPs, the relationship between RCF and compound physicochemical properties is less clear. Polar compounds may move through roots to accumulate in aerial tissues or be blocked by the Casparian strip and accumulate in roots. Ionizable compounds may be subject to additional processes like ion trapping and electrostatic interactions with cell walls. For example, RCF values from a single soil correlated positively with the root lipid content of five types of vegetables for carbamazepine (neutral, log K_{ow} = 2.45), but not for diphenhydramine (cationic, pK_a = 9.08, log K_{ow} = 3.44) or triclocarban (neutral, log K_{ow} = 4.9).[153] Correlation was similarly not observed between root lipid content and RCF for carrot uptake of triclosan (neutral in the pH 5.5 soil used, log K_{ow} = 4.7) from soil.[143] To account for the pH-dependent speciation of compounds, $D_{ow} = \alpha_{neutral} \cdot K_{ow,neutral}$ is often used, where $\alpha_{neutral}$ is the fraction of the compound present as the neutral species and $K_{ow,neutral}$ is the *n*-octanol-water partition coefficient for the neutral species. This approach may yield a more accurate measure of lipophilicity for ionizable compounds than K_{ow}, although the assumption of no partitioning of the charged species may be a limitation. Positive correlation between log RCF and log D_{ow} was reported for nine pharmaceuticals and flame retardants in roots of lettuce and strawberry grown in soil (R^2 = 0.78).[152] The RCF values discussed above were calculated from concentrations in the bulk soil rather than in soil pore water and may not represent the fraction of PPCP available to

plant roots. This may contribute to the generally weak correlations. In developing relationships between compound properties and RCF, we recommend use of soil pore water concentrations rather than those in bulk soil.

To further examine the relationship between lipophilicity (as expressed by D_{ow}) and RCF, we conducted a meta-analysis of root uptake data for PPCPs from the literature (see the SI), focusing on a single vegetable (lettuce) to eliminate effects from species differences, and analyzed data for hydroponic and soil studies separately to account for differences in uptake due to plant physiology and compound bioavailability. Using data from a single hydroponic study,[168] we found that RCF did not correlate with log D_{ow} for 20 compounds (encompassing a range of lipophilicities and pK_a values, and including acids, bases and neutral compounds) (Figure 2a); however, correlations for RCF were strong when data were restricted to neutral compounds (Figure 2a).

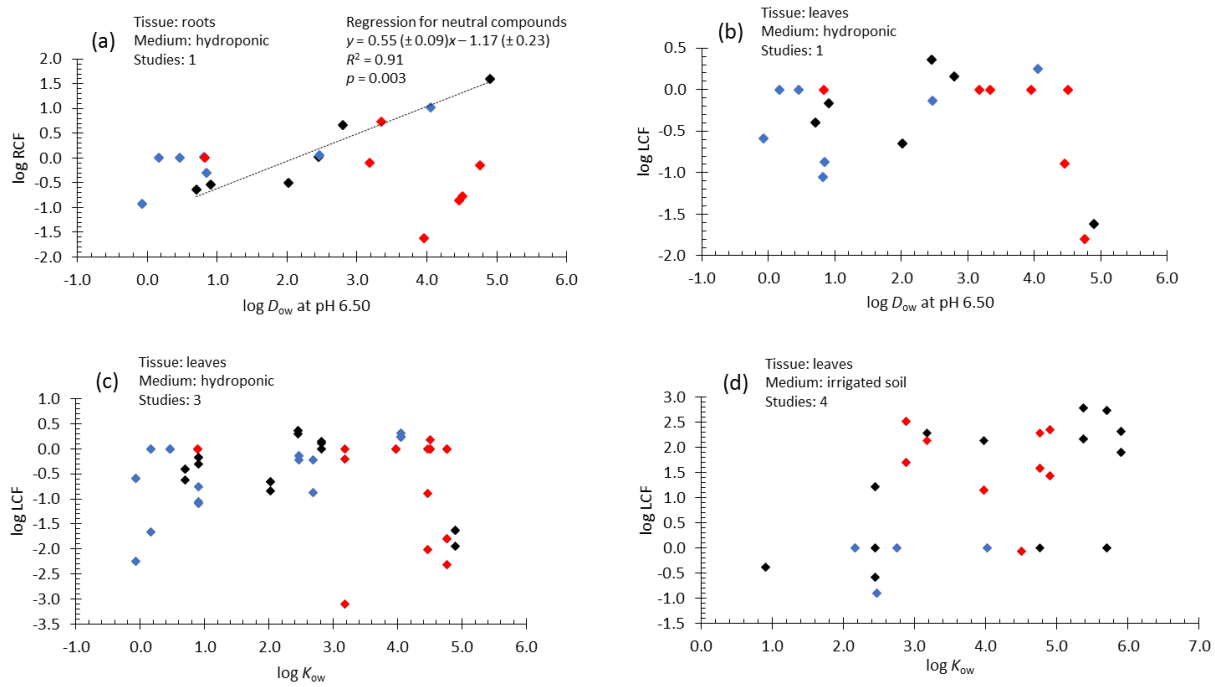

Figure 2. Correlation of lettuce uptake of PPCPs with log K_{ow} and log D_{ow} (pH = 6.5).[142,146,153,161,168,179,182,185] Correlations of **(a)** the logarithm of the root concentration factor (RCF) with D_{ow} (pH 6.5) and **(b)** log LCF for hydroponically grown lettuce in a single study (adapted from Wu et al.[168]). LCF was calculated as mole compound/kg fresh weight leaf divided by mol compound/L exposure medium. When dry weights were reported, leaf fresh weight was estimated assuming lettuce is 96% water.[260] In **(a)** and **(b)**, lettuce was grown in nutrient solutions containing 0.5 $\mu g \cdot L^{-1}$ PPCPs. Trends or lack thereof were similar for 5 $\mu g \cdot L^{-1}$ exposures and for uptake by spinach, cucumber, and pepper. Inclusion of all measured PPCPs yielded poor correlation between LCF and log D_{ow} (R^2 = 0.045, p = 0.37), indicating factors other than lipophilicity are important for the uptake of ionized PPCPs. The correlation for RCF was strong when the data set was restricted to neutral compounds (R^2 = 0.91, p = 0.003). Correlations of the logarithm of leaf concentration factor (LCF) with log K_{ow} for **(c)** multiple studies using hydroponic methods and **(d)** multiple studies using soil irrigated with PPCP-amended water. Using D_{ow} to account for the speciation of ionizable PPCPs in the exposure medium may have yielded stronger correlations, but could not be done for the full data set because 30% of the studies did not report the pH or the exposure medium. Specific compound and study details are available in the SI. Neutral, basic and acidic compounds are indicated respectively in black, blue, and red.

ACCUMULATION OF PPCPS IN AERIAL TISSUES

Translocation to Aboveground Tissues. Compounds reaching the vascular tissue can be transported to shoots, leaves, and fruit via the xylem or phloem. Xylem vessels form the transpiration stream, transporting water, inorganic nutrients, and organic substances from roots to shoots and leaves. Increased transpiration correlates with removal of PPCPs from hydroponic nutrient solution, especially for cationic and neutral PPCPs.[155] Xylem sap contains a variety proteins[169,170] that may play a role in organic contaminant transport. Major latex-like proteins are implicated in the translocation of hydrophobic organic pollutants t,[171] and may contribute to that of PPCPs. For example, cucurbit xylem sap contains proteins that facilitate translocation of dieldrin from roots to shoots.[172] Zucchini root-to-shoot transfer of some PPCPs is higher than that of soybean and a closely related squash,[173] at least partly attributable to increased solubilization of contaminants in the zucchini xylem sap.[173] An osmotically generated pressure gradient drives transport through phloem, carrying photosynthetically derived carbohydrates from leaves to roots, and contributing to the movement of proteins, secondary metabolites, and hormones to buds and fruits.[174] Phloem represents an important translocation pathway for many herbicides[175–177] and is expected to be important in PPCP translocation to some edible tissues. The PPCPs most likely to accumulate in aerial tissues are those entering the root via either passive diffusion into the symplastic pathway or active uptake into cells. These compounds are unimpeded by the Casparian in their transit to the vasculature, and may end up in leaves if transported by the xylem, or fruits if transported by the phloem.

The distinction between compounds mobile in xylem and those mobile in phloem is not clear cut; all phloem-mobile compounds can also move in xylem, and even compounds exhibiting predominately xylem mobility can enter the phloem.[177] Whether xylem movement to leaves or

phloem movement to fruits is the dominant transport route for herbicides depends on the compound's ability to cross membranes. Compounds easily crossing membranes rapidly equilibrate between phloem and xylem, and thus move predominantly in the xylem because of its much larger water flow.[177] Rules for how herbicide physicochemical properties affect transport have been identified,[177] although the majority of these compounds are applied to and taken up by leaves instead of roots. Highly lipophilic compounds sorb to lipids and are not readily transported through plants. For non-ionized compounds, those with log $K_{ow} < 0$ are ambimobile (mobile in both the xylem and phloem) and compounds of intermediate lipophilicity ($0 < \log K_{ow} < 3$) are only xylem mobile. Acids with $pK_a < 7$ and $\log K_{ow} < 3$ tend to remain in the phloem due to ion-trapping mechanisms and can move to fruits. For bases with $pK_a > 7$, those with $\log K_{ow} < 0$ tend to be ambimobile and those with $0 < \log K_{ow} < 4$ tend to move in xylem.

Studies on Translocation to Aerial Tissues. The ability of a contaminant to translocate from roots to shoots is often described using the translocation stream concentration factor (TSCF), the ratio of compound concentration in the xylem sap to that in the exposure medium. The TSCF can be a useful value to predict in-plant distribution of compounds across species. For example, PPCPs with lower or higher TSCF values in cucumber (viz. trimethoprim, sulfonamides vs. carbamazepine) were detected at relatively high concentrations in pea roots and cotyledons or pea leaves, respectively.[157] TSCF values for the relatively hydrophobic neutral compounds endosulfan and triclocarban were lower than those of a more hydrophilic compound (caffeine) in zucchini, squash, and soybean,[173] indicating that if hydrophilic compounds can cross the Casparian strip, they may be able to move to leaves at a faster rate than more hydrophobic compounds.

While TSCF is a useful parameter for characterizing translocation, few studies of PPCP uptake have reported it due to the difficulty of collecting xylem sap for analysis. The translocation factor (TF; the ratio of leaf concentration factor (LCF) to RCF) is sometimes used as an alternative metric that accounts for both xylem and phloem transport.[152] For most PPCPs measured in both the roots and leaves of a single plant, reported LCF values are smaller than RCF values (TF < 1), with reported LCF values typically in the range of ~0.01 to ~100.[138,141,142,147,149–154,157–160] Carbamazepine is notable for frequently displaying higher bioconcentration in aerial tissues than in roots, but even carbamazepine LCF values span a range of several orders of magnitude.[25–27,145,151,155–158,168,178–183] Whether other PPCPs have high TF values is not yet known, as many compounds have been reported in only one study and many studies report concentrations only for edible tissues. To our knowledge, no studies have investigated the mechanisms of carbamazepine uptake and translocation; passive diffusion into the vascular system is assumed because the compound is uncharged and has intermediate hydrophobicity.

While translocation of cationic PPCPs is expected to be limited due to difficulty bypassing the Casparian strip and cation-exchange interactions with negatively charged cell walls, plant uptake to aerial tissues from soil has been demonstrated for all studied basic (cationic) compounds.[26,136,138,142,145,151,158,183–185] Many have also shown uptake from hydroponic systems,[155–157,168,182,186] although some remained mostly in the roots in both types of experiment.[151,186]

The accumulation of many PPCPs in fruits tends to be lower than in leaves and roots,[25,185,187] indicating translocation primarily via xylem. Notable exceptions are the weak acids bezafibrate, ketoprofen, and naproxen,[27] which, like weakly acidic herbicides, travel predominantly in the phloem when unable to rapidly cross membranes.[177] Another exception may be fluoroquinolone antibiotics, which were found in higher concentrations in fruits than in leafy

vegetables and have intermediate hydrophobicity ($-0.4 < \log K_{ow} < 1.1$) and multiple moieties that ionize at environmentally relevant pH values.[188] Although the comparison is between different species, it may suggest translocation primarily via phloem, as ion trapping is expected to affect fluoroquinolones (their carboxylate group (pK_a ~6) and a secondary or tertiary amine (pK_a ~8-9) make them anionic or zwitterionic at the pH of plant compartments). Accumulation in seeds has received scant study, but appears to be driven by the ability of the compound to be transported in phloem. Metformin, a hydrophilic basic compound, accumulates in a variety of seeds,[136,138] whereas triclosan, a lipophilic phenolic compound, has been reported to accumulate to low levels (bioconcentration factors relative to bulk soil < 1) in seeds of soybeans grown on biosolids-amended soil,[160] but was undetectable in seeds of barley grown on spiked soil.[143]

Correlations between Accumulation in Aerial Tissues and Compound Properties. Neutral compounds with log K_{ow} values between −1 and 5 are considered mobile in the transpiration stream[109,165] (i.e., expected to move to aerial tissues if they enter the xylem). A nearly sigmoidal relationship appears to exist between TSCF and log K_{ow} for neutral compounds for hydroponically grown plants.[189,190] However, TSCF values do not correlate with log K_{ow} when TSCF values of both neutral and ionizable compounds are compiled from the literature.[189] Correlations between TSCF and lipophilicity may be poor because of factors like metabolism in plant roots, energy-dependent uptake processes, and for ionizable compounds, ion trapping in the phloem and electrostatic interactions with cell walls.

For organophosphate flame retardants, TF decreases with increasing K_{ow}.[152] However, for PPCPs, TF does not correlate well with compound lipophilicity, even within a single plant species.[168] This may stem in part from not distinguishing between association with the root exterior and uptake into roots and using bulk soil concentrations in RCF calculations. Some evidence exists

that cationic compounds have higher TF values than do anionic compounds,[155,168] but correlations between TF and D_{ow} are poor.[152,157,168] Translocation factors vary by plant species[147,152,154,155,168,191] and variety[192] and do not correlate well with transpiration rates,[155] indicating that translocation to aerial tissues is affected by plant properties beyond plant water use efficiency.

Leaf concentration factors or concentrations within leaf tissue are more commonly reported than TF values. For both nonpolar and polar/ionizable compounds, LCF rarely correlate with compound properties. Experiments with barley uptake of non-ionized chemicals (e.g., *O*-methylcarbamoyloximes, substituted phenylureas[166]) suggested that LCF correlated with log K_{ow},[166] but experiments with a wider range of compound structures and plants indicate poor correlation.[155,168,180,193] Our analysis of published data on lettuce uptake demonstrated LCF values did not correlate with log D_{ow} for 20 PPCPs using data from a single hydroponic study[168] (Figure 2b). Similarly, no correlation was found between log K_{ow} and LCF for hydroponic or soil-grown lettuce for neutral, acidic, or basic compounds using data from multiple studies (Figure 2c-d). Correlations between LCF and lipophilicity are expected to be poor due factors including *in planta* metabolism, ion trapping (phloem vs. xylem transport), and electrostatic interactions with cell walls.

TRANSFORMATION AND SEQUESTRATION *IN PLANTA*

Contaminants taken up by plants may be transformed or sequestered by plant metabolic processes, which are similar to those of the hepatic detoxification system leading to the appellation the "green liver".[194] Exogenous lipophilic compounds are often oxidized, reduced, or hydrolyzed to enhance their reactivity and polarity (phase I metabolism), and then conjugated with a polar molecule (e.g., sugars, amino acids, malonic acid, glutathione; phase II metabolism).[194] The resulting conjugates can bind to insoluble components (e.g., lignin, polysaccharides) or be stored in cell vacuoles (an energy-dependent process called sequestration).[195] Sequestration of PPCP conjugates may provide an additional mechanism of bioaccumulation beyond passive partitioning into lipids and other phases. For example, sequestration of the herbicide glyphosate by resistant weeds is temperature-dependent,[196] driven by an ATP-binding cassette transporter,[197] and results in glyphosate accumulation over time.[198] Once in the vacuole, xenobiotics may be further transformed by peroxidases.[195] In some cases, compounds may be secreted from cells instead of sequestered,[195] as was observed for bimane dye conjugates[199] and triclosan.[200] Plant transformation may yield products more toxic than parent compounds, potentially impacting the accuracy of human health risk assessments that rely only on the concentrations of the parent compound. Bioactivation of xenobiotics other than PPCPs have been reviewed.[201–203] Transformation of many pesticides by plant enzymes leads to increased mutagenicity or toxicity;[204] these enzymes also likely function on PPCPs.

Transformations occurring in mammals can provide clues about reactions PPCPs may undergo *in planta,* since many enzymes responsible for transforming xenobiotics, including cytochromes P450 (CYP450s) are conserved across phyla.[205] Carbamazepine is likely transformed *in planta* to 10,11-epoxycarbamazepine, a potentially genotoxic metabolite considered more toxic

than carbamazepine[145,206] and formed by mammalian liver enzymes and in wastewater treatment sludge.[207,208] Analysis of soil-plant systems for carbamazepine and two transformation products (10,11-epoxycarbamazepine and 10,11-dihydro-10,11-dihydroxy-carbamazepine) demonstrated the presence of both metabolites in soils, leaves, and fruits, but only 10,11-epoxycarbamazepine in roots.[27,145] The 10,11-epoxycarbamazepine-to-carbamazepine ratio was much higher in leaves than in roots, leading to the hypothesis that carbamazepine is metabolized primarily in leaves. Metabolism and/or uptake of metabolites may also occur in the roots. Sequential activation by plant and animal metabolic systems (after ingestion) may also result in toxic transformation products not observed with either system independently.[204]

Many PPCPs are susceptible to direct photolysis and indirect photodegradation in natural waters,[76,209–211] leading to the hypothesis that some PPCPs may also be subject to photodegradation in plant leaves.[152] Breakdown via photolysis would decrease bioaccumulation of parent compounds, but may not reduce risk of consumption of exposed crops if breakdown products are toxic. We are not aware of any studies confirming photodegradation of contaminants within plants.

Transformation of PPCPs by plants affects estimates of uptake, but aside from carbamazepine, few studies on plant metabolism of PPCPs have been published. Phase I metabolite epimers of tetracycline in pinto bean leaves have been reported.[187,212] Chlortetracycline is conjugated with glutathione by the phase II enzyme glutathione *S*-transferase in maize.[187,213] Barley,[214] horseradish hairy root cell cultures,[214] and bulrush[215] hydroxylated diclofenac to 4′-OH-diclofenac, a hypothesized mammalian hepatotoxin,[216] in a concentration-dependent manner. Subsequent conjugation with glucopyranoside did not correlate with diclofenac concentration.[214] Eight phase II triclosan conjugates were identified in carrots and carrot cell cultures.[200] Triclosan metabolism in horseradish root cultures produced ≥ 33 phase I and II metabolites.[217] The human

health risk posed by carbamazepine, diclofenac, and triclosan in crops was considered low in studies that did not account for metabolites.[160,218–220] These examples indicate further study of plant metabolism is warranted, both to accurately estimate PPCP uptake and to identify potentially toxic transformation products. Most prior descriptive studies have accounted for only parent compounds, and many have concluded (possibly erroneously) that uptake was limited. Ignoring PPCP metabolites may underestimate the extent of their uptake into plants, contributing to difficulties in developing predictive uptake models. Future studies should focus not only on quantifying parent compounds, but also identifying and quantifying transformation products.

Phytotoxicity of PPCPs. Effluent-irrigated crops are exposed to PPCPs as mixtures. PPCPs or their metabolites may induce (toxic) effects in plants that impact uptake, translocation, and transformation processes. Phytotoxicity studies have been previously reviewed,[140] but knowledge gaps remain. Published terrestrial phytotoxicity studies have focused mainly on tetracyclines and sulfonamides, and little information is available on other compound classes. Existing studies tested toxicity of individual compounds, and most employed concentrations much higher than are environmentally relevant.[140]

Mixtures of antibiotics are often used in medicine due to their synergistic effects against bacteria. These same effects may impact plants, as plant and bacteria share some biosynthetic pathways, including the folate pathway inhibited by sulfonamides and trimethoprim.[221] Toxicity of NSAID mixtures and β-blocker mixtures is additive in algae.[222–224] Many PPCPs commonly found in RWW interact significantly with each other in mammalian systems, and could have similar effects on plants. For example, carbamazepine induces several human CYP450s which are responsible for breaking down pharmaceuticals, while ciprofloxacin, fluoxetine, and diphenhydramine inhibit human CYP450s.[225] Although the CYP450s in plants and humans are not

directly equivalent, plants have nearly 250 CYP450 genes,[205] some of which have been implicated in phase 1 metabolism of herbicides.[226] Understanding mixture toxicity may be important for predicting plant bioaccumulation of PPCPs.

PREDICTING PLANT UPTAKE OF PPCPS

Treated wastewater may contain hundreds of PPCPs, and new pharmaceuticals are continually entering clinical use. For example, in most parts of the world tricyclic antidepressants have been largely supplanted by other classes of antidepressants. Of these, plant uptake of only fluoxetine has been studied.[151,186] This example of changing clinical drug use illustrates the need for fundamental knowledge of plant uptake processes that can be applied to risk assessment without necessitating descriptive studies on all newly developed drugs. Testing all potential wastewater-derived organic contaminants for plant uptake is impractical; predictive tools are needed for exposure and risk assessment.

Modeling based on compound properties. Plant uptake models range from single-parameter correlations (discussed above) to complex mechanistic and fugacity-based models. Most current models were developed for nonpolar, non-ionizable contaminants and do not accurately predict PPCP uptake by plants. Empirical single-parameter correlations are typically based on K_{ow}, root lipid content, or molecular mass, and have generally been developed for neutral organic compounds.[165–167,227,228] The correlations for neutral contaminants are not expected to be applicable to the many PPCPs that ionize at environmentally relevant pH values; for PPCPs, partitioning to non-lipid components such as proteins and carbohydrates is expected to be important.[229,230] Fewer correlations have been developed for polar/ionizable compounds, but some studies attempting to correlate plant uptake of selected PPCPs with plant or chemical properties have yielded correlations specific to the datasets from which they were generated (discussed above).

The limited ability of log K_{ow}, even when adjusted for compound speciation, to accurately predict uptake of organic compounds by plants has prompted development of relationships based

on multiple chemical descriptors. Limmer and Burken[231] used a desirability function to evaluate molecular descriptors commonly applied for drug discovery and transmembrane migration in mammals for their ability to predict organic contaminant TSCFs. Using TSCF measurements from the literature, they developed a weighted quantitative estimate of plant translocation ($QEPT_w$) from molecular descriptors, finding K_{ow}, molecular mass, and number of H-bond donors (HBD) to be most predictive. The $QEPT_w$ exhibited improved accuracy for hydrophilic compounds (log K_{ow} < 1) relative to methods relying solely on log K_{ow}, but prediction of TSCF remained poor.

We evaluated the utility of the desirability model for predicting plant uptake of PPCPs, hypothesizing that the relatively large error in the model as initially developed[231] stemmed in part from variability in the development dataset, which included results from studies of plants from 21 genera and obtained under different growth conditions. We followed the same approach to derive a weighted quantitative estimate of plant bioaccumulation ($QEPB_w$), but constrained the dataset used (Table S4) to LCF data for lettuce grown under hydroponic conditions by a single research group (see the SI for details, TSCF data were unavailable). Our analysis indicated that log K_{ow}, the number of rotatable bonds, and HBD were the strongest predictors of uptake of the PPCPs (Table S6). Our results contrast with those of Limmer and Burken:[231] number of rotatable bonds were an important predictor and molecular mass was not. We attribute this difference to the larger fraction of compounds with few (<5) rotatable bonds and larger range of compound masses in the initial dataset compared with ours.

Lacking a separate validation data set, we cross-validated our results. Despite reducing variability by focusing on hydroponic studies of just one crop type, the predictive value of the $QEPB_w$ appeared poor (Figure S2), similar to that of the original $QEPT_w$. Our analysis relied on data from studies using the same exposure time and comparable growth conditions, so additional

factors must underlie the poor predictive ability of the model. *In planta* transformations may have confounded the analysis; carbamazepine, diclofenac, and triclosan were included in the development dataset and can be metabolized by plants.[27,145,200,215,217,232] Furthermore, if any compounds undergo active uptake or sequestration, small differences in protein levels between breeds could affect bioaccumulation. For analyses including ionizable compounds, additional descriptors that account for speciation, charge, or specific functional groups may warrant inclusion. We note that the model constrains the TSCF (or LCF) output to the closed interval of 0 to 1, which is mathematically necessary, but does not reflect a constraint in uptake, potentially limiting model accuracy and usefulness. As implemented to date, this approach cannot accurately predict plant uptake of organic contaminants.

Poly-parameter linear free energy relationships have been successfully applied to predict contaminant uptake into major biological phases (viz. storage lipids,[233] phospholipid membranes,[116] proteins[234,235]) and whole organisms/tissues[116,236] and may hold promise for predicting the accumulation of PPCPs not metabolized *in planta* or taken up by active processes. Such relationships may be applicable to root uptake of neutral molecules if their assumptions (i.e., no metabolism, equilibrium between plant and exposure concentrations) are met. However, generally applicable pp-LFERs have not yet developed for ionizable contaminants.[21,22]

Compartmental Models. More complex models[109,237–250] for plant uptake of neutral organic contaminants have been developed that consider plant and environmental parameters such as root lipid fraction and SOM, and attempt to incorporate the complexity of uptake processes into their formulation by focusing on specific uptake pathways, compound classes, or plant types. Although compartmental models include a more complete set of plant, environmental, and chemical properties, they still suffer from poor accuracy and predictive power. Collins et al.[251]

tested nine models for non-ionizable contaminants by comparing predictions to experimental study results chosen to reflect a range of chemical properties and uptake pathways, and found most models over-predicted root concentrations by at least an order of magnitude. Currently, only a single study has specifically modelled uptake of PPCPs with more than a simple correlation and compared results with experimental values; this study found both tested models over-predicted concentrations for many compounds.[252] Inaccuracies in current model predictions are due mainly to underlying conceptual uncertainties.[252,253] To yield accurate and useful predictions, complex models have high data demands, requiring parameters like root volume and rate constants for plant growth, compound metabolism, and contaminant loss from soil due to processes other than plant uptake. A sensitivity analysis performed on the compartmental model of Goktas and Aral[254] for neutral organic contaminants indicated that predicted tissue concentrations were most sensitive to the retardation factor, TSCF, and contaminant half-life within the plant. Many current models fail to account for processes such as metabolism, phytotoxicity, growth dilution, and contaminant physicochemical properties beyond K_{ow}, and to our knowledge, no models currently include energy-dependent uptake processes.

The few models developed specifically for ionizable compounds[193,255–257] have not been as fully developed and validated as those for neutral compounds. Knowledge gaps include the effects of multiple ionizable moieties, contributions of energy-dependent uptake processes, membrane permeability in passive uptake processes, electrostatic interactions with membranes and cell walls, *in planta* transformations, and rhizosphere effects (e.g., plant-induced pH changes, degradation by microorganisms). For more details on compartmental models, we direct readers to reviews focused on modeling.[109,115,258]

RECOMMENDATIONS FOR FUTURE RESEARCH

The existing literature allows some trends in uptake to be deduced, but most studies did not report important information on environmental parameters that is necessary for more rigorous analysis. Comparisons among studies to deduce trends in uptake due to chemical, soil, or plant properties is rendered difficult by variations in plant growth conditions, analytical methods, species/cultivars studied, and data reporting practices (e.g., fresh vs. dry weight, treatment of censored data). Table 1 lists suggested minimum data to be provided in future uptake studies, along with the fraction of existing studies reporting each parameter. Inclusion of this information in future studies will contribute to a deeper understanding of the process of plant uptake and help facilitate the development of predictive models.

We have noted knowledge gaps and provided recommendations for future research throughout this review; here we summarize these data gaps and suggested future directions:

- Rhizosphere Processes. Root exudates and rhizosphere microbiota are important factors governing plant uptake for metals, polychlorinated biphenyls, and PAHs. Their effect on uptake of ionizable organic compounds warrants investigation. Root exudates may change the pH of the rhizosphere, changing speciation and availability of many PPCPs. Soil microbiota may transform PPCPs and make them less available to plants. Beneficial symbionts may be affected by PPCPs and in turn alter plant processes.
- Uptake mechanisms. That plants can take up PPCPs is well established. The underlying mechanisms, however, are not as evident. Distinguishing between uptake mechanisms and translocation is necessary to distinguish between sorption to roots, accumulation within the plant, passive uptake, and (potential) active uptake mechanisms. Root uptake pathways, phloem transport to edible parts, and *in planta* mobility of conjugated metabolites are areas

needing investigation. Interactions between contaminants and plant components such as cell membranes and walls also merit further study. We are especially interested in uptake mechanisms for cationic PPCPs, as the literature suggests they are taken up more frequently than expected from their physicochemical properties.

- Transformation *in planta*. Little information exists on plant metabolism of PPCPs. In many cases, resulting transformation products are still unknown. Accounting for metabolized fractions of PPCPs may significantly alter estimates of uptake and human exposure (e.g., if parent compounds are released from conjugates in the body), as well as impact the development and accuracy of predictive models. Whether metabolites are toxic and their fate within plants also merit study.
- Effects of PPCPs on Plants. Wastewater-derived micropollutants may affect plant physiology and alter normal biochemical pathways. Many PPCPs are biologically active, but little information exists on their effects on plants. Even if a PPCP itself represents minimal direct toxicological risk for human consumption of contaminated crops, it could up- or down-regulate phytohormones or other endogenous plant compounds that can affect human health.
- Prediction of Plant Uptake. PPCPs are structurally heterogeneous, and different compound classes exhibit different uptake patterns. Developing models for individual compound classes may therefore represent a more promising interim approach than considering all PPCPs together. Likewise, plants differ widely in their physiology, and different taxa may need to be treated separately. Identifying and reporting key parameters responsible for influencing uptake and their values will also improve model validation and utility.

Table 1. Suggested minimum data set for plant uptake studies and fraction of currently published studies on plant uptake of PPCPs reporting listed parameters (*n* = number of studies).

Plant Properties	**Fraction of Studies**	***n***
variety	0.43	35
% water	0.03	35
% lipid	0.11	35
plant health metrics	0.26	35
age at first exposure	0.77	35
Environment Properties		
exposure duration	0.69	35
temperature	0.57	35
humidity	0.23	35
Hydroponics		
solution pH	0.70	10
solution volume	0.80	10
frequency of solution renewal	0.80	10
inclusion of no-plant control	0.20	10
Soil Properties		
soil pH	0.57	25
soil texture	0.56	25
water content	0.24	25
% OM or OC	0.84	25
cation exchange capacity	0.29	25
mineralogy	0.04	25
nutrient concentrations	0.16	25
biosolids properties	0.56	9
inclusion of no-plant control	0.27	25
Irrigation		
amount	0.06	25
frequency	0.14	25
Analysis		
LODs/LOQs	0.77	35
Frequency of detection in plant tissue	0.11	35

A large body of literature exists for organic contaminant uptake by plants from both the theoretical and descriptive perspectives (largely for only neutral compounds for the former), but current models and experimental data are not sufficiently complementary to facilitate quantitative predictions of uptake for unstudied PPCPs. Few mechanistically focused experimental studies have been published. Many compounds have the potential to be taken up by plants, but failure to

report essential data and site-specific nature of most descriptive studies limits the broad application of their results. Plants are living organisms that interact with and sometimes alter their environments, and model accuracy may be improved by incorporating these interactions. Well-controlled experiments that systematically vary important properties will be key to understanding plant uptake of PPCPs and improving the capabilities of predictive models.

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SUPPORTING INFORMATION AVAILABLE

Discussion of risk assessment of consumption of PPCP-contaminated crops, details of meta-analysis methods, and tables and figures related to the QEPB model development.

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SUPPORTING INFORMATION

FOR CHAPTER ONE

ROOT UPTAKE OF PHARMACEUTICAL AND PERSONAL CARE PRODUCT INGREDIENTS

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SUMMARY

The SI is 14 pages long and includes 5 tables and 2 figures. It is split into 4 sections; Section S1 discusses risk analysis of consumption of PPCP-contaminated crops, Section S2 discusses a particular study in detail, Section S3 details out literature data compilation methods, and Section S4 discusses our QEPB model development. Tables S1, S2, and S3 show the literature data we used to generate Figure 2 (in the main text). Tables S4 and S5 show the optimized values for our QEPB model. Figure S1 shows histograms of leaf concentration factor literature data for the molecular descriptors used to generate the QEPB model. Figure S2 shows a comparison between uptake data and QEPB predictions.

SECTION S1: PLANT UPTAKE OF PPCPS IN RELATION TO HUMAN EXPOSURE LEVELS OF CONCERN

Table S1 summarizes recent literature reports of detected concentrations of PPCPs in realistic field scenarios. This is not a comprehensive summary of the literature; we only aim to give a sense of real environmental concentrations. For more information, we direct readers to recent reviews of PPCPs in plants.[1–3] While using realistic field scenarios more accurately represents risk assessment for a specific place/crop/set of PPCPs, real environments are less useful for developing an understanding of underlying mechanisms driving uptake, which is necessary because of the sheer number of possible combinations of current and future PPCPs and plants and environmental conditions.

Table S1. Examples of environmental levels of PPCPs and corresponding concentrations in crop plants. All studies used realistic field and growing conditions, not spiked growth media. n.d. = no detection (includes all <LOD and <LOQ values).

Compound Class (compound)	Irrigation Water ($\mu g \cdot L^{-1}$)	Soil ($\mu g \cdot kg^{-1}$)	Biosolids ($\mu g \cdot kg^{-1}$)	Crop	Crop ($\mu g \cdot kg^{-1}$)	Reference
Tetracyclines (chlortetracycline)	-	240	4000 – 9000	winter wheat	n.d. - 874	[4]
Macrolides (erythromycin)	0.004 – 0.01	1.1 – 4.4	-	Chinese white cabbage, water spinach, Chinese radish, corn, rice	n.d. – 2.2	[5]
Fluoroquinolones	-	0.1- 651.6	-	tomato, cucumber, spinach, pepper, eggplant	2.0 – 661.0	[6]
Sulfonamides (sulfadiazine)	-	90	145000 - 250000	winter wheat	n.d. – 487	[4]
NSAIDs	n.d. – 22.4	-	-	lettuce, carrot	n.d. - 113	[7]
Tricyclic Psychoactive Drugs (carbamazepine)	0.06 – 0.369	-	-	lettuce, carrot	n.d. - 52	[7]
Miscellaneous Basic Drugs (caffeine)	0.295- 0.789	-	-	apple tree, alfalfa	0.114-110.7	[8]
Personal Care Product Ingredients	n.d. - 0.543	-	-	apple tree, alfalfa	0.024 – 67.6	[8]

Several groups have assessed measured PPCP concentrations in vegetables with respect to human exposure and the extent to which plant uptake of PPCPs warrants concern. Modeling of crop uptake and subsequent human exposure to a range of PPCPs showed intake of pharmaceuticals to be < 15 ng·person^{-1}·d^{-1} and intake of personal care product ingredients to be < 250 ng·person^{-1}·d^{-1}.[9] However, the estimated BCFs were in general much higher than those observed in experimental uptake studies. Exposure to triclosan and triclocarban via consumption of contaminated crops, as evaluated using literature uptake results, appears to be of low concern, because even high concentrations reported in edible tissues represent a small fraction of acceptable daily intake levels for adults and children.[10,11] Similarly, the potential contribution of vegetable material grown on animal-manure amended soil to the total acceptable daily intake of eight veterinary pharmaceuticals was found to be < 10% for all compounds tested,[11] the estimated values of daily human exposure of five antibiotics in five different crops were much lower than the minimum therapeutic doses and below the recommended ADI values,[5] and the estimated per capita annual PPCP exposure for seven compounds in eight different crops is more than three orders of magnitude smaller than a single medical dose for one compound.[12] Large-scale testing of mushrooms, vegetables, aquaculture products, and animal tissues collected from sites in the United Kingdom and aquaculture products from Southeast Asia showed detectable levels of musk compounds and antibiotics in some samples, but mostly at low ng/g concentrations, suggesting limited contamination of target chemicals in realistic food-producing systems.[13] Supply chain modeling indicates that milk and meat products may also be a source of PPCP exposure, but that the level of the human exposure via these pathways does not exceed the ADIs for the compounds.[14] However, PPCP uptake may pose more risk under certain conditions. For example, aquatic vegetables grown in undiluted swine manure, as is common in southeast Asia, exhibit

bioaccumulation of oxytetracycline that corresponds to a significant fraction (>48%) of the compound's ADI.[15]Although exposure risk for most individual compounds appears low, little is known about the effects of chronic low dose exposures, especially in regards to mixture toxicities. Additionally, some PPCPs or PPCP metabolites may be of concern if they are highly toxic. For example, compounds such as carbamazepine and caffeine would require an adult to consume hundreds of kilograms daily of sweet potatoes or carrots grown in soil irrigated with treated wastewater to reach the threshold of toxicological concern level, but potentially toxic levels of the carbamazepine metabolite 10,11-epoxycarbamazepine and the anti-convulsant lamotrigine are reached at a much lower daily consumption, as the threshold for toxic concern level of lamotrigine would be surpassed for a 70 kg adult by consuming two carrots a day (180 g carrot/day) and for a 25 kg child by consuming half a carrot a day (60 g carrot/day).[16] Furthermore, if metabolites revert back to parent compounds during digestion of contaminated crops, exposure may be higher than predicted. For example, triclosan is glycosylated in roots,[17,18] resulting in relatively weakly conjugated bonds with sugars that may easily be broken by gut bacteria in the large intestine. In light of uncertainty in chronic, low-dose mixture toxicity and variation in RWW and biosolid application, regulations for use, and PPCP concentrations, we conclude that exposure to PPCPs from contaminated crops may represent a significant exposure pathway for at least some compounds and merits continued research.

SECTION S2: NOTES ON SABOURIN ET AL., 2012

The results of one study stand in contrast to many of those described in the main text.[19] Plants were grown in soil that had received amendment of biosolids containing many PPCPs one year prior to planting in accordance with the regulations in Ontario, Canada. Compounds in the biosolids included several commonly taken up PPCPs, such as carbamazepine, along with many that have not been included in other studies. There were no compounds detected in both the biosolids and the experimental plants that were not also present at comparable levels in the control plants. This result suggests reduction in bioavailability or dissipation of the PPCPs during the intervening time period, and indicates that a wait-time between biosolids application and planting could minimize PPCP exposure risk in some environments. However, the time elapsed between biosolids application and planting may not reflect practices in other regions. Additionally, the authors do not measure PPCP concentrations remaining in the soil after the one year wait period, and uptake due to contamination in irrigation water is not addressed, although control plants had detectable levels of some compounds. Because of the uncertainty in the compound exposure levels in this study, we chose not to include it in the qualitative and quantitative analyses in our review (with the exception of the discussion of amitriptyline).

SECTION S3: SUGGESTED MINIMUM DATA FOR APPLIED UPTAKE STUDIES

Suggested essential minimum data that should be reported to facilitate inter-study comparisons and aid understanding of plant uptake are presented in Table 1, along with the fraction of currently published studies that report each parameter. Failure to report critical experimental data leads to much considerable uncertainty in inter-study trends. This is demonstrated in both our data compilation (Figure 3a-b; Figure S2) and that of Limmer and Burken.[20] Without accounting for differences in exposure times, transpiration rates, etc., neither analysis was able to find a strong correlation between compound molecular descriptors and plant uptake. Here, we clarify the meaning and describe the importance of each parameter.

Plant Properties. There are many varieties within a given crop species, and these varieties may have different lipid contents, water use efficiencies (transpiration rates), or efficiencies of enzymes for degradation of xenobiotics. Plants may also express different ratios of degradation enzymes or other important proteins (i.e., transport proteins) at different stages of their life cycle or when under stress. Feedback loops, in which exposure to one contaminant changes plant gene expression, leading to changes in uptake of other compounds, may be possible, although to our knowledge this has not yet been studied. These plant properties may affect measured uptake and bioaccumulation, leading to unexplainable differences in reported values when they are not measured. Additionally, while gross plant parameters such as total lipid and water content are important for broadly applicable models, species-specific parameters such as root endodermis structure and xylem sap protein content may also need to be considered. Current models typically assume default values for such parameters, although sensitivity analysis on several models for uptake of neutral organic hydrophobic contaminants has shown that parameter variability changes the simulated plant accumulation by approximately two orders of magnitude from fifth to 95th

percentile, with the most sensitive parameter differing by compound and including transpiration and growth rates.[21] Depending on the extraction procedure (see below), concentrations within plant tissues may be reported as per wet or dry weight, so knowing the water content of the tissues facilitates inter-study comparison without introducing assumptions about water content.

Environmental Properties. Important environmental parameters to report include temperature, humidity, water and nutrient availability, which are important when comparing transpiration rates and uptake kinetics**.** Temperature and humidity affect transpiration rates, which are generally assumed to control passive uptake, and have been shown to loosely correlate with uptake.[22] These factors, as well as water and nutrient availability, can also affect plant health, which can in turn affect uptake (see above). Exposure duration affects bioaccumulation, with longer exposures leading to increased bioaccumulation; predictions of bioaccumulation therefore relies on knowing exposure durations in the datasets used in model development and validation.

Hydroponics. Solution pH controls ionization state of ionizable contaminants; neutral and ionized forms may have different uptake rates. No plant controls are important for establishing whether contaminants are disappearing from solution for reasons other than plant uptake – sorption to the solution container, abiotic degradation via hydrolysis or photodegradation, or degradation by microorganisms may all contribute to lower than expected exposure levels, altering concentration factor calculations. Solution volume and frequency of renewal also contribute to contaminant and nutrient availability to the plant.

Soil Properties. Contaminant-soil interactions - including sorption/desorption processes, degradation, and formation of bound residues - control the availability of the contaminant to the plant (see main text section Processes Impacting Plant Uptake of PPCPs). These interactions are

governed by contaminant physicochemical properties and soil properties. Therefore, reporting only the general classification of soil used (i.e., "sandy loam") is not enough to predict availability from soil. Soil interactions depend on the class of the PPCP, organic matter/carbon amount and properties, cation exchange capacity, mineralogy (composition of inorganic minerals), and particle size distribution. Soil nutrient and water contents may contribute to plant-induced rhizosphere changes in pH or changes within the plant due to water availability, including transpiration rates. For studies using biosolids-amended soils, properties of the biosolids (water and OM content, nutrient content, pH) are important for determining contaminant availability from biosolids. Addition of biosolids also changes bulk soil properties, altering contaminant availability from soil as well. As with hydroponic studies, no-plant controls are important for establishing whether contaminants are disappearing from the system for reasons other than plant uptake – sorption/binding to soil particles, abiotic degradation via hydrolysis or photodegradation, or degradation by microorganisms may all contribute to lower than expected exposure levels, altering concentration factor calculations.

Irrigation. For soil studies, irrigation amount and frequency may drastically change total exposure levels, especially when exposure is via contaminated irrigation water. Calculating bioconcentration factors is also not possible without this information, as growth medium PPCP concentrations are needed. For studies where PPCPs are directly spiked into soil or added with biosolids, irrigation amount and frequency may contribute to leaching rates, soil water content, and plant gene expression changes induced by water stress.

Analysis. Reporting extraction and detection method parameters such as limits of detection and quantification (LODs/LOQs) and recoveries allows comparison between data from different methods. Frequency of detection is also important to report, as different groups have

treated censored data in different ways and including no detection samples in concentration averages decreases means.

SECTION S4: DATA COMPILATION

Figure 3. (a) and (b): We compiled raw data from eight PPCP uptake studies using lettuce, and organized them by growth media. We chose lettuce because it was the most commonly used vegetable in descriptive uptake studies. We defined hydroponic studies as those experiments where plants were exposed to PPCPs via water or liquid nutrient solution without soil, and irrigated soil studies as those experiments where plants were grown in soil and exposed to PPCPs via irrigation (as compared to experiments where the soil was spiked directly with PPCPs or amended with biosolids containing PPCPs; we did not include soil experiments other than irrigated soil due to the variability in methods of reporting soil concentrations). For each compound in each study, we determined the exposure concentration ($\mu g \cdot L^{-1}$ of either hydroponic solution or irrigation solution; for irrigated soil studies we were unable to account for frequency and amount of irrigation due to variability in or lack of reporting) and the leaf concentration ($ng \cdot g^{-1}$ fresh weight equivalent, assuming lettuce is 96% water[23]). We used these data to calculate LCF ($\frac{\text{leaf concentration}}{\text{exposure concentration}}$) for each compound in each study. In plotting these data, we defined acids as compounds ionizing form an anion between pH 4 and 10, bases as those ionizing to form a cation in this pH range, and neutral compounds as those not ionizing in this pH range.

Table S2. Hydroponic lettuce data from the literature (used to produce Figure 2a). Neutral, basic and acidic compounds are indicated respectively in black, blue, and red.

Compound	pK_a*	log K_{ow}*	Medium Concentration ($\mu g \cdot L^{-1}$)	Leaf Concentration ($ng \cdot g_{fw}^{-1}$)	LCF	Source
acetaminophen	9.38	0.46	0.5	0	0	[24]
			0.5	0	0	[25]
			5	0	0	[25]
atenolol	9.6	0.16	0.5	0	0	[24]
			0.5	0	0	[25]
			5	0.108	0.02	[25]
atorvastatin	4.46	4.46[26]	0.5	0.064	0.1	[24]
			0.5	0.064	0.1	[25]
			5	0.048	0.01	[25]
caffeine	10.4	-0.07	0.5	0.128	0.3	[24]
			0.5	0.128	0.3	[25]

			5	0.028	0.01	[25]
carbamazepine	13.94	2.45	0.5	1.148	2	[24]
			0.5	1.16	2	[25]
			5	10	2	[25]
DEET	0.67[25]	2.02	0.5	0.112	0.2	[24]
			0.5	0.112	0.2	[25]
			5	0.72	0.1	[25]
diazepam	3.4	2.8	0.5	0.712	1	[24]
			0.5	0.72	1	[25]
			5	6.4	1	[25]
diclofenac	4.15	4.51	0.5	0	0	[24]
			0.5	0	0	[25]
			5	0	0	[25]
			0.2374	0.362	1.52	[27]
dilantin	8.33	2.47	0.5	0.368	0.7	[24]
			0.5	0.368	0.7	[25]
			5	3.04	0.6	[25]
diuron	8.33	2.68	0.5	0.068	0.1	[25]
			5	3	0.6	[25]
fluoxetine	9.7[28]	4.05	0.5	0.84	2	[24]
			0.5	0.88	2	[25]
			5	10.4	2	[25]
gemfibrozil	4.5	4.77	0.5	0.008	0.02	[24]
			0.5	0.008	0.02	[25]
			5	0.024	0.005	[25]
ibuprofen	4.91	3.97	0.5	0	0	[24]
			0.5	0	0	[25]
			5	0	0	[25]
meprobamate	15.17[‡]	0.7	0.5	0.2	0.4	[24]
			0.5	0.2	0.4	[25]
			5	1.2	0.2	[25]
naproxen	4.15	3.18	0.5	0	0	[24]
			5	0.004	0.0008	[25]
			0.1782	0.1124	0.6308	[25]
primidone	11.5[‡]	0.91	0.5	0.34	0.7	[24]
			0.5	0.34	0.7	[25]
			5	2.44	0.5	[25]
sulfamethoxazole	5.7[29]	0.89	0.5	0	0	[24]
			0.5	0	0	[25]
			5	0	0	[25]
triclocarban	12.7[30]	4.9	0.5	0.012	0.02	[24]
			0.5	0.012	0.02	[25]
			5	0.056	0.01	[25]
triclosan	7.9	4.76	0.5	0	0	[24]
			0.5	0	0	[25]
			5	0	0	[25]
trimethoprim	7.12	0.91	0.5	0.088	0.2	[24]
			0.5	0.044	0.09	[25]
			5	0.4	0.08	[25]

*from https://www.ncbi.nlm.nih.gov/pccompound unless otherwise noted

[‡]http://www.drugbank.ca/

Table S3. Irrigated soil lettuce data from the literature (used to produce Figure 2b). Neutral, basic and acidic compounds are indicated respectively in black, blue, and red.

Compound	pK_a*	log K_{ow}*	Medium Concentration (µg·L^{-1})	Leaf Concentration (ng·g$_{fw}^{-1}$)	LCF	Source
ambrettolide		5.37[‡]	0.497	75	150	[7]
			0.261	159	609	[7]
azithromycin	8.74	4.02	1	0	0	[31]
carbamazepine	13.94	2.45	0.369	0	0	[7]
			0.061	1	16	[7]
			0.225	0.058	0.26	[12]
clindamycin	7.6	2.16	1	0	0	[31]
clofibric acid	3.2[32]	2.88[32]	0.35	18	51	[7]
			0.003	1	300	[7]
diclofenac	4.15	4.51	22.41	19	0.85	[7]
dilantin	8.33	2.47	0.203	0.026	0.13	[12]
flunixin	5.82[33]	4.9[‡]	0.367	10	30	[7]
			0.027	6	200	[7]
galaxolide		5.9[#]	0.451	36	79	[7]
			0.153	32	210	[7]
ibuprofen	4.91	3.97	0.35	5	10	[7]
			0.043	6	100	[7]
naproxen	4.15	3.18	0.576	113	196	[7]
primidone	11.5[**]	0.91	0.175	0.072	0.41	[12]
roxithromycin	9.2[34]	2.75[35]	1	0	0	[31]
tonalide		5.7[#]	0.226	124	549	[7]
			0.117	0	0	[7]
triclosan	7.9	4.76	0.233	9	40	[7]
			0.007	0	0	[7]

[*] from https://www.ncbi.nlm.nih.gov/pccompound unless otherwise noted

[‡] predicted using EPIsuite http://www.epa.gov/opptintr/exposure/pubs/episuite.htm

[#] http://toxnet.nlm.nih.gov/

[**] http://www.drugbank.ca/

Figure 3 (c) and (d): We found some errors in the pH-dependent *n*-octanol-water partitioning coefficients (D_{ow}) calculated by Wu et al.,[25] so we recalculated them using the same pK_a values except for that of sulfamethoxazole (this compound has pK_a values at 5.7 and 1.85;[29] Wu et al. used the 1.85 value only). Using the same K_{ow} values as Wu et al., we calculated D_{ow} using the equation $\log D_{ow} = \log K_{ow} + \log \frac{1}{1+10^{i(\text{pH-p}K_a)}}$ where $i = -1$ for acids and $i = 1$ for bases and pH = 6.5. Acids were defined as compounds ionizing form between pH 4 and 10, bases were defined as those ionizing to form a cation in this pH range, and neutral compounds were defined as those not ionizing in this pH range. Using the raw data supplied by Wu et al. in their Supplemental Information, we calculated RCF as $RCF = \frac{\text{average concentration detected in roots}}{0.5\ \mu g/L}$, where average concentration detected in roots = 0 for no detection of the compound.

Table S4. Corrected data from Wu et al.[25] Neutral, basic and acidic compounds are indicated respectively in black, blue, and red.

Compound	pK_a*	log K_{ow}*	reported log D_{ow}	recalculated log D_{ow}	LCF	RCF
Acetaminophen	9.38	0.46	0.46	0.46	0	0
Caffeine	10.4	-0.07	-3.97	-0.07	0.3	0.1
Meprobamate	15.17‡	0.7	-8.43	0.70	0.4	0.2
Primidone	11.5‡	0.91	-4.21	0.91	0.7	0.3
Sulfamethoxazole	5.7[29]	0.89	0.89	0.83	0	0
Atenolol	9.6	0.16	-2.94	0.16	0	0
Trimethoprim	7.12	0.91	0.2	0.82	0.09	1
DEET	0.67[25]	2.02	2.18	2.02	0.2	0.3
Carbamazepine	13.94	2.45	2.45	2.45	2	1
Dilantin	8.33	2.47	0.63	2.46	0.7	1
Diuron	8.33	2.68	2.68	0.84	0.1	0.5
Naproxen	4.15	3.18	0.83	3.18	0	0.8
Diazepam	3.4	2.8	2.82	2.80	1	5
Fluoxetine	9.7[28]	4.05	0.46	4.05	2	10
Atorvastatin	4.46	4.46[26]	4.36	4.46	0.1	0.1
Ibuprofen	4.91	3.97	2.37	3.96	0	0.02
Gemfibrozil	4.5	4.77	3.01	4.77	0.02	0.7
Triclosan	7.9	4.76	4.74	3.34	0	6
Diclofenac	4.15	4.51	2.16	4.51	0	0.2
Triclocarban	12.7[30]	4.9	4.9	4.90	0.02	40

* from https://www.ncbi.nlm.nih.gov/pccompound unless otherwise noted

‡ http://www.drugbank.ca/

SECTION S5: QEPB ANALYSIS

We applied the methods of Limmer and Burken[20] to the hydroponic lettuce LCF data from the literature (see above), because we hypothesized that minimizing the variability arising from differences between plant genera may increase the utility of this type of analysis. We conducted this analysis using LCF rather than TSCF data because the TSCF dataset for PPCPs available in the literature was too limited to support QEPB analysis. The selected datasets were from studies conducted by the same group, using the same exposure period prior to harvesting and analysis (21 d starting at the seedling stage). One of the studies[27] used ^{14}C-diclofenac and did not distinguish between parent compound and metabolites. Such studies were excluded by Limmer & Burken, but we included this study because the one data point used from this study does not seem to alter our conclusions. Molecular descriptors (log K_{ow}, number of hydrogen bond donors and acceptors, molecular mass, number of rotatable bonds, polar surface area) were obtained using the ACD/PhysChem Suite as implemented by ChemSpider.[36] We averaged LCF values for each unique compound (n = 20), and computed weighted histograms of each molecular descriptor, with weights based on the average compound LCF, via the following algorithm (Figure S1):

1. We fixed a number of bins based on the descriptor values. For example, PSA was split into m = 5 bins, on intervals (19.9,38.4], (38.4,56.8], (56.8,75.2], (75.2,93.6], (93.6,112], and with counts 6, 6, 3, 1, 4, respectively.
2. For each bin, instead of counting how many observations belong to the interval, we weighted the bin by adding up the average LCF value for compounds with the corresponding descriptor within the specified interval range.

To each weighted histogram we fitted the asymmetric double-sigmoidal function

$$D(x) = a + \frac{b}{1 + \exp\left(-\frac{x - c + \frac{d}{2}}{e}\right)}\left(1 - \frac{1}{1 + \exp\left(-\frac{x - c - \frac{d}{2}}{f}\right)}\right)$$

where $D(x)$ is the desirability function for each molecular descriptor, x, and a–f are fitting parameters, by minimizing the sum of squares $\sum_{i=1}^{m}(h_i - D(x_i))^2$ were m is the number of bins, i indexes the bins, h_i is the value taken by the i-th bin, and x_i is the bin center. Standard errors of the fitting parameters were estimated using a bootstrapping procedure,[37] wherein the following algorithm was repeated 1000 times:

1. A random sample of the dataset compounds (n = 20, which is the same size as the original dataset) was collected with replacement (so each individual compound can appear more than once).
2. The whole algorithm was run with the new dataset, and a - f and the weights for each resampled dataset were registered.
3. The standard deviation of the registered parameters is approximately the true standard error

Desirability functions were combined to calculate the quantitative estimate of plant bioaccumulation ($QEPB_i$) given a set of weights, such that

$$QEPB_i = \exp\left(\frac{\sum_j w_j \, log\left(D_j(x_i)\right)}{\sum_j w_j}\right)$$

where w_j is a weighting factor belonging to [0,1] and D is the desirability function for molecular descriptor x_i. Weights were determined by maximizing the Shannon entropy (SE), and are given in Table S6.

To maximize SE, we first did a grid search in [0, 1] with increments of 0.2 (thus evaluating 46,656 cases), then used a constrained-optimization algorithm with initial values based on the best guess from the grid search. We report both the maximum SE weights ($QEPB_{max}$; SE = 8.9117818) and the average of the top 100 weights from the grid search ($QEPB_{100}$; SE = 8.8191322). Since we lacked a separate validation data set, we cross-validated our results; for each compound, we removed that compound from the dataset, ran the whole procedure on the remaining compounds, and estimated the LCF of the removed compound by the predicted $QEPB_w$ (Figure S2).

Table S6 presents the optimized desirability function weightings for each molecular descriptor with standard errors approximated from a bootstrapping procedure by resampling the data.[37] Our errors reflect both the small data set and the large amount of variability in uptake results even for one crop type under similar growing conditions. In our analysis, molecular mass and polar surface area had the most variability relative to the optimized weightings (Table S5), which may be due to the inclusion of the compound atorvastatin. This compound is significantly larger than the other compounds, but we chose to retain it in the analysis because of our small dataset size; its exclusion did not substantially alter the weighting of the $QEPB_w$ output.

Table S5. Optimized fitting parameter values for log K_{ow}, number of hydrogen bonding donors (HBD), number of hydrogen bonding acceptors (HBA), molecular mass (MW), number of rotatable bonds (ROT), and polar surface area (PSA). Standard errors were computed using a bootstrapping method and are reported in parentheses.

	a	*b*	*c*	*d*	*e*	*f*
log K_{ow}	1.551	4.574	3.197	0.871	0.009	6.009
	(0.82)	(2.61)	(1.60)	(2.68)	(0.02)	(4.28)
HBD	1.154	4.497	1.604	1.428	0.221	0.004
	(0.80)	(2.56)	(0.76)	(1.51)	(2.27)	(0.01)
HBA	1.291	5.135	0.007	7.887	6.142	0.044
	(0.59)	(2.41)	(2.13)	(3.89)	(2.50)	(0.01)
MW	-0.149	38.533	850.525	-1033.458	809.102	3.176
	(1.70)	(10.09)	(289.01)	(456.87)	(714.13)	(112.61)
ROT	-0.057	57.989	-13.156	19.729	42.095	3.210
	(0.85)	(7.14)	(6.64)	(9.32)	(8.11)	(0.44)
PSA	1.263	5.775	-49.589	206.196	158.061	4.068
	(0.96)	(7.11)	(90.04)	(152.77)	(76.90)	(9.95)

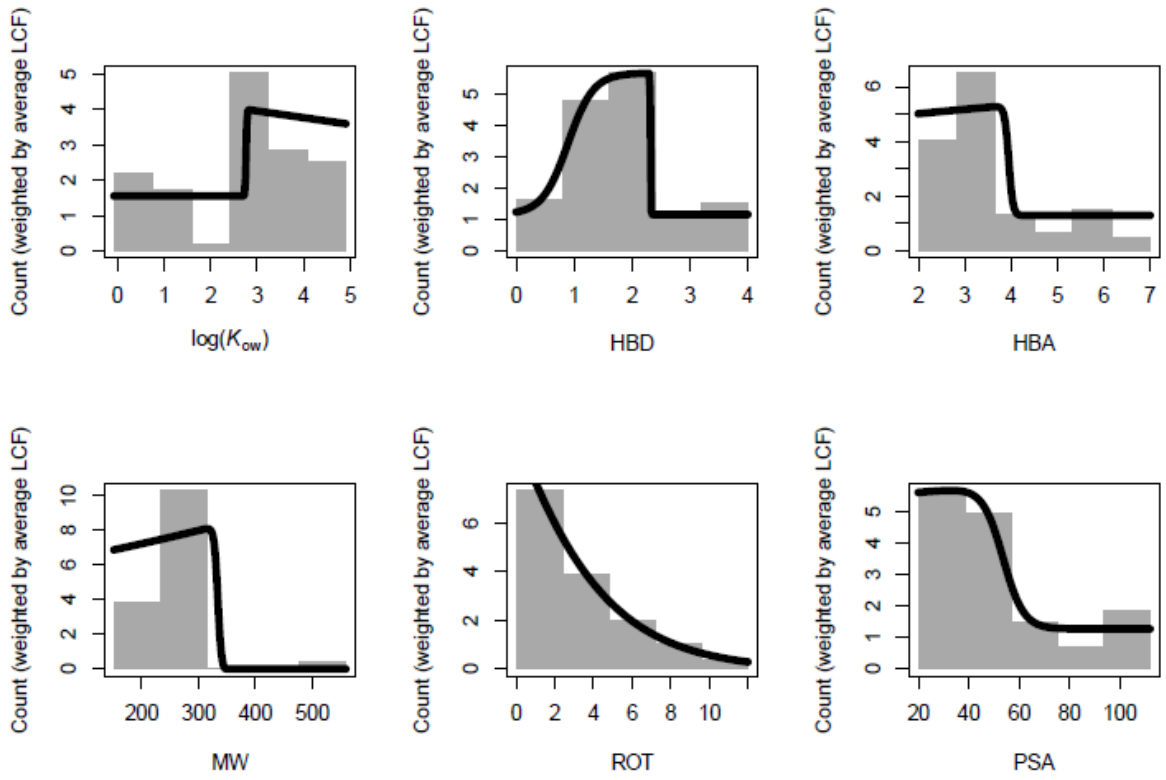

Figure S1. Histograms of LCF values for hydrophobicity (log K_{ow}), number of hydrogen bonding donors (HBD), number of hydrogen bonding acceptors (HBA), molecular mass (MW), number of rotatable bonds (ROT), and polar surface area (PSA). Solid curves are the fitted desirability functions.

Table S6. Optimized desirability function weightings for the quantitative estimate of plant bioaccumulation (QEPB) or translocation (QEPT). QEPB/T_{100} is an average of the 100 highest-scoring weights. Standard errors are indicated in parentheses (note that because the model is restricted to $D(x) \in [1,0]$, the standard errors do not correspond to ranges).

	source	log K_{ow}	HBD	HBA	MW	ROT	PSA
$QEPB_{max}$	this study	0.48	0.1	0	0	0.42	0
		(0.26)	(0.2)	(0.12)	(0.22)	(0.2)	(0.23)
$QEPB_{100}$	this study	0.79	0.29	0.03	0.03	0.77	0.03
		(0.33)	(0.29)	(0.21)	(0.31)	(0.32)	(0.32)
$QEPT_{max}$	[20]	0.65	0.75	0	0.9	0	0
$QEPT_{100}$	[20]	0.56	0.64	0	0.76	0	0

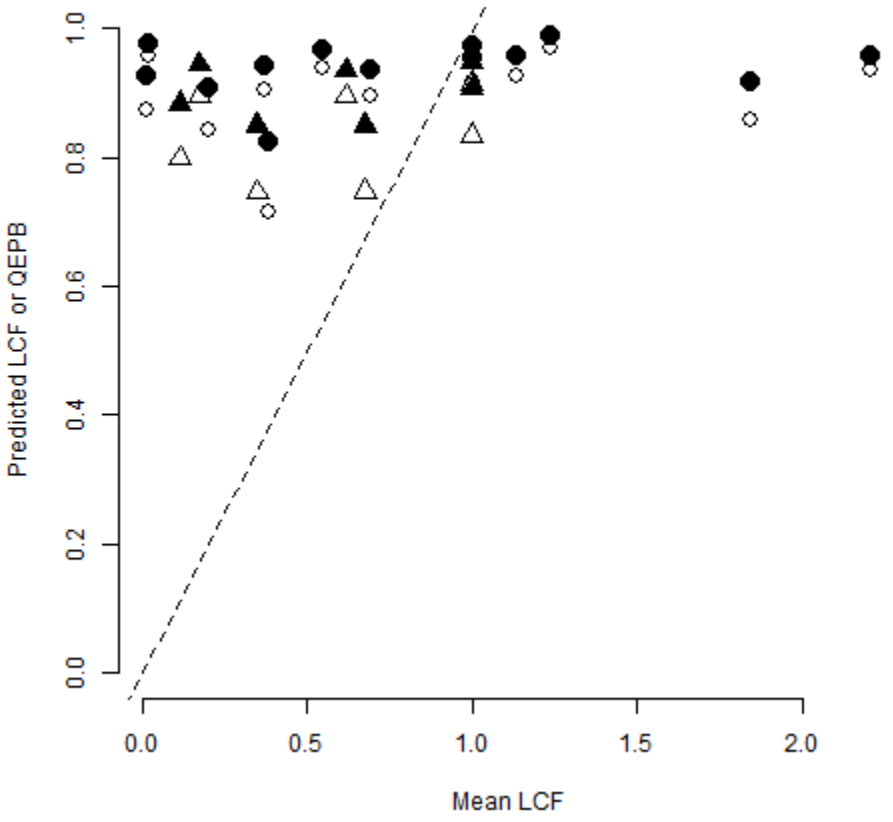

Figure S2. Cross-validation of QEPB LCF predictions compared against LCF measurements. $QEPB_{max}$ calculated values are indicated in black and $QEPB_{100}$ calculated values are indicated in white. Triangles represent hydrophilic compounds with a log K_{ow} of < 1.

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CHAPTER TWO

PLANT-INDUCED CHANGES TO RHIZOSPHERE PH INFLUENCE UPTAKE OF IONIZABLE ORGANIC CONTAMINANTS

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ABSTRACT

Many ionizable organic contaminants (IOCs) are present in treated wastewater used to irrigate edible crops in arid regions. Previous studies have shown that IOCs can accumulate in plants under field conditions. The phytoavailability of IOCs with pK_a values between 4 and 9 may be affected by the pH of the rhizosphere (the water and soil within 2-3 mm of the root surface). Plants can alter rhizosphere pH by 2 to 3 units in either direction in response to nutrient availability. The effects of nutrient availability and rhizosphere pH on plant accumulation of IOCs has not been previously considered. Here we show that plant-driven changes in rhizosphere pH alter accumulation of lamotrigine (pK_a of conjugate base = 5.7), but do not affect accumulation of carbamazepine, a non-ionizable contaminant. When plants received nutrient solution containing only nitrate, rather than both nitrate and ammonium, the rhizosphere pH was 1.5-2.5 units higher. We found strong correlations between the amount of neutral lamotrigine available in pore water and accumulation in roots and above-ground tissues. Future studies on plant uptake of IOCs should consider rhizosphere pH separately from bulk soil pH and report concentrations of nutrients available to the plants.

INTRODUCTION

Ionizable organic contaminants (IOCs), including many pharmaceuticals, are not completely removed by conventional municipal wastewater treatment processes.[1] Use of reclaimed wastewater and wastewater effluent-dominated water sources for irrigation of food crops has become a common practice in arid regions worldwide[2] and is expected to increase in importance as the global climate warms, population increases, and demand for freshwater rises. Thus, the potential risks associated with food crop accumulation of IOCs from reclaimed water used for irrigation warrants evaluation.

Crop plants can accumulate IOCs in edible tissues at environmentally relevant concentrations.[3] The ionizable phenyltriazine anti-epileptic drug lamotrigine (LTG, pK_a of conjugate acid = 5.7)[4] and the non-ionizable tricyclic anticonvulsant carbamazepine (CBZ) have both been detected in reclaimed wastewater and accumulate in effluent-irrigated plants.[5] Both LTG and 0,11-epoxycarbamazepine, the primary CBZ metabolite, can accumulate in carrots irrigated with reclaimed wastewater to levels that exceed the threshold of toxicological concern at normal consumption levels.[5] The threshold for toxicological concern is a conservative estimate used for compounds present at very low concentrations for which minimal toxicological data are available, and serves as an indicator of when additional study of toxic effects is warranted.[6,7]

Understanding the controls on IOC availability to plants is important for identifying conditions that may lead to accumulation of IOCs to unacceptable levels in food crops. Current models have had limited success predicting the accumulation of these compounds.[3] Sorption to soil and plant roots and interactions with lipid membranes in plants differ for the neutral and ionized forms of molecules and are important factors controlling IOC availability for root uptake and movement within the plant.[3,8–10] For IOCs with pK_a values between 4 and 9, the pH of the soil

pore water surrounding the plant roots (the rhizosphere) and at the root surface is a key variable to consider. Plants can alter rhizosphere pH by 2 to 3 units in either direction up to 2-3 mm from the root surface in response to nitrogen, phosphorus, and iron availability.[11–13] Thus, estimating IOC uptake based on bulk soil pH may mischaracterize the speciation of IOCs in the zone where they are immediately available for transport into to plant roots and lead to inaccurate prediction of phytoavailablilty. Plant-driven alteration of rhizosphere pH can affect accumulation of copper from contaminated soils by wheat, tomato, and rapeseed.[14–16] To our knowledge, the impact of rhizosphere pH on IOC accumulation has not been previously reported.

In this study, we examine the effects of plant-driven rhizosphere pH changes on wheat accumulation of LTG and CBZ. We cultivated durum wheat (*Triticum durum)* with different forms of nitrogen in a growth system designed to isolate the rhizosphere.[14–16] We measured pH and pharmaceutical concentrations in the rhizosphere and related them to the phytoaccumulation of CBZ and LTG. We tested both an ionizable and a non-ionizable contaminant to discriminate between effects on accumulation due to changes in rhizosphere pH and those due to other changes to the plants caused by the different nitrogen sources.

MATERIALS AND METHODS

Plant Growth. Durum wheat seeds were sterilized, rinsed, soaked, and then allowed to germinate in a damp paper towel. After 2-5 days, sprouted seeds were transferred to growth cells (**Figure 1**) based on the RHIZOtest.[15,17,18] Four seeds were placed inside each cell, and the roots grew into a planar mat against a layer of 30 μm nylon mesh. Plants were cultured hydroponically for 21 days.[15] The first 7 days after the seeds were hydrated, plants were provided only with ultrapure (≥ 18 MΩ·cm, Barnstead GenPure Pro) water. Commencing on day 8, the water was replaced with nutrient solution (pH 5.7) containing nitrate as the sole nitrogen source (details in the Supporting Information (SI) §S.1.2).

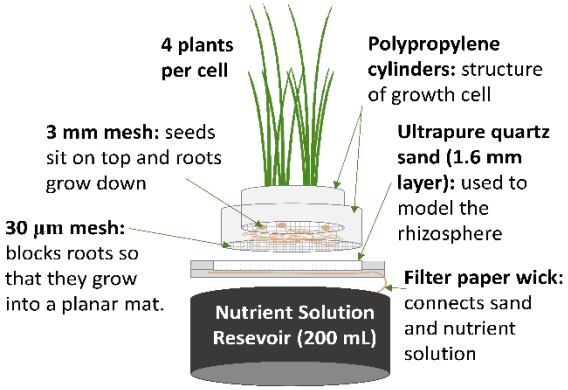

Figure 1. Exploded view of a full rhizosphere setup. Growth cells were composed of two polypropylene cylinders (i.d. 25 mm and 35 mm) with 3 mm polypropylene mesh stretched across the bottom of the smaller and 30 μm nylon mesh attached to the bottom of the larger. The smaller cylinder was glued inside the larger to leave a 3.5 mm gap between the coarse and fine meshes. Seeds were placed on top of the coarse mesh such that the roots grew down to form a planar mat on top of the fine mesh. On day 22 post-hydration, growth cells were placed into full rhizosphere setups with a 1.6 mm layer of sand to represent the rhizosphere. A strip of cellulose filter paper placed under the layer of sand connected the sand layer to the nutrient solution reservoir. Solution was replenished every 2 days. Nutrient solution was amended with lamotrigine or carbamazepine for a final concentration of 100 $\mu g \cdot L^{-1}$.

On day 22 post-hydration, each growth cell was transferred to a rhizosphere setup **(Figure 1)**, with the model rhizosphere consisting of 5.00 g ultrapure silica sand. A strip of cellulose filter paper connected the sand to a nutrient solution reservoir (replenished every 2 days). Sand and

paper were saturated with nutrient solution for the experimental duration. The nutrient solution contained 100 $\mu g \cdot L^{-1}$ LTG or CBZ. Half the rhizosphere setups received nutrient solution with nitrate as the sole nitrogen source; the other half received a solution containing a molar 1:2 ammonium-to-nitrate ratio (details in §S.1.2). Nutrient solutions had equivalent ionic strength and were adjusted to pH 5.7. Solution composition was chosen to produce differences in rhizosphere pH while minimizing effects on plant growth. Each treatment (pharmaceutical + nutrient solution combination) was replicated nine times; controls lacking pharmaceuticals or lacking plants were each conducted in triplicate. One replicate consisted of a growth cell containing four plants. After 8 days of exposure, above-ground tissues (mostly leaves), roots, and rhizosphere sand were collected, frozen at –80 °C, freeze dried, and stored at –80 °C until extraction. Plant and sand masses were measured before and after freeze drying.

Extraction and Analysis by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Freeze-dried sand from each replicate was extracted with 10 mM $CaCl_2$.[19] Freeze-dried plant samples were ground, spiked with mass-labeled internal standards, and subjected to accelerated solvent extraction with methanol (10,300 kPa, 80 °C). Extracts were evaporated to dryness and reconstituted in 80% ultrapure water, 20% acetonitrile, 0.1% acetic acid.

We measured LTG, CBZ, and the CBZ metabolites 10,11-epoxycarbamazepine and 10,11-*trans*-dihydroxycarbamazepine by LC-MS/MS (Agilent 1260 HPLC, Waters Xterra MS C18 column, Agilent 6460 triple quadrupole mass spectrometer, ESI+ source) in extracts of above-ground tissues and roots, sand extracts, and nutrient solution at the beginning and end of one two-day replenishment cycle. Further details on extraction and analysis methods are in SI §S.1.3.

RESULTS

Plant Manipulation of Rhizosphere pH. Wheat plants altered pore water pH in the model rhizospheres in response to the nitrogen source provided. The pH of the solutions in the nutrient solution reservoirs was initially 5.7 ± 0.05 and changed by no more than 0.1 pH unit at the end of each two-day replenishment cycle. In treatments containing wheat plants, provision of nitrate as the sole nitrogen source resulted in elevation of pore water pH by 1.5-2.5 pH units relative to those supplied with ammonium + nitrate (**Figure 2**). Exposure to CBZ and LTG did not affect pore water pH (Figure S2). The fresh masses of roots and leaves and the mass of water transpired did not differ among treatments (Table S4).

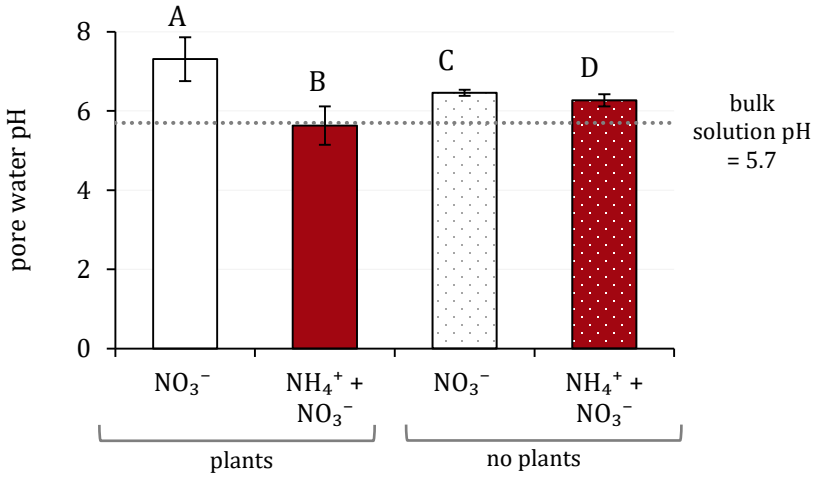

Figure 2. Wheat plants altered pore water pH in model rhizospheres in response to the form of nitrogen provided. Plants raised pore water pH when supplied with nitrate as the sole nitrogen source. Pore water pH in control rhizosphere setups lacking plants differed slightly between nitrogen source treatments. The dotted line indicates the pH of the initial nutrient solution. The pH in the nutrient solution reservoirs at the end of each solution replenishment cycle varied from the initial value by ≤ 0.1 pH unit. Letters indicate statistical significance based on an ANOVA with Welch's correction and Games-Howell *post hoc* analysis ($p < 0.05$). Bars represent mean values; error bars indicate one standard deviation ($n = 21$ for treatments with plants; $n = 9$ for treatments lacking plants).

Effect of Rhizosphere pH on Plant Accumulation of Pharmaceuticals. We tested the effect of rhizosphere pH on the accumulation of CBZ (non-ionizable) and LTG (pK_a = 5.7)[4] in wheat roots and leaves. CBZ accumulation was not affected by differences in nitrogen form provided or rhizosphere pH (**Figure 3**). Furthermore, the nitrogen source and rhizosphere pH did not affect accumulation of CBZ metabolites in plant roots or leaves. Metabolites accounted for less than 2% of total CBZ measured (§S.3.3).

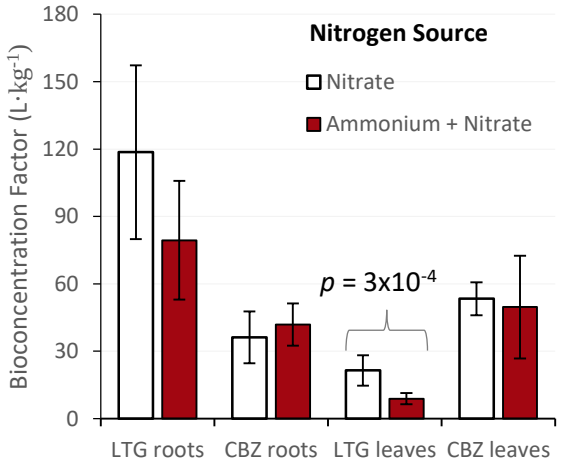

Figure 3. Bioconcentration of lamotrigine (LTG) and carbamazepine (CBZ) in the roots and leaves of wheat plants supplied with the indicated nitrogen sources. The amount of LTG that accumulated in the leaves of plants provided with only nitrate exceeded that in plants receiving both ammonium and nitrate by a factor exceeding two. Bioconcentration factors were calculated by dividing the concentration in the plant roots or leaves by that in the pore water at the end of the exposure period. Welch's *t*-test was used for pairwise comparison between treatments. Error bars indicate one standard deviation. Total CBZ and LTG in plant tissue accounted for less than 4% of the compound added to the system. Concentrations of LTG, CBZ in pore water did not vary between nitrate only plants and ammonium + nitrate plants (Figure S3).

In contrast, significantly more LTG accumulated in the above-ground tissues of plants supplied with nitrate as the sole nitrogen source (pH 7.4 ± 0.6) than in those provided with ammonium and nitrate (pH 5.7 ± 0.4) (**Figure 3**). Nitrogen source did not affect accumulation of LTG in plant roots. Concentrations of LTG in plant tissues correlated with the concentration of uncharged LTG in pore water (**Figure 4**). The concentration of uncharged LTG in pore water was

calculated via the Henderson-Hasselbalch equation using the model rhizosphere pH, the LTG pore-water concentration, and the pK_a of the conjugate base of LTG (5.7).[4] Correlations between LTG in plant tissues and total LTG in pore water were not statistically significant ($p > 0.05$; Table S5). The concentration of CBZ in plant tissues did not correlate with CBZ in pore water ($p > 0.05$, Table S5).

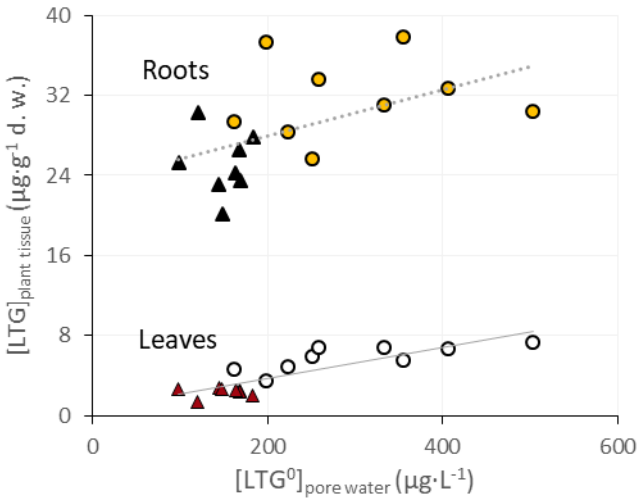

Figure 4. Lamotrigine (LTG) accumulation in wheat leaves and roots correlated with the concentration of the neutral LTG species in pore water. White and yellow circles correspond to plants provided with nitrate as the sole nitrogen source. Red and black triangles correspond to plants provided with ammonium + nitrate. The bottom line shows a linear regression of the concentration of LTG in leaves against the concentration of neutral LTG in pore water ($R^2 = 0.73$). The slope is 0.015 ± 0.002 ($p = 1.4 \times 10^{-5}$), the *y*-intercept does not differ from zero ($p = 0.31$). The top line shows a linear regression of LTG concentration in plant roots against the concentration of neutral LTG in pore water ($R^2 = 0.27$). The slope is 0.023 ± 0.009 ($p = 0.032$), the *y*-intercept is 23 ± 2 ($p = 9.7 \times 10^{-8}$).

DISCUSSION

Plants alter rhizosphere pH to maintain electrochemical equilibrium as they take in nutrients. Energy for uptake of cations is provided via counter-transport of H^+ out of root cells, and uptake of anions is accompanied by co-transport of H^+ into root cells.[13] Therefore, uptake of cationic ammonium decreases rhizosphere pH, while uptake of anionic nitrate increases rhizosphere pH (**Figure 5**).[12] Most plants modulate rhizosphere pH in response to the form of nitrogen available, as nitrogen accounts for up to 80% of ions taken in by plants.[13] The pH values in our rhizosphere models (**Figure 2**) are consistent with these processes.

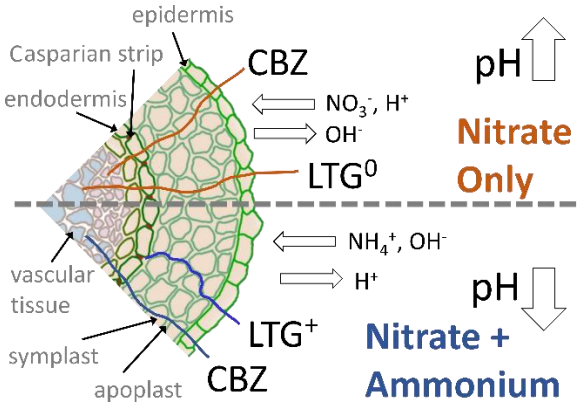

Figure 5. Part of a root cross section, with carbamazepine (CBZ) and the neutral lamotrigine species (LTG^0) moving symplastically through the root to reach the vascular tissue and the lamotrigine cation (LTG^+) moving apoplastically and being blocked by the Casparian strip. Movement of ions to maintain cation-anion balance in the root with different nitrogen sources and the resulting pH change is also shown.

To be transported through a plant, a molecule must reach the root vascular tissue (**Figure 5)**. Molecules can enter roots via symplastic or apoplastic pathways. In the symplastic route, the molecules cross a cell membrane to enter a root cell, then travel to the vascular tissue through interconnecting channels between cells.[20] In the apoplastic route, molecules move through intercellular space (the apoplast) and are blocked from entering the vascular tissue by the Casparian

strip, a waxy barrier.[20] Therefore, the likelihood of a molecule reaching the vascular tissue and being transported to above-ground tissues depends on its ability to cross cell membranes.[3,10] Generally, neutral organic molecules have much higher membrane permeability than charged ones.[10] Therefore, we expect the neutral form of an IOC to readily enter the vascular tissue and be transported through the plant, while the ionized form remains primarily in the apoplast.

Our results are consistent with this expectation. Plant accumulation of non-ionizable CBZ was not affected by rhizosphere pH, and a large fraction of the compound was transported to above-ground tissues (**Figure 3**). We hypothesize that CBZ travels symplastically through plant roots (**Figure 5**). Plant accumulation of LTG was affected by rhizosphere pH, with more LTG accumulating when a larger fraction of LTG in the rhizosphere was uncharged (**Figures 3** and **4**). The large correlation coefficient for the relationship between LTG accumulation in leaves and concentration of the neutral LTG species in the rhizosphere (**Figure 4**) indicates that LTG speciation is the main variable controlling its access to the vascular tissue. The non-statistically significant *y*-intercept in the correlation is consistent with ionized LTG not being transported to leaves. The higher *y*-intercept for the correlation between LTG accumulation in roots and neutral LTG in the rhizosphere suggests that ionized LTG was can adsorb to or enter root tissue, but to a smaller extent than neutral LTG. We hypothesize that ionized LTG did not enter root cells, and remained in the apoplastic space and sorbed to the root surface, preventing transport to leaves, whereas neutral LTG was transported symplastically to some extent (**Figure 5**). Lamotrigine accumulation in leaves was lower than that of CBZ even when >99% LTG was in the neutral form; we therefore hypothesize that CBZ enters root cells more easily than neutral LTG.

Broader Implications. We have provided direct evidence that plant-driven changes in rhizosphere pH impact accumulation of an IOC in plant leaves, a process that we expect also occurs

in the field. However, the impact of the conditions in our simple model system on our results must be considered. We employed ultrapure quartz sand with low buffering capacity and lower sorption capacity for CBZ and LTG than has been reported for field soils.[21] Sorption to the quartz sand had little impact on our findings (details in §S4). This may not be the case for field soils with higher K_d values. The magnitude and spatial extent of rhizosphere pH change depends on the buffering capacity of the soil. Nonetheless, even in a soil with high buffering capacity due to inclusion of 30% $CaCO_3$, chickpeas (*Cicer arietinum*) produced a pH change of ~2 units within 1 mm of the root surface.[13] Overall, we expect plant-driven changes in rhizosphere pH to be important under field conditions for the accumulation of IOCs with pK_a values between 4 and 9, though pH-dependent sorption to soil constituents and high soil buffering capacity may alter trends in phytoavailability.

Our experiment used durum wheat, a species known to significantly alter rhizosphere pH in response to nitrogen source.[15] Nitrogen source-driven changes in rhizosphere pH have been documented in many species including maize, sorghum, chickpea, Norway spruce, white lupine, white clover, tomato, and rapeseed.[11,14] Species demonstrated to alter rhizosphere pH include graminaceous and non-graminaceous monocots, dicots, and species with and without N_2-fixing symbionts.[11] We expect the effect demonstrated in this study to be important across a wide range of plant species, although the magnitude of plant-driven changes in rhizosphere pH is species-specific and can vary among cultivars.[11]

Based on our results, we suggest future studies on plant accumulation of IOCs report the nutrients available to the plants, especially the main form of nitrogen supplied, and measure the rhizosphere pH if possible. Differences in speciation at rhizosphere vs. bulk soil pH may contribute to the limited success in predicting plant uptake of IOCs.[3] Species-specific information on how

available nutrients modulate rhizosphere pH may be necessary to accurately predict IOC bioaccumulation.

ASSOCIATED CONTENT

Supporting information (SI) is available. SI contains methodological details and additional results noted in the main text.

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

FOR CHAPTER TWO

PLANT-INDUCED CHANGES TO RHIZOSPHERE PH INFLUENCE UPTAKE OF IONIZABLE ORGANIC CONTAMINANTS

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S.1. SUPPLEMENTAL METHODS

S.1.1. Chemical Sources. Carbamazepine (99% purity) was obtained from ACRŌS Organics. Lamotrigine (98% purity) was obtained from Comb and Blocks. All mass-labeled internal standards were obtained from CDN isotopes. IOTA quartz sand (Unimin Corporation, New Canaan, CT) was used as the model rhizosphere. Nutrient solution salts were obtained from a variety of sources. We obtained KNO_3 from Fisher, $CaNO_3$ from ACRŌS Organics, KH_2PO_4 from Alfa Aesar, $MgSO_4$, $(NH_4)_2SO_4$, and KOH from dot Scientific, K_2SO_4 from Strem Chemicals, and Murashige and Skoog micronutrient solution from Caisson Laboratories.

S.1.2. Plant growth methods

S.1.2.1. Growth cell construction. Coarse polypropylene mesh (pore size ~3 mm) was stretched across the bottom of a polypropylene cylinder (i.d. 25 mm) and glued at the top. A layer of fine nylon mesh (pore size 30 μm) was attached to the bottom of another polypropylene cylinder (i.d. 35 mm). The smaller cylinder was inserted into the larger and glued at the top to leave a 3.5 mm gap between the coarse and fine meshes. Hot melt glue was used as the adhesive. Coarse mesh and cylinders were made of polypropylene to minimize sorption of CBZ and LTG. A cartoon of the growth cell is shown in Figure 1 in the main text. Growth cell design and construction is modeled after the setup used for the RHIZOtest.[1–3]

S.1.2.2. Seed sterilization, soaking, and germination. No. 1 premium hard amber desert durum wheat seeds (Ramona Farms Heritage Collection) were sterilized in 70% ethanol (60 s), rinsed three times with ultrapure water, soaked in 4% sodium hypochlorite (20 min), and rinsed five times with ultrapure water. Seeds were then soaked in ultrapure water for 5-6 h in the dark before transfer to a folded, damp paper towel. The paper towel was kept in a sealed plastic bag while the seeds germinated (2-4 d).

S.1.2.3. Plant growth and rhizosphere setup. Seeds were placed in the growth cells on top of the coarse mesh such that the roots would grow down through the course mesh and form a planar mat between the layers of mesh. Each cell contained five to six seeds but was thinned to four plants prior to the exposure period. One growth cell represented one replicate. For the first 21 d after seed sterilization, cells were submerged in water (first 7 d) or solution containing nitrate as the sole nitrogen source (days 8-21) up to the level of the coarse mesh (replenished twice daily). Nutrient solution contained 6 mM NO_3^-, 2.5 mM K^+, 2 mM Ca^{2+}, 3 mM Mg^{2+}, 3 mM SO_4^{2-}, 0.5 mM $H_2PO_4^-$ and 10 mL·L^{-1} Murashige and Skoog micronutrients,[4] and was adjusted to pH 5.7 ± 0.05 using KOH.

On day 28, cells were transferred from the hydroponic setup to rhizosphere setups where each cell was placed on a layer of rhizosphere sand and connected to a container of nutrient solution via a strip of cellulose filter paper (grade 222, Ahlstrom). The rhizosphere sand consisted of 5.00 ± 0.05 g of ultrapure silica sand (between sieve sizes 60-120), which formed a ~1.6 mm thick layer under the fine mesh and root mat. Water, nutrients, pharmaceuticals, and root exudates could pass freely through the fine mesh, although roots and sand could not. CBZ and LTG were added to the nutrient solution reservoir and wicked up the filter paper with the nutrient solution to reach the plants.

Solution reservoirs and the structures supporting the sand and filter paper were constructed of polypropylene to minimize losses of CBZ, LTG, and nutrients due to sorption. Lids were place loosely on nutrient solution containers to allow the filter paper to exit, but to minimize evaporation. Each cell was connected to a separate nutrient solution reservoir, and the outsides of the containers and lids were painted black to minimize nutrient solution exposure to light. Each setup was weighed at the beginning and end of each 2-day replenishment cycle to measure

evapotranspiration. Transpiration was differentiated from evaporation by subtracting the average mass lost from setups lacking plants from the mass lost by the setups containing plants.

Half the setups received nutrient solution with nitrate as the sole nitrogen source (same as the solution used for days 8-21). The other half were provided a solution containing both ammonium and nitrate of the following composition: 2 mM NH_4^+, 4 mM NO_3^-, 1.5 mM K^+, 2 mM Ca^{2+}, 2.25 mM Mg^{2+}, 3.25 mM SO_4^{2-}, 0.5 mM $H_2PO_4^-$, and 10 mL·L^{-1} Murashige and Skoog micronutrients. Nutrient solutions had equivalent ionic strength and were adjusted to pH 5.7 ± 0.05 using KOH.

S.1.3. Extraction and Analysis Methods

S.1.3.1. Rhizosphere Extraction. Freeze dried sand (4.000 ± 0.005 g) from each replicate was equilibrated with 8.00 mL of 10 mM $CaCl_2$ for 3 h and sedimented by centrifugation (20 min, 4637*g*). An aliquot (1 mL) of the supernatant was withdrawn and filtered through a 0.2 μm PTFE filter in preparation for analysis by LC-MS/MS analysis. We measured the pH of the remaining solution. Pore water concentrations of LTG, CBZ, and CBZ metabolites was calculated using the equation:

$$C_{i\text{pore water}} = \frac{C_{i\text{extract}} V_{\text{extract}} M_{\text{sand,total}}}{M_{\text{sand,extract}} M_{\text{porewater,total}}} \quad \text{(S1)}$$

where C_i is concentration of compound *i* (mass per unit volume for the extract and mass per unit mass for the pore water), *V* is volume, and *M* is mass.

S.1.3.2. Plant Extraction. Freeze dried plant tissues were ground with a mortar and pestle, then extracted using Accelerated Solvent Extraction (ASE) with 100% methanol. Sample masses are shown in Table S1. Each ASE cell contained a glass fiber filter and 1.0 g fluorosil at the bottom, over which was placed the tissue sample followed by another 1.0 g fluorosil. We used an ASE 200

(Dionex) with 11 mL stainless steel cells. Samples were spiked with mass labeled internal standards (Table S1) and sat at room temperature overnight prior to extraction. Internal standards used were carbamazepine-d_{10}, 10,11 epoxycarbamazepine-d_{10}, and lamotrigine-$^{13}C_3$ purchased from CDN Isotopes. The ASE cycle (completed twice per sample) included a 5 min preheat, 5 min heat, 5 min static, 60% volume flush, and 120 s purge with a pressure of 10,300 kPa and temperature of 80 °C. Extracts were evaporated to dryness then reconstituted in 80% ultrapure water, 20% acetonitrile, 0.1% acetic acid. Leaves from plants exposed to LTG were reconstituted with 1 mL solution; all other samples were reconstituted in 5 mL solution. All samples were sonicated 10 min in reconstitution solvent, centrifuged (20 min, 17000*g*), and filtered through 0.2 μm PTFE filters.

S.1.3.3. LC-MS/MS Analysis. We used a Waters Xterra MS C18 3.5 μm 2.1 × 100 mm column. Mobile phases for nutrient solution and plant extract analysis were 100% acetonitrile (organic phase) and 0.1% formic acid in 10% acetonitrile (aqueous phase). A buffered aqueous mobile phase (10 mM ammonium acetate, 1% acetic acid, 2.5% acetonitrile, 2.5% methanol) was used for the sand extracts to prevent variation in pH during analysis. A gradient of 5% to 95% organic phase was used for nutrient solution and plant extract analysis, and a gradient of 10% to 95% organic phase was used for sand extract analysis. Column temperature was held at 30 °C. Internal standard-based calibration and measurement was used for plant extracts only. Sand extracts and nutrient solution did not contain internal standards. Ion masses used for detection via LC-MS/MS are listed in **Table S1.**

Table S1. Mass-to-charge ratios (*m*/*z*) for precursor, quantitative and confirmatory ions

	Ion *m*/*z*		
Compound	**Precursor**	**Quantitative**	**Confirmatory**
lamotrigine	256	43.1	108.9, 58.1
lamotrigine-$^{13}C_3$	259	44.1	59.1
carbamazepine	237.1	194.1	165
carbamazepine-d_{10}	247.2	204.1	173.1
10,11-epoxycarbamazepine	253.1	180.1	210.1
10,11-epoxycarbamazepine-d_{10}	263.3	188.1	220.1
trans 10,11-dihydroxycarbamazepine	271	180.1	210.1

S.1.3.4. Extraction Recoveries and Limits of Detection/Quantification. Extraction recoveries are shown in **Table S2**. Compound recoveries are based on a calibration using internal standard (IS) ratios. Internal standard recoveries are absolute recoveries based on an external calibration. Recovery samples (n = 4) consisted of blank plant tissue with analytes and IS spiked prior to ASE. Spike levels are listed in **Table S2**. For both CBZ metabolites we used 10,11 epoxycarbamazepine-d_{10} as the IS.

Table S2. Recovery of analytes from plant tissues.*

		lamotrigine	carbamazepine (CBZ)	10,11-epoxy-CBZ	10,11 trans-dihydroxy-CBZ
recovery (%)	compound	108 ± 8	91 ± 9	109 ± 8	–
	IS	49 ± 2	79 ± 2	120 ± 5	NA
spike (ng sample^{-1})	compound	400	1500	20	1
	IS	20	100	20	NA
sample mass (g_{dw})		0.10 ± 0.001	0.10 ± 0.001	0.10 ± 0.001	0.10 ± 0.001
recovery (%)	compound	100 ± 5	102 ± 6	100 ± 10	90 ± 20
	IS	91 ± 2	101 ± 5	140 ± 10	NA
spike (ng sample^{-1})	compound	625	375	1	0.25
	IS	100	100	20	NA
sample mass (g_{dw})		0.04 ± 0.02	0.025 ± 0.003	0.025 ± 0.003	0.025 ± 0.003

** Abbreviations: IS, internal standard; NA, not applicable.*

Limits of detection (LOD) and quantification (LOQ) (**Table S3**) were determined by running a low concentration sample seven consecutive times and taking the standard deviation of the measurements (σ). The LOD and LOQ was calculated using the equations:[5]

$$LOD = 3\sigma \quad \text{(S2)}$$

$$LOQ = 10\sigma \quad \text{(S3)}$$

Table S3. Limits of detection and quantification

compound	plant tissue (ng/g dw)				pore water (ng/g)		nutrient solution (ng/mL)	
	leaves		roots					
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
lamotrigine	8	27	33	111	8.3	27.5	0.22	0.88
carbamazepine (CBZ)	8	25	6	20	2.4	7.9	0.12	0.48
10,11-epoxy- CBZ	2	8	2	5	0.2	0.7	0.002	0.006
trans 10,11-dihydroxy-CBZ	3	11	4	13	0.6	1.9	0.02	0.08

S.1.3.5. Statistical Analyses. We used Microsoft Excel Professional Plus 2013 to conduct Welch's two sample *t*-tests and linear regressions. We used JMP Pro 12.2.0 (SAS) for ANOVAs with Tukey's post hoc analysis for comparison of data with equal variance. We used R package userfriendlyscience (version 0.6-1) to conduct ANOVAs with Welch's correction and Games-Howell *post hoc* analysis for data with unequal variance. All comparisons were made at the $\alpha = 0.05$ level of significance.

S.2. SAND ADSORPTION EXPERIMENTS.

We conducted a series of experiments to assess LTG and CBZ adsorption to the quartz sand used as the growth substrate. We equilibrated 8.00 g sand for 24 h with 4.00 mL of solution containing 100 $\mu g \cdot L^{-1}$ CBZ or LTG. Though LTG and CBZ concentrations increased during the plant exposure experiments, we used the starting concentration in the nutrient solution reservoir to represent a maximum sorption scenario. After equilibration, samples were centrifuged for 20 minutes at 4637 *g* and filtered through 0.2 μm PTFE filters. Then pH was measured in each sample and LTG and CBZ concentrations were measured using HPLC-MS/MS as described above.

Distribution coefficients (K_d) were determined by dividing LTG and CBZ concentrations in sand by the concentrations in solution equilibrated with sand. We calculated sand concentrations based on the difference between solution samples equilibrated with and without sand. The missing fraction in the sand containing samples was assumed to be adsorbed to the sand.

We tested CBZ and LTG sorption to sand at initial pH values of 4.5 and 8 in a KCl solution of equivalent ionic strength to the nutrient solutions. We used a KCl solution as maximum sorption scenario, as the Ca^{2+} and Mg^{2+} ions in the nutrient solution would be expected to cause more competition for binding sites on the sand than K^+ alone. We did not detect depletion of CBZ from solution due to adsorption. In contrast, the adsorption of LTG to quartz sand exhibited pronounced dependence on pH. To further investigate LTG sorption under conditions relevant for the plant exposure experiments, we used background solutions with the cation composition and ionic strength of each of the nutrient solutions over the pH range of LTG-containing samples for each nutrient solution (**Figure S1**).

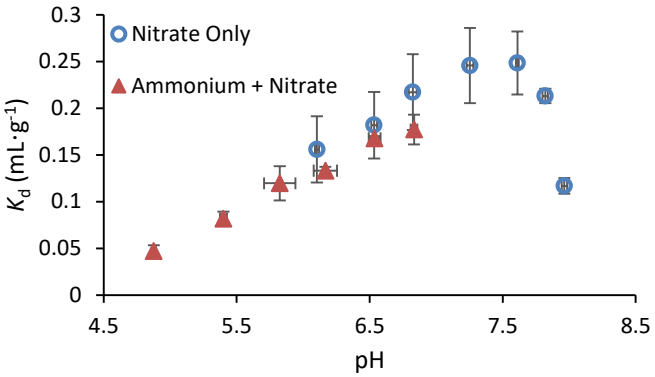

Figure S1. pH-Adsorption envelope for lamotrigine adsorption to quartz sand. Distribution coefficients (K_d) are plotted as a function of equilibrium pH. Lamotrigine adsorption to quartz was strongly dependent on pH but varied minimally between the solutions for the points where both were tested. We linearly interpolated between plotted K_d values to calculate the lamotrigine available in rhizosphere pore water. Adsorption corrected values for pore water concentration and bioconcentration factors are used throughout the paper. Error bars represent one standard deviation (n = 3) and do not always extend beyond the data points.

We found that LTG sorption to quartz sand depended strongly on pH. Measured K_d values ranged from 0.047 to 0.24 L·kg^{-1}. Sorption increased with pH up to pH 7, and then steeply decreased as pH increased beyond 7.5. This trend was confirmed in three separate sorption experiments across the pH range. Quartz possesses a point of zero charge at ~2 and would therefore bear a net negative charge over the pH range surveyed. Lamotrigine exists as a cation at pH values below its pK_a of 5.7. The reduced adsorption at lower pH values is hypothesized to be due to competition for sorption sites between LTG$^+$ and the other cations in solution. The decline in adsorption as pH exceeds 7.5 is attributed to the increasing density of Si–O$^-$ groups on the quartz surface. The abundance of anionic Si–O$^-$ groups exceeds that of neutral Si–OH groups at pH ~7.[6] Neutral LTG molecules may bond to neutral Si-OH groups via van der waals forces, while cations in the background solution can likely outcompete neutral LTG to bond with anionic Si–O$^-$. Further investigation of LTG sorption to quartz warrants investigation, but is beyond the scope of the present study.

We note that the extent of adsorption observed in these adsorption experiments may differ somewhat from that occurring in the rhizosphere set up as root exudates can have biotic and abiotically driven effects on sorption of organic contaminants in the rhizosphere.[7,8] Surface active compounds in root exudates can promote desorption of neutral organic contaminants from rhizosphere soils, though root exudate effects on desorption of IOCs from soils has not been investigated to our knowledge.[7,9–12] Root exudate driven changes in sorption may alter compound availability to plants in ways that are not captured by simple sorption experiments. This topic warrants future study, although it lies beyond the scope of the present study.

S.2.1. Correction of exposure concentrations for adsorption. We linearly interpolated between the K_d values plotted in **Figure S1** to account for the fraction of LTG adsorbed to the sand in both our extraction process and during the plant exposure experiment. All of the LTG pore water concentrations and bioconcentration factors presented in the main text and supplemental information account for LTG adsorption to the sand in the model rhizosphere. We found that correcting the data for adsorption to the sand did not affect the trends present in the LTG data.

S.3. SUPPLEMENTAL RESULTS

S.3.1. Pore Water and Nutrient Solution Analysis. Bulk nutrient solution from days 4-6 of the exposure period was analyzed at the beginning and end of the replenishment cycle. Nutrient solution concentrations of CBZ and LTG varied <15% between the beginning and end of replenishment cycles. 10,11-Epoxycarbamazepine was present in the nutrient solutions at the beginning and end of the exposure period at < 0.02% of the total CBZ concentration. 10,11-*Trans*-dihydroxycarbamazepine was below detection in all nutrient solution samples.

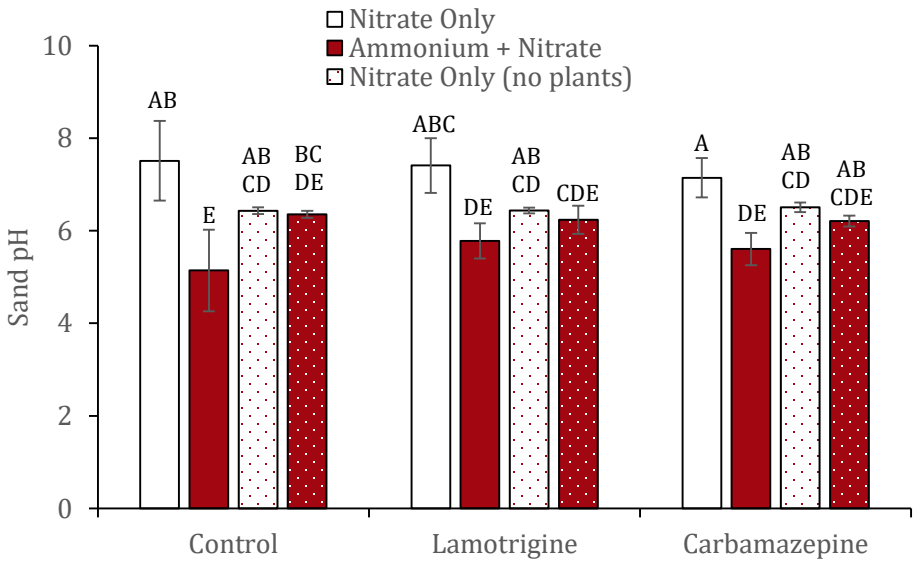

Figure S2. Pore water pH in sand with and without plants. The addition of CBZ or LTG did not affect pH for any treatment. Treatments containing plants with nitrate as the sole nitrogen source had higher pH than those containing plants with ammonium and nitrate, and some treatments that lacked plants. In some cases, the ammonium + nitrate treatments with plant had lower pH than the corresponding treatments lacking plants. Fewer differences were found among the control samples due to the lower number of replicates. Letters indicate statistically significant differences (ANOVA with Welch's correction and Games-Howell *post hoc* analysis, p <0.05). Error bars denote one standard deviation.

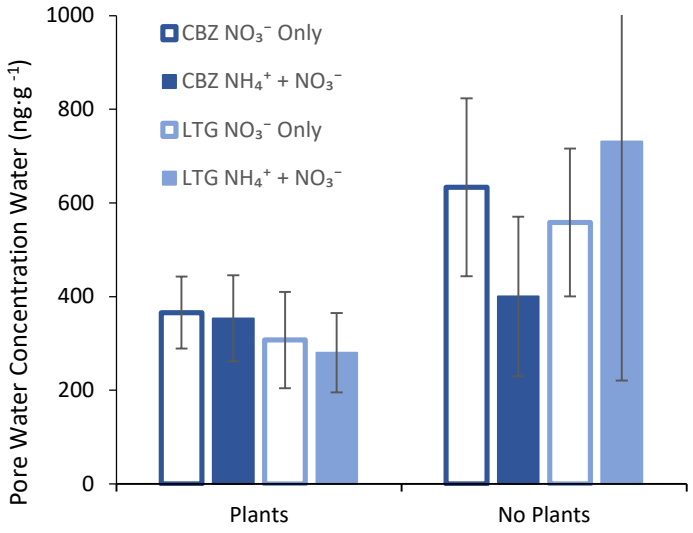

Figure S3. Total CBZ and LTG in pore water. Error bars indicate one standard deviation. Pore water concentrations did not differ within the CBZ or LTG treatments (ANOVA with Welch's correction, p >0.05). CBZ and LTG accumulate in pore water as water evaporates and/or transpires more quickly than the compound is taken up. Therefore, pore water concentrations are higher than the starting bulk solution concentration (100 $\mu g \cdot L^{-1}$). Variation within treatments is due to the differences in transpiration and evaporation among samples; bulk solution concentrations exhibited little variation.

We found 10,11-epoxycarbamazepine at up to 0.9 $ng \cdot g^{-1}$ in pore water, but it was below the limit of detection in five samples and below the limit of quantification in an additional 10 samples (n = 18). 10,11-*Trans*-dihydroxycarbamazepine was below the limit of detection in all pore water samples.

S3.2. Plant Mass and Transpiration. Root fresh masses, leaf fresh masses, leaf dry masses, and the mass of transpired water (Table S5) did not differ among treatments (p > 0.05, ANOVA). Dry mass of plant roots was higher for nitrate only controls relative to nitrate only CBZ exposed plants and ammonium + nitrate CBZ and LTG exposed plants (p < 0.05, ANOVA with Tukey's post hoc analysis). We hypothesize that this difference is due to variation in plant mass prior to the exposure period. Transpiration values are the total amount transpired over the 8 day exposure period.

Table S4. Masses of plant tissues and transpired water

exposure	nutrient solution	leaf mass f.w.	leaf mass d.w.	root mass f.w.	root mass d.w.	transpired water mass (g)
Control	NO_3-	1.0 ± 0.3	0.08 ± 0.02	1.3 ± 0.2	0.19 ± 0.02	101 ± 9
Control	NH_4^+ + NO_3-	1.0 ± 0.1	0.06 ± 0.02	1.5 ± 0.3	0.20 ± 0.04	90 ± 10
CBZ	NO_3-	1.2 ± 0.4	0.05 ± 0.01	1.1 ± 0.2	0.16 ± 0.03	80 ± 20
CBZ	NH_4^+ + NO_3-	1.1 ± 0.2	0.04 ± 0.01	1.2 ± 0.2	0.17 ± 0.03	80 ± 20
LTG	NO_3-	1.3 ± 0.5	0.05 ± 0.02	1.2 ± 0.3	0.18 ± 0.04	80 ± 20
LTG	NH_4^+ + NO_3-	1.0 ± 0.4	0.04 ± 0.01	1.2 ± 0.3	0.16 ± 0.03	80 ± 20

S.3.3. Plant Accumulation of Carbamazepine Metabolites. 10,11-Epoxycarbamazepine was present above the limit of quantification in all samples from plants exposed to CBZ. The 10,11-epoxycarbamazepine concentrations in leaves and roots were respectively 220 ± 90 ng·g^{-1} dry weight and 40 ± 20 ng·g^{-1} dry weight. Concentrations were not affected by nitrogen source or rhizosphere pH. 10,11-Epoxycarbamazepine remained below 2% of the total CBZ measured in leaves and below 0.6% of the total CBZ measured in roots.

10,11-*Trans*-dihydroxycarbamazepine was present above the limit of detection in all but one sample from plants exposed to CBZ. The 10,11-*trans*-dihydroxycarbamazepine concentration exceeded the limit of quantitation in four root and ten leaf samples (n = 18). We found concentrations of 10,11-*trans*-dihydroxycarbamazepine up to 23 ng·g^{-1} dry weight in leaves and 26 ng·g^{-1} dry weight in roots.

S.3.4. Correlation Between Pore Water Concentrations and Plant Accumulation. LTG accumulation in leaves and roots correlated with the concentration of neutral LTG in rhizosphere pore water. Correlations between total CBZ and LTG concentration in pore water and concentration in the plants are not statistically significant ($p > 0.05$)

Table S5. Correlation coefficients for plant-pore water regressions

slope	*Y-intercept*	R^2	*p*-value
$[CBZ]_{leavess}$	$[CBZ]_{pore\ water}$	0.02	0.56
$[CBZ]_{roots}$	$[CBZ]_{pore\ water}$	0.11	0.18
$[LTG]_{leaves}$	$[LTG_{Total}]_{pore\ water}$	0.19	0.07
$[LTG]_{roots}$	$[LTG_{Total}]_{pore\ water}$	0.01	0.65
$[LTG]_{leaves}$	$[LTG^0]_{pore\ water}$	0.73	1.4×10^{-5}
$[LTG]_{roots}$	$[LTG^0]_{pore\ water}$	0.27	0.03

S.3.5. Temperature and Humidity Measurements. Temperature and humidity readings are shown in Figure S3. Temperature cycled between 19 and 26 °C. Temperature was higher during the day due to the light cycle, although fans constantly cycled air through the growth chamber. Dips in temperature are present when the chamber was opened for plants to be removed for solution renewal every 2 days. Relative humidity varied between 9 % and 38 %. These low relative humidity values are characteristic for Madison, WI in March, where the experiment took place.

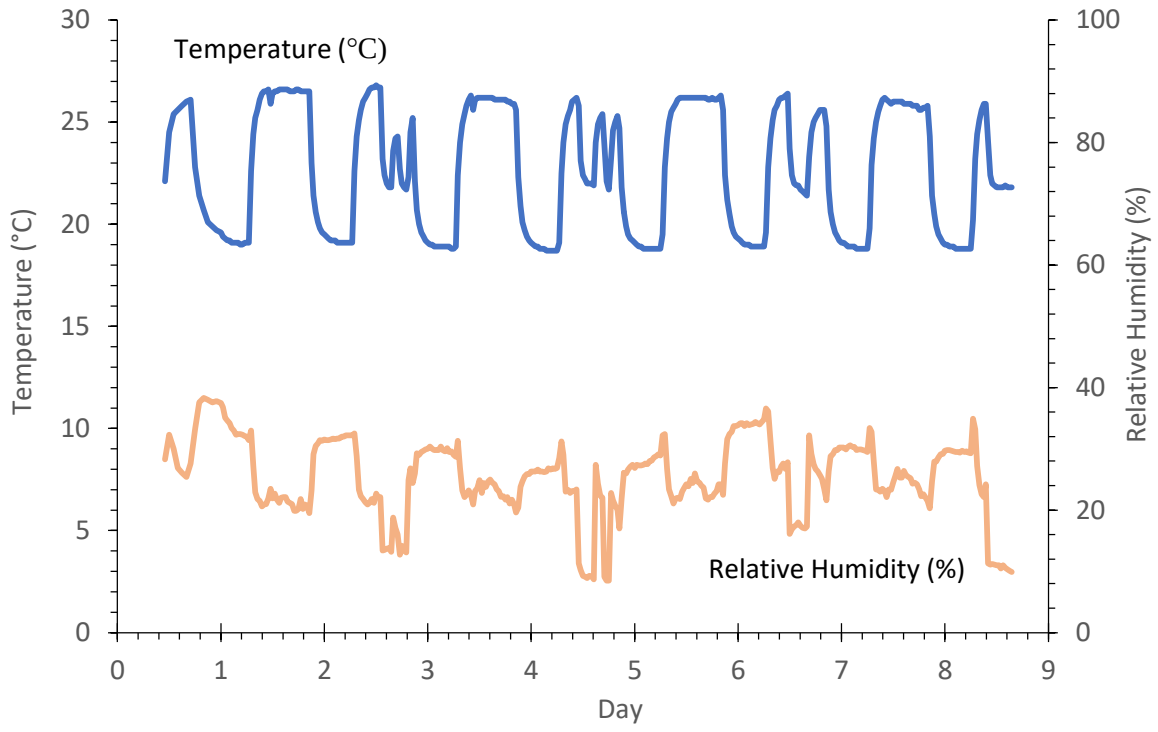

Figure S4. Temperature and humidity variation over time. Temperature and humidity measurements were taken every 30 min using an automated system for the duration of the exposure period.

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CHAPTER THREE

EFFECTS OF TRANSPIRATION AND BINARY MIXTURES ON ACCUMULATION OF PHARMACEUTICALS BY SPINACH

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ABSTRACT

Many pharmaceuticals are present in reclaimed wastewater and effluent-dominated water bodies used to irrigate edible crops. Previous research has shown that pharmaceuticals can accumulate to detectable levels in plants irrigated with reclaimed wastewater, but plant driven-processes that control differences in accumulation between compounds are not yet well understood. Here, we test the effects of binary compound mixtures and transpiration on spinach accumulation and metabolism of four pharmaceuticals commonly found in treated wastewater effluent. We found that fluoxetine and amitriptyline decreased the accumulation of the primary and potentially toxic metabolite of carbamazepine, 10,11-epoxycarbamazepine. Compound accumulation in spinach plants strongly correlated with predicted accumulation using a simple model based on transpiration and exposure concentrations, although the ratio between predicted and actual accumulation varied among tested compounds. Amitriptyline and fluoxetine have similar physico-chemical properties, but they exhibited different trends in uptake. We hypothesize that passive ion transporters in root cell membranes impact compound transport into the plant and cause some of the observed differences between compounds. Our findings highlight the need to consider plant physiology and mixture effects in studying the accumulation of polar and ionizable organic contaminants and their metabolites.

KEY WORDS

Plants, pharmaceuticals, transpiration, mixtures, carbamazepine, lamotrigine, reclaimed wastewater, effluent irrigation

INTRODUCTION

Water scarcity is a growing concern as world population expands and climate change makes freshwater availability more unpredictable.[1] Reuse of treated wastewater (i.e., reclaimed wastewater) is an important strategy to reduce demand on freshwater resources. In arid agricultural areas such as in Israel and California, USA, irrigation of crops with reclaimed wastewater is already widely practiced.[2] While wastewater reuse is necessary for addressing water scarcity, not all wastewater-derived organic contaminants are effectively removed during conventional treatment processes. Reclaimed wastewater therefore contains a large variety of organic contaminants, including human pharmaceuticals, and the presence of these compounds has become ubiquitous in surface waters worldwide.[3,4]

A large body of literature shows that crop plants can take up and accumulate pharmaceuticals under field conditions.[5,6] Nonetheless, predicting the accumulation of these polar and ionizable compounds by plants has proven difficult.[5] Predicting accumulation is important because new drugs are constantly being introduced to the market and monitoring agricultural produce for all potential contaminants is not practical. Most attempts to predict pharmaceutical accumulation in whole plants or in specific tissues have been based on correlations with contaminant physico-chemical properties.[5] Such approaches are based the assumption that accumulation in the plant is driven by passive processes such as diffusion and partitioning. Modeling plant uptake of persistent, hydrophobic contaminants based on the logarithm of the *n*-octanol-water partition coefficient (log K_{ow}) has worked well,[7,8] but the same approach has not proved sufficient for polar and ionizable organic compounds that may undergo *in planta* metabolism.[5]

Most pharmaceuticals are polar or ionizable, have reactive functional groups, and are degraded in mammalian metabolic pathways to some extent. Plant metabolic pathways for xenobiotic compounds have similarities to mammalian systems. Plants produce many enzymes belonging to the same classes as those responsible for pharmaceutical metabolism in humans, including cytochromes P450 (CYP450s), glutathione-*S*-transferases (GSTs), and uridine 5'-diphospho-glucuronosyltransferases (UGTs).[5] *In planta* transformation of pharmaceuticals is therefore considered likely.[5] Though metabolism of most compouds has not yet been studied, carbamazepine, diclofenac, and ibuprofen are transformed in plants to the same metabolites that are formed in humans.[9–12] In mammals, some pharmaceuticals can affect the metabolism of other compounds by inducing or inhibiting metabolism enzymes. For example, carbamazepine induces the activity of several CYP450 and UGT enzymes to the extent that doses of other medications need to be adjusted for patients to whom carbamazepine is administered.[13,14] In the field, plants are exposed to complex mixtures of pharmaceuticals, which may alter compound accumulation and metabolism relative to exposures to single compounds in controlled studies.[5] This topic has received minimal attention, but warrants investigation.

The accumulation of water soluble contaminants (such as ionized compounds) is driven partially by water flow into and through the plant via evapotranspiration.[5,15,16] While multiple studies have examined the relationship between transpiration and phytoremediation of non-polar, volatile organic compounds,[17,18] to our knowledge, only two previous studies have examined the influence of transpiration on plant accumulation of polar and ionizable, nonvolatile organic compounds. Dodgen et al.[15] found weak correlation between transpiration and bioconcentration factors (concentration in plant divided by concentration in growth media) across three crop species and multiple neutral, cationic, and anionic pharmaceuticals. However, they did not provide

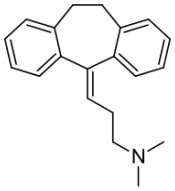
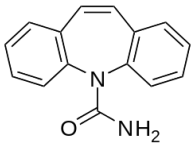
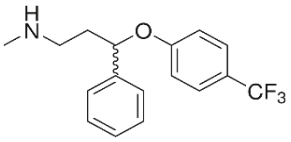
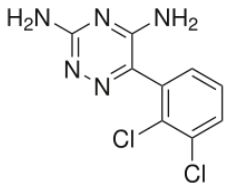
analyses of individual crop species or pharmaceuticals, and their reported correlations, while statistically significant, are too weak to be used in a predictive capacity ($R^2 < 0.13$).[15] Recently, Lamshoeft et al.[16] found much stronger correlation between water uptake and contaminant mass in wheat plants. However, their analysis focused on compounds that are very hydrophilic, with log K_{ow} values ranging from –1.54 to 1.88, and while their main analysis included compounds with molecular mass up to 369, compounds with molecular mass over 200 showed reduced uptake relative to the smaller compounds they tested. Pharmaceuticals, which tend to be larger and more hydrophobic than most of the compounds studied by Lamshoeft et al., may not be as similar to each other and may show reduced uptake or different distribution within the plant. Additionally, the analysis in Lamshoeft et al.[16] was based on radiolabeled compounds and did not account for possible transformation of the analytes, which we expect to be important for pharmaceuticals taken up by plants.

The objectives of this study were to investigate the effects of binary mixtures on pharmaceutical accumulation and metabolism and to evaluate the relationship between transpiration and compound accumulation in spinach plants. We used a mass-balance approach to determine the relationship between plant uptake of water and pharmaceuticals, the fraction transformed within the experimental system, and whether metabolism takes place in solution or in the plant. The compounds we used were carbamazepine (CBZ), lamotrigine (LTG), amitriptyline (AMI), and fluoxetine (FLX). We selected these compounds because they have been detected in treated wastewater,[19,20] and have shown accumulation in plants in previous studies.[21–24] Additionally, CBZ induces the enzymes responsible for metabolizing LTG, AMI, and FLX in mammalian systems.[25]

MATERIALS AND METHODS

Materials. Carbamazepine (99% purity), $NH_4H_2PO_4$ and $CaNO_3$ were obtained from ACRŌS Organics. Lamotrigine (98% purity) was purchased from Comb and Blocks. Fluoxetine hydrochloride (>95% purity) was acquired from Matrix Scientific. Amitriptyline hydrochloride (≥ 98% purity) was obtained from Sigma Aldrich. The mass-labeled internal standards carbamazepine-d10, 10,11-epoxycarbamazepine-d10, lamotrigine $^{13}C_3$, amitriptyline-d6, and fluoxetine-d6 were procured from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). We bought KNO_3 from Fisher, KH_2PO_4 from Alfa Aesar, $MgSO_4$ and KOH from DOT Scientific. The Murashige and Skoog micronutrient solution was from Caisson Laboratories. DMSO was obtained from Macron Fine Chemicals. Nutrient solutions were prepared in ultrapure water (≥ 18 MΩ·cm; Thermo Scientific GenPure Pro system). Structures and selected physico-chemical properties of AMI, CBZ, FLX, and LTG are shown in Table 1.

Table 1. Structures and physico-chemical properties of study compounds.

compound	CAS number	structure	molecular mass	pK_a [a]	log (K_{ow}) [a]	Henry's Law Constant (atm·m^3·mol^{-1}) [b]
amitriptyline	50-48-6		277.403	9.4	4.92	$6.85 \cdot 10^{-8}$
carbamazepine	298-46-4		236.269	–	2.45	$1.08 \cdot 10^{-10}$
fluoxetine	54910-89-3		309.326	9.8	4.05	$8.90 \cdot 10^{-8}$
lamotrigine	84057-84-1		256.091	5.87	2.5	$2.22 \cdot 10^{-11}$

[a] From DrugBank.ca. Experimental values used when available.
[b] Compiled from ChemSpider (EPISuite predictions used).

Plant Growth and Exposure Experiments. Tyee Hybrid Spinach seeds (Jung Garden Center) were sterilized in 70% ethanol (60 s), rinsed three times with ultrapure water, soaked in 4% sodium hypochlorite (20 min), and rinsed five times with ultrapure water. Seeds were then soaked in ultrapure water for 5-7 h in the dark before transfer to a folded, damp paper towel. The paper towel was kept in a sealed plastic bag while the seeds germinated (2-3 d). After germination, sprouts were transferred to aerated hydroponic solution. We used a modified Hoagland's solution that contained 14 mM NO_3^-, 6 mM K^+, 4 mM Ca^{2+}, 2 mM Mg^{2+}, 2 mM SO_4^{2-}, 0.5 mM $H_2PO_4^-$,

0.5 mM NH_4^+, and 10 mL·L^{-1} Murashige and Skoog micronutrients, and was adjusted to pH 5.7 ± 0.05 using KOH. Each plant was grown in an individual nutrient solution container with ~400 mL nutrient solution. Plants grew hydroponically in this solution for 7-8 weeks prior to exposure experiments. Nutrient solution was replenished regularly throughout the growth period. Light was provided by Verilux natural spectrum 48" T12 fluorescent bulbs and varied from 35-80 $\mu mol_{photons} \cdot m^{-2} \cdot s^{-1}$ throughout the growth area.

To test the effects of binary mixtures, we exposed spinach plants to CBZ, LTG, AMI, FLX, or to mixtures of CBZ with one of the latter three pharmaceuticals for 7 days. Exposure concentrations were 1 μg·L^{-1} or 100 μg·L^{-1} (equal concentrations in mixture exposures). Only the high exposure concentration was tested for FLX. We chose CBZ as the basis for our mixtures due to its known effects on the metabolism of other pharmaceuticals in mammalian systems.[13,14]

We also examined CBZ accumulation as a function of time for 14 days. In these experiments, we exposed plants to 100 μg·L^{-1} CBZ and sacrificed plants to measure CBZ accumulation after 1, 3, 7, and 14 d (spiked nutrient solution replaced on day 7). Our experiments included control plants not exposed to pharmaceuticals and control solutions which contained pharmaceuticals but no plants. Temperature and humidity were monitored every 30 min (Figure S1). Nutrient solution was not aerated during the exposure period to minimize variability in evaporation rates. We did not observe any plant health effects caused by lack of aeration of the roots.

During the exposure experiments, individual plants were placed in polypropylene tubs holding 400 mL of nutrient solution each. Pharmaceuticals were added in DMSO, with 40 μL per tub added for the mixture experiments and 20 μL per tub for the CBZ time series. Vehicle (DMSO alone) was added to pharmaceutical-free control solutions. Each tub of solution was mixed and

sampled before the plant was added and at the end of the exposure period. Transpiration (water uptake by the plant) was determined by measuring the mass of the nutrient solution at the beginning and end of the exposure period. Evaporation (as separate from transpiration) was estimated by measuring evaporation from control tubs without plants situated near each plant-containing tub. After the exposure period, above-ground tissues (leaves) and roots were collected, and separately frozen at –80 °C, freeze dried, and stored at –80 °C until extraction. Plant masses were measured before and after lyophilization. Plant mass and transpiration data for all experiments are provided in Table S4. Additional experimental methods information is provided in section S1.1.

Extraction and Analysis. Freeze dried plant samples were ground with a mortar and pestle, spiked with mass-labeled internal standards, allowed to sit overnight at room temperature, and subjected to Accelerated Solvent Extraction (ASE) with 100% methanol. Each ASE cell contained a glass fiber filter and 1.0 g fluorosil at the bottom, over which was placed the tissue sample (0.200 g for leaves and 0.050 g for roots) followed by another 1.0 g fluorosil. We used an ASE 200 with 11 mL stainless steel cells. The ASE cycle (completed twice per sample) comprised a 5 min preheat, 5 min heat, 5 min static extraction, 60% volume flush, and 120 s purge with a pressure of 10.3 kPa and temperature of 80 °C. Extracts were evaporated to dryness, then reconstituted to 1 mL in 80% ultrapure water, 20% acetonitrile, 0.1% acetic acid (for LTG, CBZ, and CBZ metabolite analysis) or 20% ultrapure water, 80% acetonitrile, 0.1% acetic acid (for AMI, FLX, CBZ, and CBZ metabolite analysis). All samples were sonicated 10 min in reconstitution solvent, centrifuged (20 min, 17000*g*), and filtered through 0.2 μm PTFE filters.

We measured AMI, FLX, LTG, CBZ, and the CBZ metabolites 10,11-epoxycarbamazepine (epCBZ) and 10,11-*trans*-dihydroxycarbamazepine (diOH-CBZ) by liquid chromatography with tandem mass spectrometry (Agilent 1260 HPLC equipped with a Waters

Xterra MS C18 3.5 μm, 2.1 × 100 mm column, Agilent 6460 triple quadrupole mass spectrometer, ESI+ source) in leaf and root extracts and starting and ending nutrient solutions. Mobile phases were 100% acetonitrile and 0.1% formic acid in 10% acetonitrile. We used a gradient of 5% to 95% organic phase and column temperature was held at 30 °C. Internal standard-based calibration and measurement was used for plant extracts only. Nutrient solution samples did not contain internal standards. Extraction recoveries, limits of quantification and detection, and ion masses used for measurement are provided in section S1.2.

RESULTS AND DISCUSSION

Compound Accumulation and Mixture Effects. We found that accumulation and tissue distribution within spinach plants varied among compounds. Amitriptyline exhibited the highest overall accumulation, CBZ had the highest concentration in leaves, and LTG and FLX remained mainly in the roots (Figure 1). The presence of CBZ did not affect plant accumulation of AMI, FLX, and LTG (t-tests, $p > 0.05$). Likewise, CBZ accumulation was not affected by the presence of the other compounds (*t*-tests, $p > 0.05$).

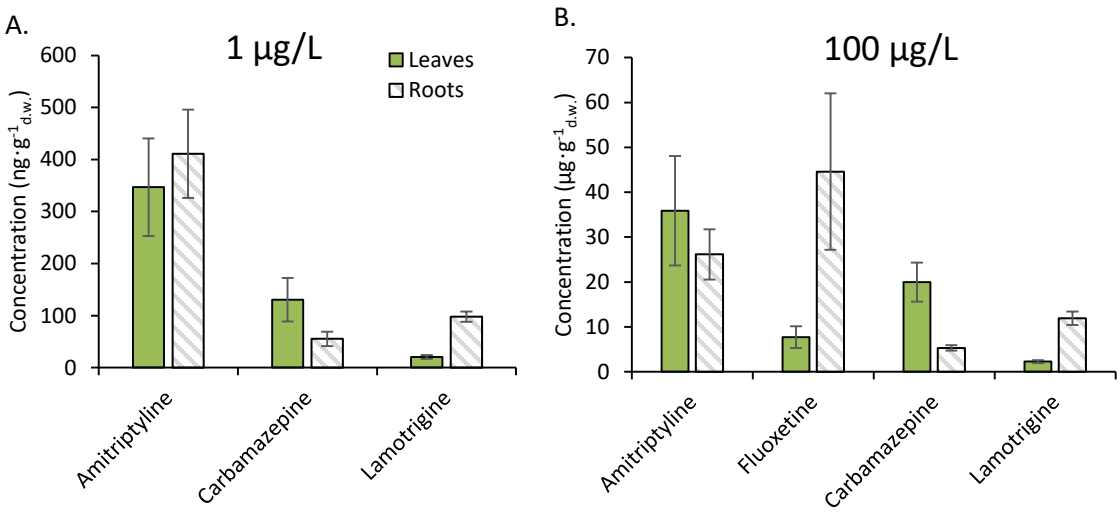


Figure 1. Concentrations of parent compounds in leaves and roots of spinach plants exposed to (A) 1 µg·L^{-1} or (B) 100 µg·L^{-1} of the indicated compounds. Data from mixture and single compound treatments are combined, as no significant differences were found. Error bars represent one standard deviation ($n \geq 7$).

In contrast, leaf accumulation of epCBZ, the primary metabolite of CBZ in plants and humans,[9,26] was lower in plants co-exposed to AMI or FLX (Figure 2). Lamotrigine did not affect metabolite accumulation (*t*-tests, $p > 0.05$). Leaf concentrations of epCBZ were higher than those in roots by a factor of 12 ± 8 in the 100 µg·L^{-1} exposure, indicating that CBZ metabolism likely occurs in the leaves. This is consistent with past analysis of CBZ metabolism in plants.[9,24,23] Concentrations of epCBZ were below the limit of quantification in the roots of plants exposed to

1 μg·L^{-1} CBZ. Concentrations of diOH-CBZ exceeded the limit of detection only in the leaves of plants exposure to 100 μg·L^{-1} CBZ. The presence of other pharmaceuticals did not affect diOH-CBZ accumulation (Dunnett's test, $p > 0.05$).

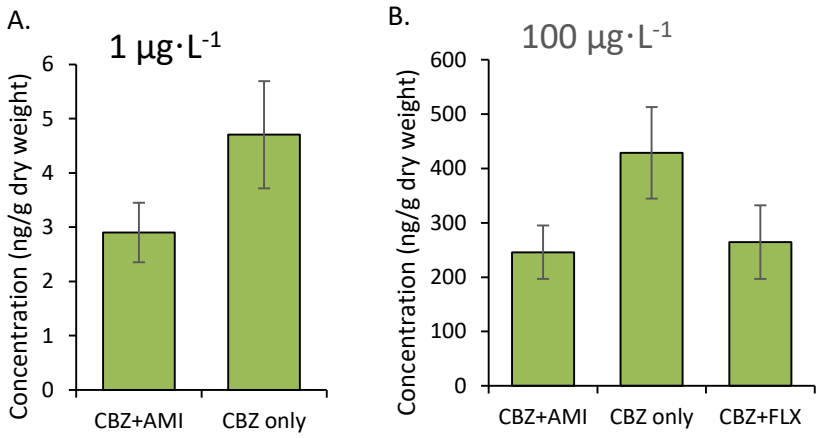


Figure 2. Accumulation of 10,11-epoxycarbamazepine in spinach leaves is lower when plants are exposed to carbamazepine (CBZ) alongside amitriptyline (AMI) or fluoxetine (FLX) at the 1 μg·L^{-1} (A) and 100 μg·L^{-1} (B) exposure levels ($p < 0.05$ Dunnett's tests). Error bars represent one standard deviation ($n \geq 4$).

Plant metabolism of LTG, AMI, and FLX has not been previously studied to our knowledge. We estimated the extent of metabolism for all four compounds using a mass balance approach. For CBZ and LTG, we did not detect any compound loss for mixture or single compound treatments, i.e. all of the compound initially added to the nutrient solution was detected in either the plant or the nutrient solution at the end of the exposure period, within the standard deviations of the measurements (Figure 3). The CBZ mass balance calculations include measured metabolites. For FLX and AMI (100 μg·L^{-1} exposure only), we observed compound mass lost, but single compound and mixture treatments did not differ (*t*-tests, $p > 0.05$). Metabolites of AMI, and FLX have not been previously reported in plants, but we hypothesize that they would be similar to those formed in mammalian systems as is true for other pharmaceuticals.[9–12]

We note that the mixture effects on CBZ metabolism were too small to be detected by a mass balance approach. Metabolites comprised < 4% of total CBZ measured in all treatments, and the amount of metabolites measured was less than the variation observed in CBZ measurements. As the metabolites make up such a small portion of total CBZ measured, and CBZ itself was not affected by the presence of other pharmaceuticals, single and dual exposure data are combined for the remainder of the analyses in this manuscript.

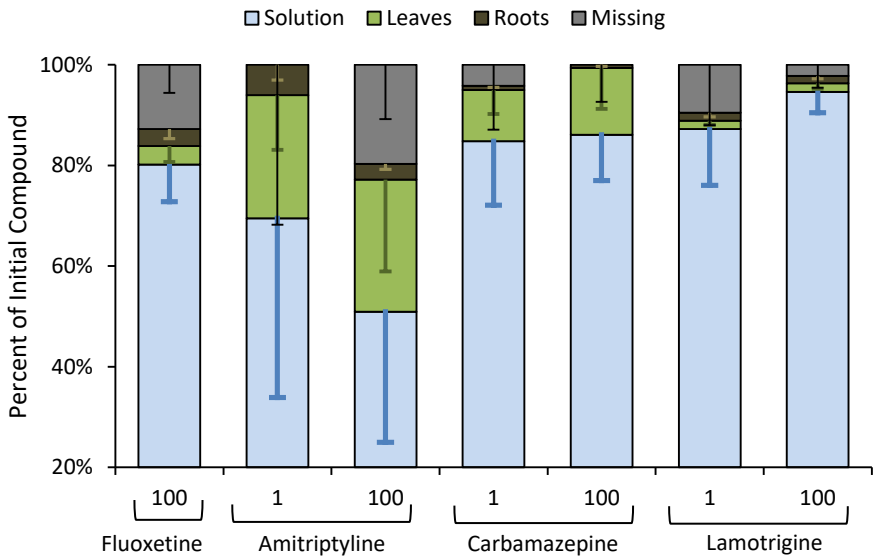


Figure 3. Mass balance for compounds taken up by spinach plants exposed for 7 days. The "missing" fraction denotes the difference between the initial amount of compound added to nutrient solution and the amount detected in nutrient solution and plants and the end of the exposure period. We found measureable loss of amitriptyline (AMI) and fluoxetine (FLX) in the 100 $\mu g \cdot L^{-1}$ exposure, but not for carbamazepine (CBZ) or lamotrigine (LTG) at either exposure concentration or AMI at 1 $\mu g \cdot L^{-1}$. Each bar includes mixture and single compound treatments. CBZ data includes measured CBZ metabolites. Error bars represent one standard deviation (n > 7).

The lack of mixture effects on the overall mass balance of our uptake experiments suggests that the mechanism for spinach uptake of these pharmaceuticals is not competitive, as the presence of one compound does not inhibit accumulation of others from solution to plant. However, our epCBZ data suggest that competition may exist for metabolic enzymes. In mammalian systems,

LTG is metabolized primarily by UGT enzymes, while AMI and CBZ are metabolized by both CYP450 and UGT enzymes, and FLX is metabolized by CYP450s only.[25] The interaction effects we observe between CBZ and AMI/FLX may be due to competition for CYP450s, as CBZ and LTG did not affect metabolism of each other in our experiments. Alternatively, AMI and FLX may inhibit the enzyme that coverts CBZ to epCBZ or induce the enzyme(s) that transform(s) epCBZ. The four compounds used in this study have many inhibitory and inductive effects on the enzymes responsible for drug metabolism in humans. Fluoxetine inhibits several CYP450s, including those responsible for metabolizing AMI, CBZ, and itself.[25] Carbamazepine induces the CYP450s responsible for metabolizing AMI, FLX, and itself, as well as the UGT enzyme that primarily metabolizes LTG.[25] In this study, CBZ metabolism is low enough that the mixture effects on metabolism did not impact the amount of CBZ measured in the plants, but this may not be the case in systems where CBZ is metabolized to a greater extent, as has occurred in other plant species.[9]

Transpiration Drives Compound Accumulation. If compound accumulation in the plant was driven solely by transpiration, no barriers hindered the compounds from entering the plant as water flowed in, no compound metabolism occurred in the plant, and no compound was volatilized from plant tissue, the mass of compound in the plant would equal the volume of water transpired multiplied by the concentration in the nutrient solution. We term this value as predicted accumulation (PA)

$$PA = \frac{C_{\text{solution-d0}} + C_{\text{solution-d7}}}{2} \times V_{\text{transpired}} \quad (1)$$

Equation 1 shows with PA in units of mass per plant, $C_{\text{solution-d0}}$ and $C_{\text{solution-d7}}$ as concentrations in nutrient solution at the beginning and end of the exposure period in units of mass per volume, and $V_{\text{transpired}}$ as the volume transpired by the plant. We calculated PA for each plant in our experiments

and compared this value to the actual accumulation (AA; total compound measured in the plant, which for CBZ includes measured metabolites). Both PA and AA vary significantly within treatments due to the range of plant sizes. Therefore, we investigated the correlation between PA and AA for each compound (Figure 4). We found that PA correlates most strongly with the mass of contaminant accumulation in the whole plant (Table S5).

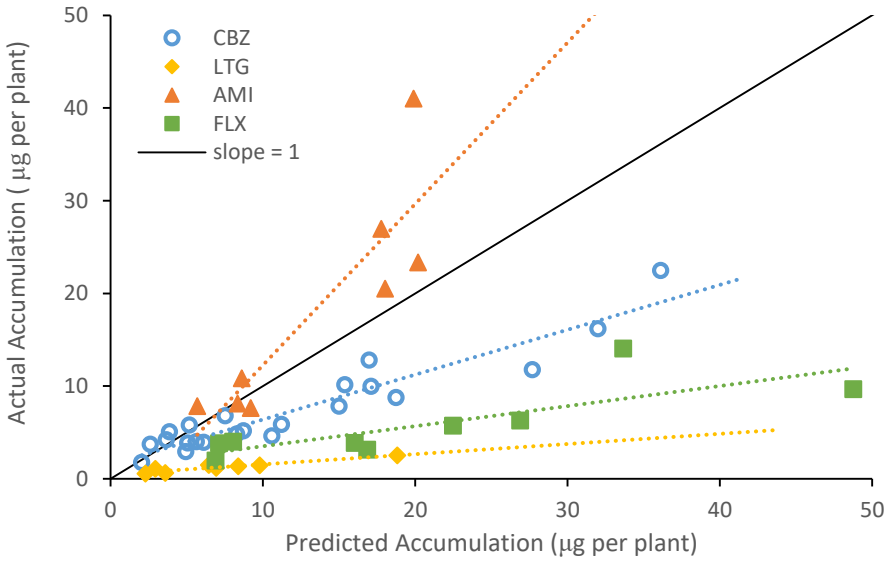


Figure 4. Predicted and actual accumulation for plants exposed to 100 µg·L^{-1} of carbamazepine (CBZ), lamotrigine (LTG), amitriptyline (AMI), and/or fluoxetine (FLX). Predicted accumulation correlates with actual accumulation ($p \leq 0.01$), but the correlation slope varies considerably among compounds and none of them are close to one (black line). Predicted accumulation is calculated using equation 1. Actual accumulation is the total mass of compound measured in plant roots and leaves.

Predicted and actual accumulation was correlated for all compounds ($p \leq 0.01$). However, regression slopes varied significantly, with AA higher than PA for AMI and lower than PA for CBZ, FLX, and LTG. We found no differences between regression slopes for plants exposed to 1 µg·L^{-1} vs. 100 µg·L^{-1} (Figure S2, Table S6). High exposure data are shown, as more compounds were tested at this exposure concentration.

For the compounds where AA is lower than PA, one might expect that some of the gap is due to in *planta* metabolism of the compounds. Based on the overall mass balance in our

experiments, we found measurable losses of AMI and FLX in the high exposure treatments, but not for CBZ and LTG nor AMI at the low exposure (Figure 3, loss indicates fraction of initial compound supplied not detected in plant or nutrient solution at the end of the exposure period). Fluoxetine losses from plant exposure treatments and no-plant controls did not differ ($p > 0.05$). Therefore, we hypothesize that the shallow slopes of the CBZ, FLX, and LTG regressions were not due to compound metabolism, and the missing fraction FLX was degraded in solution or sorbed to container walls. For AMI, we found significant compound loss in the 100 $\mu g \cdot L^{-1}$ exposure. More AMI was lost from solution than was detected in plant tissue, and the proportion of AMI lost from solution correlated strongly with the proportion of solution transpired (Figure 5). Amitriptyline was not degraded in no-plant controls. This evidence indicates that AMI is metabolized *in planta* to some extent. Phytovolatilization is unlikely for any of the studied compounds due to their low air–water partition coefficients (Table 1).

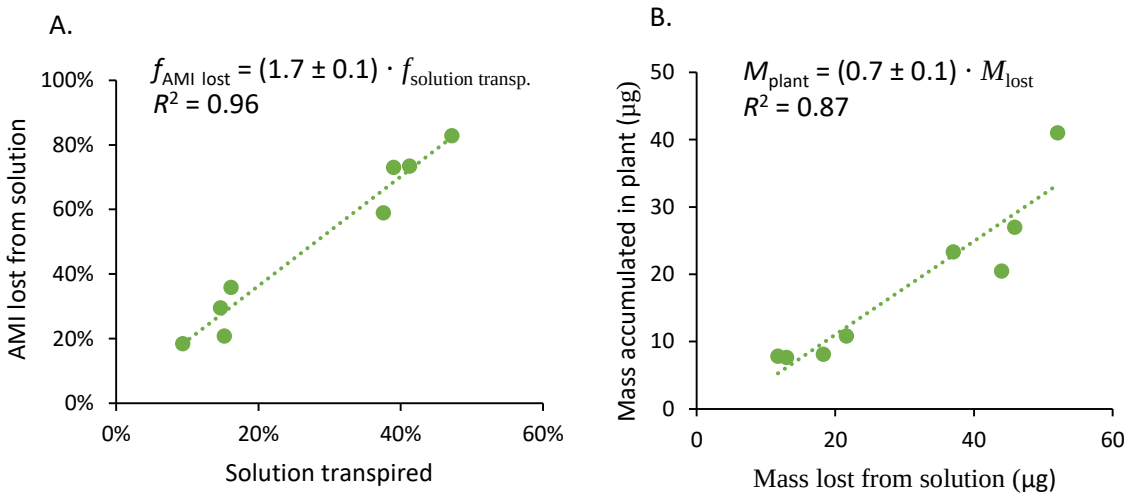


Figure 5. Amitriptyline (AMI) loss from solution correlated with (A) transpiration and (B) accumulation in the plant ($p < 0.001$). Strong correlation between transpiration and loss from solution indicates that uptake into the plant is the main mechanism for loss from solution. A larger fraction of AMI was lost from solution relative to the fraction of water transpired, indicating mechanisms beyond transpiration are important for AMI uptake. However, more AMI mass was lost from solution than accumulated in the plant, indicating AMI degradation in the plant. Equations show regression slope ± standard error. *Y*-intercepts are not statistically significant.

Plant Uptake Processes. Despite *in planta* metabolism of AMI, we measured more AMI in the plant than would be predicted based on transpiration alone. For CBZ, FLX, and LTG, we measured less accumulation than would be expected based on transpiration. Accumulation of molecules in plants is driven by several processes in addition to transpiration. Dissolved molecules can enter the apoplast (space between root cells) via water influx to the roots (the start of transpiration) or diffusion from the solution surrounding the roots. Once in the apoplast, molecules can sorb to the negatively charged cell walls and membranes, cross a root cell membrane to enter the symplast (inside of the cell), or stay in the dissolved phase.[27] Molecules that stay dissolved in the apoplast can travel through the root, but are blocked from entering the vascular tissue and main transpiration flow to the leaves by the Casparian strip, a waxy barrier. To reach the vascular tissue, molecules must enter at least one root cell and travel symplastically through interconnecting channels between cells, which provides a pathway to circumvent the Casparian strip (Figure 6).[5,28]

All of the compounds tested in this study are too large for immediate diffusion across root cell membranes.[27] However, as a neutral molecule, CBZ would be expected to be able to diffuse across significantly faster that AMI, FLX, and LTG, which are primarily positively charged at the apoplast pH (~5.5, Figure 6).[5,27] As LTG and FLX are found primarily in the roots, we hypothesize that they are blocked from easily crossing root cell membranes and stay primarily in the apoplast. As positively charged molecules, they likely sorb to the negatively charged cell walls within the apoplast as well. While the molecules enter the root with water influx, there is also diffusive flux out of the root as higher concentrations build up in the apoplast, causing the low slopes seen in Figure 4. We hypothesize that carbamazepine is able to enter the symplast more easily than LTG and FLX, and is therefore taken up to a larger extent and a larger fraction is found in the leaves, although diffusion across the cell membrane happens more slowly than water flow through the root, so CBZ accumulates in the plant less quickly than transpiration-driven water flow.

Amitriptyline is taken up to a larger extent than water and does not follow the same trend as the other compounds. Based on the log K_{ow} and pK_a values of AMI, we would expect it to accumulate similarly to FLX, but this is not the case. We hypothesize that AMI transport into root cells is facilitated by an passive ion transporter protein (Figure 6). Transporters across cell membranes such as ion channels or uniporters provide a pathway for positively charged ions to enter plant root cells without needing to diffuse through the membrane.[27] The negative membrane potential of root cells drives accumulation of positive ions in the symplast without energy expenditure.[27] As AMI is depleted from the apoplast, more diffuses in from the solution outside the root, which could happen at a faster rate than would be anticipated based on water movement into the root.

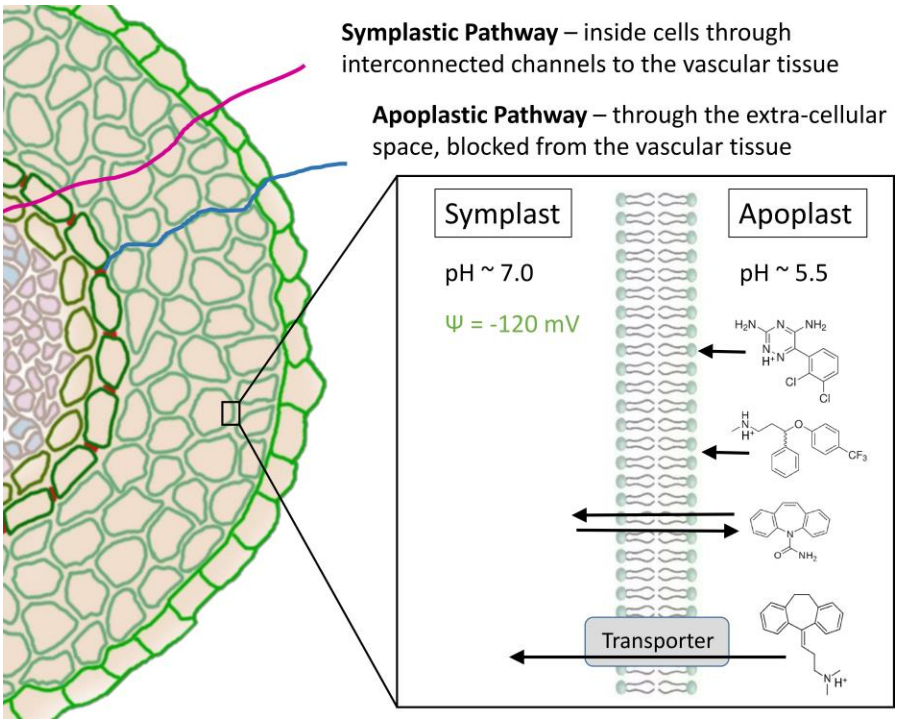


Figure 6. Schematic showing the symplastic and apoplastic pathways for a molecule to reach the vascular tissue of a plant root. Molecules that cross a cell membrane and move between cells through interconneted channels (symplastic pathway) are able to reach the vascular tissue in the center of the root and be transported with the transpiration stream to the leaves. Molecules that diffuse into the intercellular space but do not cross into root cells (apoplastic pathway) are blocked from entering the vasuclar tissue by the Casparian strip, a waxy barrier. As shown in the insert, carbamazepine, as a neutral molecule can diffuse across cell membranes to travel symplastically. As charged molecules, lamotrigine, fluoxetine, and amitriptyline diffuse very slowly through cell membranes. However, root cells have negative membrane potential, which drives accumulation of positive ions when they are not blocked by the cell membrane. We hypothesize that a transporter protein in spinach root cells allows amitriptyline accross the membrane to travel symplastically, while fluoxetine and lamotrigine are primarily stuck in the apoplast.

Plants have many transporters that are responsible for moving nutrients, hormones, and secondary metabolites through the plant.[27] Passive transporters are important for plant accumulation of positively charged molecules that are essential for plant nutrition such as potassium, calcium, and urea,[27] and are necessary for elongation and growth, maintaining membrane potential, and responses to stress and pathogens.[29] To our knowledge, transporter proteins that interact with xenobiotic organic cations in plants have not yet been identified, but transporters have been implicated in plant interactions with other types of xenobiotic organic

contaminants. Phenanthrene uptake into cells is mediated by a proton symporter,[30,31] and antibiotic resistance in plants has been connected to other membrane transporters such as members of the ATP-binding cassette and major facilitator superfamilies of proteins.[32,33]

Importance of Exposure Time. Our data provide sufficient evidence that accumulation of the investigated compounds in spinach is driven by transpiration. However, our 7-day exposure period does not represent field conditions, where plants would be exposed to pharmaceuticals intermittently throughout their development. We therefore tested whether the correlations with transpired water would hold for plants harvested at varying exposure time points. We conducted a time series experiment with CBZ to test accumulation over a 14 day time period. We chose CBZ because as a neutral molecule, we expected it to diffuse the fastest through cell membranes and therefore reach a steady-state concentration more rapidly than the other compounds studied, and we could measure its metabolites. While still not representative of field conditions, testing multiple time points provides insight to the broader applicability of our results.

We found that leaf and root concentrations of CBZ and its primary metabolite increased continuously over 14 days (Figure S3). However, the correlation between AA and PA remained consistent only for the first 7 days of exposure (Figure 7). These data could indicate that transpiration-driven uptake does not occur to as large an extent once a certain level of accumulation has been reached. The data also provide evidence that once pharmaceutical molecules accumulate enough in the apoplast, they may not continue enter the plant root despite continued transpiration, as they would need to move against a diffusion gradient. If this is the case, plants exposed to contaminants throughout their lifetime may show different accumulation trends than those exposed for shorter time periods in the laboratory. Also, compounds that travel through the root via different

pathways may have very different trends in accumulation over time. This is a topic that warrants further investigation, as much existing research focuses on plants exposed for only short periods.

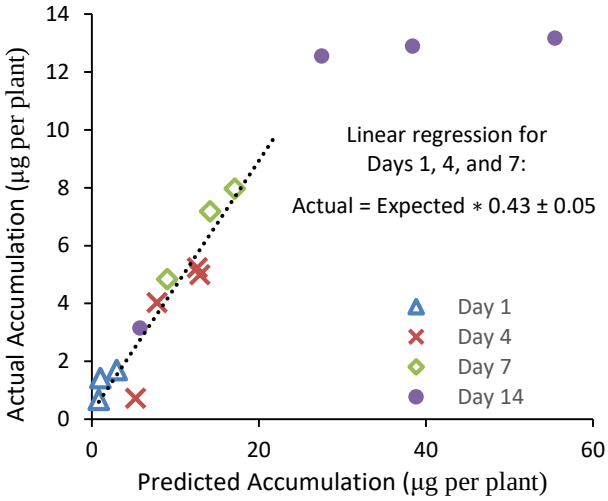


Figure 7: Predicted and actual accumulation for spinach plants exposed to 100 µg·L^{-1} carbamazepine (CBZ) harvested at various time points. Linear correlation is shown for data points for days 1-7. Plants with high transpiration harvested on day 14 did not accumulate as much CBZ as would be expected from the correlation for days 1-7.

Broader Implications. Our data provide strong evidence that the binary mixtures we tested do not affect accumulation of pharmaceuticals in spinach plants. We found evidence that AMI and FLX impact CBZ metabolism, but the measured metabolites comprised a very small fraction of total CBZ accumulated in the plant. Other plant species such as tomatoes are known to metabolize CBZ to a larger extent than spinach and other pharmaceuticals such as ibuprofen, diclofenac are degraded to a larger extent than carbamazepine.[9,11,12,34] Mixture effects may be more pronounced when metabolism is more significant, a topic that warrants additional investigation. Plants irrigated with reclaimed wastewater are exposed to complex mixtures of contaminants, and single compound exposure experiments may not produce results relevant to field conditions.

We found strong correlation between transpiration and accumulation of each compound in our study. Our results differ from those of Dodgen et al.,[15] who previously studied the relationship between transpiration and pharmaceutical uptake, in that we provide analyses for individual

compound and observed much stronger correlations. Lamshoeft et al.[16] also investigated transpiration and organic compound uptake and found strong correlation (R^2 = 0.80), but did not find clear differences among their tested compounds with molecular weights below 394. This contrasts with our results that show substantial variation among compounds. We hypothesize that variation the compounds in our study is due to their higher hydrophobicity, and because as pharmaceuticals, they are biologically active molecules that may interact with transporter proteins and metabolism enzymes that are conserved across phylogenetic kingdoms. Additionally, our analysis has more depth than previous publications on transpiration, as we considered metabolism and tested multiple exposure periods for CBZ.

Most discussion in current literature on quantifying pharmaceutical accumulation in crop plants focuses on passive processes such as diffusion across membranes, ion trapping, and sorption, in which plant transporter proteins do not play a role.[24,23,35,21] However, based on the variation between FLX and AMI uptake we see in our results, we expect that plant transporter proteins may be important for accumulation of certain pharmaceuticals (including amitriptyline), as has been previously demonstrated for phenanthrene.[30,31] If this is the case, current prediction models[36] will need to be reconceptualized to include specific plant-driven interactions with compounds. Focus on the biological aspects of plant uptake is important for increasing our understanding of plant accumulation of pharmaceuticals.

ASSOCIATED CONTENT

Supporting information (SI) is available. SI contains information on experimental design, extraction recoveries, limits of detection and quantification, additional results details as noted, and a record of temperature and humidity during plant exposure.

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Notes

The authors declare no competing financial interest.

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SUPPLIMENTAL INFORMATION FOR CHAPTER THREE

EFFECTS OF TRANSPIRATION AND BINARY MIXTURES ON ACCUMULATION OF PHARMACEUTICALS BY SPINACH: SUPPLEMENTAL INFORMATION

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Table of Contents:

S1. Supplemental Methods

S1.1. Experimental Design. We conducted three spinach uptake experiments. In the first experiment, we exposed spinach plants to 1 $\mu g \cdot L^{-1}$ or 100 $\mu g \cdot L^{-1}$ of carbamazepine (CBZ), amitriptyline (AMI), or a mixture of CBZ and AMI (1 $\mu g \cdot L^{-1}$ or 100 $\mu g \cdot L^{-1}$ each). We also included 100 $\mu g \cdot L^{-1}$ treatments for fluoxetine (FLX) and a CBZ-FLX mixture. Nutrient solution pH was kept at 5.7 for all treatments. In a separate experiment, we exposed plants to 1 $\mu g \cdot L^{-1}$ or 100 $\mu g \cdot L^{-1}$ of CBZ, lamotrigine (LTG), or a mixture (1 $\mu g \cdot L^{-1}$ or 100 $\mu g \cdot L^{-1}$ of each). Nutrient solution pH was adjusted to 7 so that most LTG would be present in its neutral phase. Each treatment included 4-5 plants for both mixture experiments. Additionally, we did a time series experiment with CBZ to test accumulation over time for 14 days (nutrient solution pH = 5.7). Three or four plants were harvested at each time point.

There was some variation in temperature and humidity between the experiments (supplemental results, Figure S1), which caused some variation in plant size and transpiration between experiments (supplemental results, Table S4). Additionally, there were some analysis methods differences between experiments. For the CBZ-LTG experiment, 80% ultrapure water, 20% acetonitrile, 0.1% acetic acid was used as the reconstitution solvent, while 20% ultrapure water, 80% acetonitrile, 0.1% acetic acid was used for the others. Additionally, only the compounds included in each experiment were measured in the relevant plants and there were small differences in the timing of the solvent gradient on the HPLC. Mass to charge ratios were the same for both methods (Table S1). The CBZ time series experiment was analyzed using a method very similar to the CBZ-AMI-FLX method. Recovery and LOD/LOQ information is provided separately for the two reconstitution solvents (Table S2). CBZ accumulation and metabolism measurements were very similar

between equivalent 7 day experiments, and data reported in the main text figures 1, 3, and 4 include plants from both mixture experiments.

S.1.2. Analytical Methods Details

Table S1. Mass-to-charge ratios (*m/z*) for mass spectral measurements

Compound	Precursor Ion (m/z)	Quantitative Ion (m/z)	Qualitative Ion(s) (m/z)
lamotrigine	256	43.1	108.9, 58.1
lamotrigine-$^{13}C_3$	259	44.1	59.1
carbamazepine	237.1	194.1	165
carbamazepine-d_{10}	247.2	204.1	173.1
10,11-epoxycarbamazepine	253.1	180.1	210.1
10,11-epoxycarbamazepine-d_{10}	263.3	188.1	220.1
trans 10,11-dihydroxycarbamazepine	271	180.1	210.1
amitriptyline	278.41	91	105
amitriptyline-d_6	284.4	91	105
fluoxetine	310.3	44.1	148.1
fluoxetine-d_6	316.0	44.1	153.6

Extraction recoveries and standard deviations are shown in Table S2. Compound recoveries are based on a calibration using internal standard (IS) ratios. A recovery of 1.00 represents 100% recovery. Recovery samples consisted of blank plant tissue with compound and IS spikes added prior to ASE. 10,11 epoxycarbamazepine-d_{10} was used as the IS for both CBZ metabolites. Measurements presented in this manuscript have not been recovery corrected.

Table S2. Extraction recoveries

Compound	Matrix	80% Acetonitrile Reconstitution			20% Acetonitrile Reconstitution		
		Spike level	Recovery	stdev	Spike level	Recovery	stdev
AMI	roots	360 $ng \cdot g^{-1}$	1.23				
		20 $\mu g \cdot g^{-1}$	0.88	0.04			
	leaves	5 $\mu g \cdot g^{-1}$	0.97	0.04			
		20 $\mu g \cdot g^{-1}$	0.96	0.01			
FLX	roots	30 $\mu g \cdot g^{-1}$	3.9	0.2			
	leaves	7.5 $\mu g \cdot g^{-1}$	0.95	0.03			
CBZ	spinach roots	40 $ng \cdot g^{-1}$	1.03		40 $ng \cdot g^{-1}$	0.91	0.02
		5 $\mu g \cdot g^{-1}$	1.14	0.02	5 $\mu g \cdot g^{-1}$	1.52	0.04
	spinach leaves	63 $ng \cdot g^{-1}$	1.4	0.2	125 $ng \cdot g^{-1}$	1.051	0.006
		20 $\mu g \cdot g^{-1}$	1.03	0.04	15 $\mu g \cdot g^{-1}$	1.37	0.09
epCBZ	spinach roots	0.4 $ng \cdot g^{-1}$	1.44		0.4 $ng \cdot g^{-1}$	1.4	0.6
		30 $ng \cdot g^{-1}$	1.04	0.03	20 $ng \cdot g^{-1}$	1.2	0.1
	spinach leaves	3 $ng \cdot g^{-1}$	1.1	0.1	3 $ng \cdot g^{-1}$	0.70	0.03
		250 $ng \cdot g^{-1}$	0.97	0.04	150 $ng \cdot g^{-1}$	0.60	0.07
hydCBZ	spinach leaves				10 $ng \cdot g^{-1}$	1.1	0.1
LTG	spinach roots				80 $ng \cdot g^{-1}$	0.97	0.04
					10 $\mu g \cdot g^{-1}$	1.256	0.006
	spinach leaves				20 $ng \cdot g^{-1}$	1.3	0.2
					2 $\mu g \cdot g^{-1}$	1.19	0.07

Limits of detection (LOD) and quantification (LOQ) were determined by running a low concentration sample 7 times in a row and taking the standard deviation of the measurements (σ). LOD and LOQ was calculated using the equations:[1]

$LOD = 3\sigma$ (S2) $LOQ = 10\sigma$ (S3)

Table S3. Limits of Detection and Quantification

Compound	80% Acetonitrile reconstitution				20% Acetonitrile reconstitution			
	Root LOD	Root LOQ	Leaf LOD	Leaf LOQ	Root LOD	Root LOQ	Leaf LOD	Leaf LOQ
Amitriptyline	3	9	2	7				
Fluoxetine	2	8	0.4	1.2				
Carbamazepine	2	6	7	24	2	5	3	9
10,11-epoxycarbamazepine	0.8	2.6	0.08	0.26	0.6	2.1	1	5
10,11-*trans*-hydroxycarbamazepine					0.3	1.0	0.2	0.6
Lamotrigine					7	23	6	19

S1.3. Statistical Analyses. We used Microsoft Excel Professional Plus 2013 to conduct *t*-tests and linear regression statistics. We used JMP Pro 12.2.0 (SAS) for ANOVA tests with Tukey's post hoc analysis and Dunnett's tests.

S2. Supplemental Results

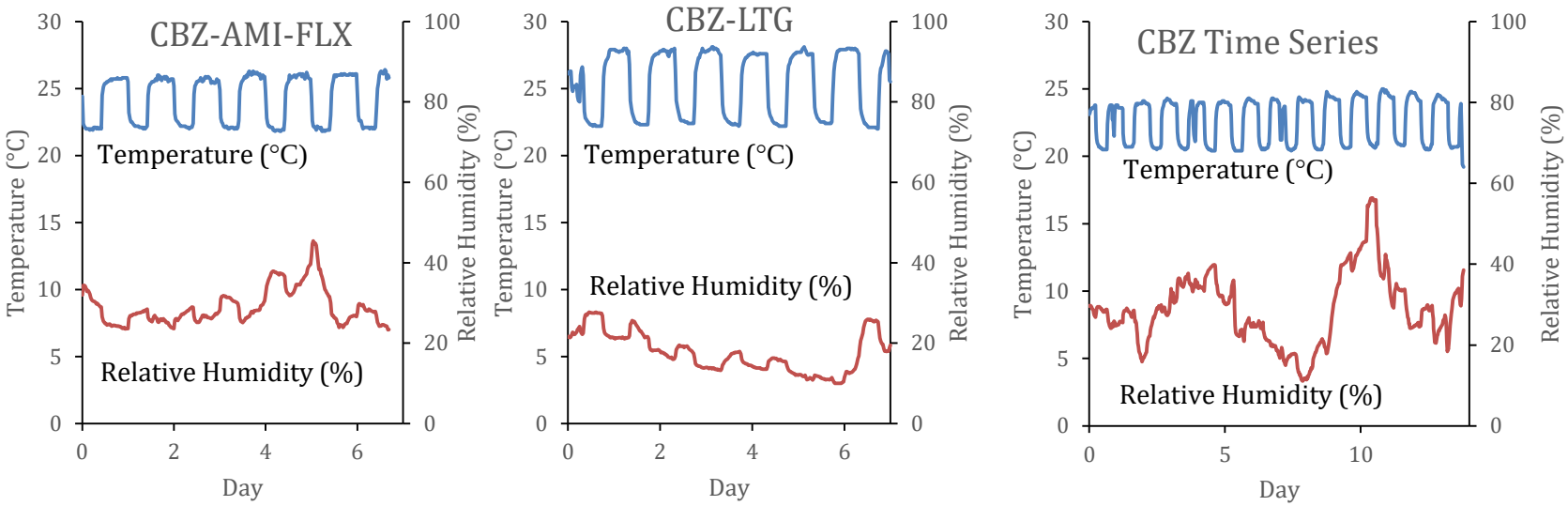

Figure S1. Temperature and humidity during the three spinach uptake experiments. Measurements were recorded every 30 minutes using an automated system for the duration of each experiment. Differences in temperature and humidity between experiments likely influenced the differences in plant size and transpiration we saw between experiments (Table S5).

Experiment	Treatment	Water Lost (g)		Transpiration (g)		Root wet mass (g)		Root dry mass (g)		Top wet mass (g)		Top dry mass (g)	
		Average	*STDEV*	Average	*STDEV*	Average	*STDEV*	Average	*STDEV*	Average	*STDEV*	Average	*STDEV*
CBZ-AMI-FLX	Control	93	*28*	80	*23*	1.7	*0.7*	0.07	*0.03*	4.3	*1.6*	0.33	*0.15*
	CBZ 1	101	*24*	91	*29*	1.8	*0.4*	0.07	*0.01*	4.6	*2.1*	0.36	*0.13*
	AMI 1	123	*69*	117	*67*	2.0	*0.8*	0.08	*0.03*	5.3	*2.7*	0.40	*0.20*
	CBZ-AMI 1	88	*23*	81	*22*	1.8	*0.5*	0.07	*0.02*	4.4	*0.8*	0.36	*0.09*
	CBZ 100	90	*36*	79	*31*	1.5	*0.5*	0.06	*0.02*	4.4	*1.7*	0.35	*0.16*
	AMI 100	117	*60*	110	*56*	1.9	*0.8*	0.08	*0.02*	5.1	*2.6*	0.41	*0.21*
	FLX 100	110	*43*	100	*34*	1.8	*1.0*	0.07	*0.04*	5.3	*3.2*	0.46	*0.30*
	CBZ-AMI 100	117	*75*	111	*73*	1.8	*1.0*	0.08	*0.04*	5.6	*3.6*	0.46	*0.31*
	CBZ-FLX 100	94	*61*	86	*64*	1.8	*0.8*	0.06	*0.03*	4.1	*2.8*	0.33	*0.21*
CBZ-LTG	Control	56	*29*	50	*28*	1.1	*0.4*	0.05	*0.01*	2.8	*1.0*	0.26	*0.08*
	CBZ 1	64	*37*	58	*40*	1.6	*0.2*	0.06	*0.01*	3.4	*1.3*	0.29	*0.09*
	LTG 1	64	*41*	57	*37*	1.7	*1.1*	0.06	*0.04*	3.4	*2.0*	0.30	*0.19*
	CBZ-LTG 1	68	*26*	60	*28*	2.0	*0.6*	0.07	*0.02*	3.9	*1.2*	0.34	*0.10*
	CBZ 100	54	*27*	45	*28*	1.2	*0.5*	0.04	*0.02*	2.5	*1.2*	0.22	*0.11*
	LTG 100	71	*24*	63	*22*	1.3	*0.4*	0.05	*0.02*	3.4	*1.3*	0.29	*0.13*
	CBZ-LTG 100	75	*57*	68	*60*	1.7	*1.1*	0.06	*0.04*	3.5	*2.6*	0.31	*0.24*
CBZ Time Series	Control	132	*60*	123	*57*	2.7	*1.1*	0.10	*0.03*	7.5	*3.1*	0.68	*0.29*
	Day 1	16	*11*	15	*11*	2.4	*0.8*	0.08	*0.03*	6.7	*2.9*	0.50	*0.17*
	Day 4	94	*31*	89	*34*	2.4	*0.9*	0.08	*0.03*	6.7	*3.5*	0.52	*0.22*
	Day 7	134	*29*	123	*35*	3.4	*0.7*	0.13	*0.03*	8.0	*2.5*	0.85	*0.30*
	Day 14	256	*141*	239	*141*	2.8	*1.1*	0.11	*0.04*	7.3	*3.2*	0.85	*0.46*

Table S4. There were no significant differences in plant masses within experiments (ANOVA, $p > 0.3$). There were no significant differences in transpiration or water lost for the CBZ-AMI-FLX or CBZ-LTG experiments (ANOVA, $p > 0.4$). As expected, water loss and transpiration increased over time in the CBZ time series experiment, with significant differences between days 1 and 14 (ANOVA, Tukey's post hoc, $p < 0.05$). The CBZ+LTG experiment has less water loss and transpiration than the others ($p < 0.0002$), and lower top wet mass ($p < 0.5$). The time series experiment has higher wet and dry root and top masses than the other experiments ($p < 0.0005$). We attribute the differences in experiments to variation in environmental conditions.

Treatment	R^2 for whole plant	R^2 for leaves only
CBZ 100	0.89	0.89
CBZ 1	0.83	0.85
AMI 100	0.77	0.77
AMI 1	0.82	0.50
FLX 100	0.63	0.57
LTG 100	0.90	0.93
LTG 1	0.66	0.36

Table S5. We compared regressions between predicted accumulation in the whole plant, and accumulation in plant leaves only. While all regressions with whole plant data were statistically significant ($p < 0.05$), regressions for AMI and LTG 1 ug/L exposures were not significant.

At first thought, it makes sense that a transpiration based prediction would correlate well with accumulation in plant leaves, as water moving through the plant travels out of the roots and dissipates from the leaves. We hypothesize that including root accumulation in the correlations improves them for some treatments because contaminant molecules that enter the plant along with water may be blocked by the Casparian strip, which protects the vascular tissue of the plant. Thus, accumulation in roots may be a dynamic, transpiration driven process as additional contaminant enters the plant with water flow, but does not get translocated above ground.

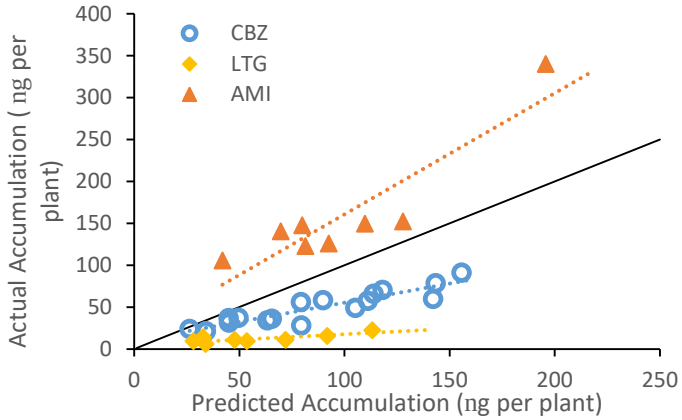

Figure S2. Predicted and actual accumulation for plants exposed to 1 µg · L^{-1}. Predicted accumulation correlates with actual accumulation, but the correlation slope varies significantly between compounds and none of them are close to one (black line). Predicted accumulation is calculated by multiplying the average concentration of compound in the nutrient solution by the volume of solution transpired. Actual accumulation is the total mass of compound found in plant roots and leaves.

	Exposure concentration (µg·L^{-1})	R^2	Slope		Intercept	
			Coefficient	*p*-value	Coefficient	*p*-value
CBZ	1	0.83	0.46 ± 0.05	3.29E-07	10 ± 5	0.07
	100	0.89	0.48 ± 0.04	4.90E-11	1.5 ± 0.58	0.015
LTG	1	0.66	0.13 ± 0.04	0.015	5 ± 3	0.12
	100	0.90	0.11 ± 0.01	3.01E-04	0.4 ± 0.1	0.013
AMI	1	0.82	1.4 ± 0.3	0.002	20 ± 30	0.61
	100	0.77	1.7 ± 0.4	0.004	5 ± 6	0.402
FLX	100	0.63	0.22 ± 0.06	0.010	1.3 ± 1.5	0.408

Table S6. Linear equations for correlations between predicted and actual accumulation. Standard error is shown for all coefficients. Slopes for 1 µg · L^{-1} regressions do not differ from slopes for 100 µg · L^{-1}.

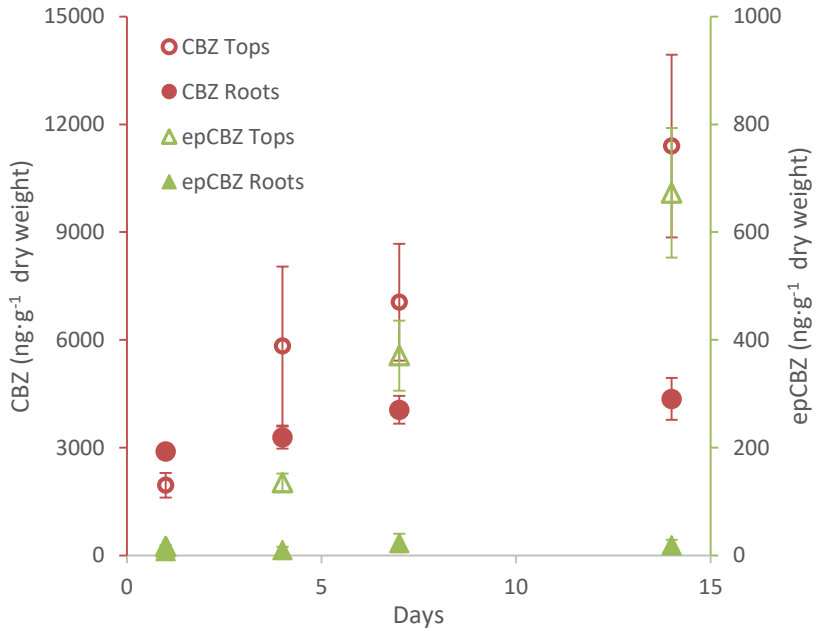

Figure S3. Concentration of carbamazepine (CBZ) and 10,11-epoxycarbamazepine (epCBZ) in spinach roots and leaves during a 14-day exposure period. Error bars show one standard deviation ($n \geq 3$).

CHAPTER FOUR

PLANT ACCUMULATION AND METABOLISM OF CARBAMAZEPINE AND LAMOTRIGINE: AN INTERSPECIES COMPARISON

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ABSTRACT

Pharmaceuticals are commonly detected contaminants in treated wastewater, which may be used to irrigate food crops in arid regions. Approaches to predict plant uptake of pharmaceuticals are necessary because monitoring produce for all potential contaminants is impractical. However, the variation among plant species in the uptake, translocation, and metabolism of pharmaceuticals is not well understood. We grew *Arabidopsis thaliana*, spinach, cucumber, and tomato plants and exposed them to the antiepileptic drugs carbamazepine and lamotrigine alone and in a mixture. We found that accumulation, mixture effects, and metabolism varied among species. Cucumber and tomato accumulated higher concentrations of carbamazepine metabolites and a significantly lower percentage of the initial compound provided was detected in the nutrient solution and plants at the end of the exposure period than for spinach and *A. thaliana*. Water uptake by the plants correlated with carbamazepine and lamotrigine loss from solution across species indicating that the loss of the compounds is likely due to transformation *in planta*. This hypothesis is supported by higher degree of similarity between human drug metabolism enzymes and those in tomato and cucumber than spinach and *A. thaliana*. We further hypothesize that photodegradation of carbamazepine and lamotrigine in plant leaves occurred to a larger extent in the cucumber and tomato plants. Our data demonstrate the importance of considering species differences when investigating plant accumulation and metabolism of pharmaceuticals, and caution that model species may not be representative of important crop species.

A NOTE ON MANUSCRIPT PROGRESS:

Chapter 4 is still a work in progress and is not at the same stage of completeness as the former chapters. Unlike Chapters 2-3, where all of the data was collected at the University of Wisconsin – Madison, Chapter 4 represents a collaborative effort between UW-Madison and the Hebrew University of Jerusalem (HUJI). Experiments were conducted in parallel at the two institutions, with data on two plant species collected at each location (total of four plant species). Efforts were made to ensure experimental and analysis methods were similar between locations, but some method validation work including measurement of extraction recoveries and limits of detection and quantification is still underway at HUJI, where they have experienced some instrument malfunction.

As you will see in the manuscript that follows, there were large differences between the data collected on the plants grown at UW-Madison (spinach and *A. thaliana*) and the plants grown at HUJI (cucumbers and tomatoes), particularly when considering mass balance. There is a large fraction of the initial compound provided that is not detected in plants or nutrient solution at the end of the exposure period for tomatoes and cucumbers. While the manuscript currently provides an explanation for this based on plant metabolism enzymes and photodegradation, it is also possible that the data also reflects problems with the analytical methods used – perhaps a low extraction recovery. We are currently working to determine if this is the case. The method validation results will allow us to assess whether extraction recovery is affecting our results and to determine a correction factor to adjust the data if necessary. Low or variable extraction recoveries would also impact most of the other data presented in the manuscript to some extent, but we expect that the major trends would not be affected.

The currently presented chapter is a second draft of a joint manuscript between the two institutions. While I have attempted to unify the style of the writing and figures, some discrepancies are still there, and there are places where additional discussion of method validation and more comparison to previous literature would improve the text. I intend to continue to work on the manuscript and add in the method validation information when available. A complete version of the manuscript and supplemental information will be submitted to the journal *Environmental Toxicology and Chemistry*.

INTRODUCTION

Pharmaceuticals are frequently detected contaminants in treated wastewater (TWW) because conventional treatment processes do not completely remove them.[1] The release of TWW into the environment has resulted in contamination of freshwater supplies worldwide.[2] Irrigation accounts for 70% of water withdrawals for human benefit and supports roughly 40% of global food production, with increases expected due to population growth and climate change.[3] While surface water and groundwater represent the largest sources of irrigation water, using TWW has become a common practice in arid and semi-arid agricultural regions worldwide such as California and Israel.[4] Thus crop plants are frequently exposed to pharmaceuticals via contaminated irrigation water.

The fate of pharmaceuticals in agricultural environments has been extensively studied and previous research has shown than plants can take up these compounds under field conditions.[5,6] Thus, human consumption of TWW-irrigated crops constitutes an exposure pathway to a variety of pharmaceuticals. While several studies have found that pharmaceutical levels in plants are unlikely to reach levels that are expected to adversely affect humans,[7–10] levels exceeding the threshold for toxicological concern for adults and children under normal consumption levels have been reported for lamotrigine (LTG) in carrots and a metabolite of carbamazepine (CBZ) in carrots, potatoes and eggplant irrigated with treated wastewater.[11,12] The threshold for toxicological concern is a conservative estimate used for compounds present at very low concentrations for which minimal toxicological data are available, and is meant to indicate when additional study of toxic effects is warranted.[13]

For CBZ, measurements of bioaccumulation and distribution within the plant vary dramatically among studies, spanning several orders of magnitude.[5] For example, reports of CBZ

bioconcentration factors (concentration in plant tissue divided by concentration in the growth medium) have ranged from 0.26 to 16 $L \cdot kg_{f.w.}^{-1}$ in lettuce leaves grown with irrigation water containing CBZ,[5,10,14] and reach 24 $L \cdot kg^{-1}$, for the leaves of hydroponically grown cucumber.[15] While LTG received far less study, bioaccumulation and distribution within the plant varies across species and among studies.[12,16–18]

Beyond simple measures of accumulation in plant tissue, a mechanistic understanding of how pharmaceuticals are taken up, translocated, and metabolized within plants is necessary for predicting plant uptake of pharmaceuticals. New drugs are constantly entering the market, and testing all potential TWW contaminants for phytoaccumulation in varying field scenarios is impractical. Predictions of phytoaccumulation of pharmaceuticals based solely on compound properties have had minimal success; mechanistic understanding is necessary to anticipate scenarios in which plant accumulation of pharmaceuticals may result in unsafe human exposure.[5] Compound transformation and the effects of mixtures on plant uptake and metabolism will be important considerations in developing mechanistic models, but these factors are not yet well understood and likely vary between species.

The objectives of this study were to quantify phytoaccumulation of two pharmaceuticals: CBZ and LTG, in four plant species, investigate CBZ and LTG metabolism in each species, and assess whether exposing the plants to a mixture of CBZ and LTG affected accumulation or metabolism of either compound. CBZ and LTG were selected based on the previous literature on their phytoaccumulation and because they have a strong mixture interaction in mammalian systems; CBZ induces the enzyme responsible for metabolizing LTG.[19] Thus these compounds are of interest to study together, as well as individually. Plant species included the genetic model plant *Arabidopsis thaliana* and crop plants spinach, tomato, and cucumber.

MATERIALS AND METHODS

Plant growth and exposure experiments. We grew *Arabidopsis thaliana* and spinach (*Spinacea oleracea*) hydroponically in the University of Wisconsin – Madison and cucumber (C*ucumis sativus*) and tomato (*Solanum lypopersicum*) at the Hebrew University of Jerusalem. *A. thaliana* seeds were sprouted in agar-filled pipette tips, spinach seeds on moist paper towels, and cucumber and tomato in calcium-saturated vermiculite. After sprouting, plants were moved to a hydroponic system with nutrient solution containing necessary macro- and micronutrients. Plants were grown until they were large enough for uptake experiments (2-3 weeks for cucumbers and tomatoes, 5-7 weeks for spinach and *A. thaliana*). Full details on materials used and plant growth methods are provided in the Supporting Information sections S1.1 and S1.2.

During the exposure experiment, nutrient solution pH was adjusted to 7 and plants were placed in polypropylene containers containing spiked solution. *A. thaliana* plants were placed in 1.500 L, spinach in 0.400 L, and tomato and cucumber in 2.200 L. Each replicate of spinach, cucumber, and tomato plants contained one plant. Each replicate of *A. thaliana* contained 10-15 plants grown together in one tub of nutrient solution. Solution was not replenished during the 7 day exposure period. Cucumber and tomato had a photo period of 16 hours and light intensity of 200 $\mu mol_{photons} \cdot m^{-2} \cdot s^{-1}$ and *A. thaliana* and spinach had a photo period of 14 hours with light intensity of 30-80 $\mu mol_{photons} \cdot m^{-2} \cdot s^{-1}$. Temperature and humidity were monitored throughout the exposure period. Nutrient solution mass was measured at the beginning and end of the exposure period to quantify evapotranspiration.

Plants were exposed to 1 or 100 $\mu g \cdot L^{-1}$ of carbamazepine, lamotrigine, individually or together. Control plants were not exposed to either compound. Control solution was placed in plant growth tubs in the growing area and contained compounds but no plants. *A. thaliana* was exposed

at only the higher concentration. *A. thaliana*, cucumber, and tomato treatments were done in triplicate. Spinach treatments had 4 replicates each. Nutrient solution samples were taken from each replicate at the beginning and end of the exposure period.

Plant roots were rinsed with water prior to harvesting. Roots and leaves were harvested separately, frozen, freeze dried, and stored frozen until analysis (–20 °C for cucumber and tomato, –80 °C for *A. thaliana* and spinach). Root and leaf masses were measured before and after freeze drying (wet and dry mass).

Extraction and Quantification of Pharmaceuticals. Freeze dried plant tissues were ground and then extracted using Accelerated Solvent Extraction with 100% methanol. Spinach and *A. thaliana* samples were spiked with mass labeled internal standards prior to extraction. Extracts were evaporated to dryness and reconstituted in a mixture of water an acetonitrile (80:20 for spinach and *A. thaliana* and 70:30 for cucumber and tomato) with 0.1% acetic acid. Mass-labeled internal standards were added to tomato and cucumber during the reconstitution step. Internal standards used were carbamazepine-d_{10}, 10,11 epoxycarbamazepine-d_{10}, and lamotrigine-$^{13}C_3$. Recovery experiments are in progress to allow us to account for the effect that adding internal standards at different times has on the data.

Pharmaceuticals and their transformation products were quantified in plant extracts and nutrient solution samples using an Agilent 1200 HLPC coupled with a 6400 series triple quadrupole mass spectrometer with an ESI source in positive mode. Internal standard-based calibration and measurement was used for plant extracts only. Nutrient solution did not contain internal standards. Compounds quantified included lamotrigine (LTG), carbamazepine (CBZ), 10,11-epoxycarbamazepine, and *trans*-10,11-dihydroxycarbamazepine in all samples and

lamotrigine-*N*-oxide in tomato and cucumber plants. A full description of extraction and analysis methods is available in section S1.3.

RESULTS AND DISCUSSION

Plant Mass and Transpiration Measurements. In general, tomato and cucumber plants were larger than spinach plants with average fresh leaf masses of 77 g, 54 g, and 3 g respectively. *A. thaliana* composite samples (10-15 plants each) had an average fresh leaf mass of 3 g. As larger plants require more water, the larger species transpired a larger volume. The average evapo-transpired water volume was 1780 mL for tomato, 1234 mL for cucumber, 65 mL for spinach, and 51 mL for *A. thaliana*. These differences emphasize the physiological variances of the four plant species. The full data set of plant masses and transpiration volumes is available in Table S5.

Fresh mass and dry mass of roots and leaves did not differ between treatments for spinach, tomato, or cucumber (ANOVA, $p > 0.05$). *A. thaliana* plants were only included in the higher concentration experiments and those exposed only to CBZ had significantly higher shoot mass than other treatments (ANOVA, $p < 0.05$). We attribute the difference to differences in plant growth before the exposure period. Transpiration did not differ among treatments within each crop (ANOVA, $p > 0.05$).

Accumulation of CBZ and LTG in Plants. Concentrations of CBZ and LTG in roots and leaves varied among species (Figure 1). For both exposure concentrations, CBZ has a higher concentration than LTG in leaves for all species, and tomato has a very high concentration of LTG in roots. Cucumber has lower accumulation of CBZ and LTG in roots and CBZ in leaves than the other species.

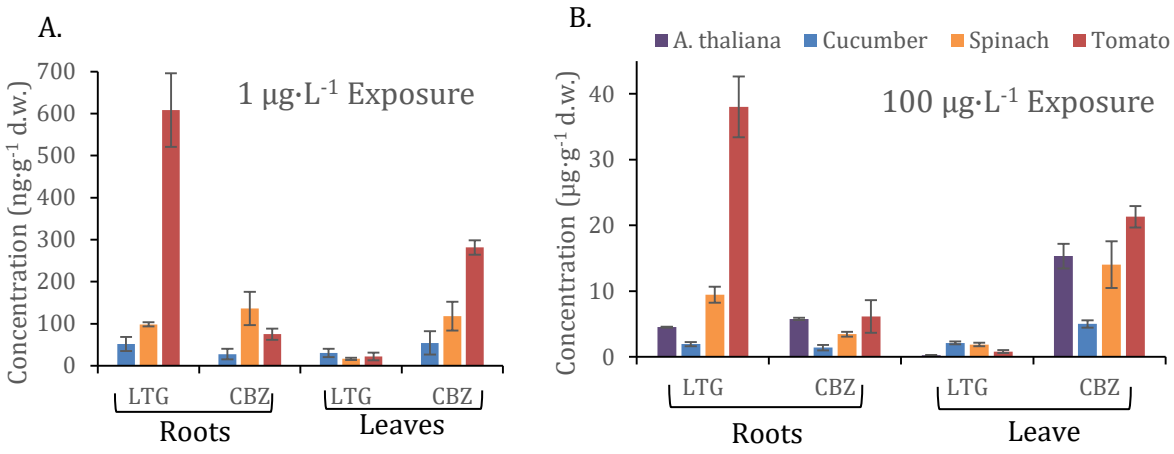

Figure 1. Measured accumulation of carbamazepine (CBZ) and lamotrigine (LTG) in roots and leaves of tested plant species for single compound exposures to (A) 1 µg·L^{-1} and (B) 100 µg·L^{-1}. Error bars show one standard deviation ($n \geq 3$).

We also analyzed the mass distribution of CBZ and LTG between the roots and leaves of each species. For CBZ, there were no significant differences between species or exposure concentrations, with ~90% of the CBZ mass accumulated in the leaves of each plant. Distribution of LTG varied significantly among species (Figure 2, ANOVA, $p < 0.05$). The relative similarity in CBZ distribution between plants and the variation in LTG distribution points to differences in how CBZ and LTG are transported through the plants. The data supports the idea that CBZ follows a similar uptake pathway in each of the tested species. Previous literature has theorized that CBZ is translocated with the transpiration stream resulting in accumulation in the leaves with lower concentrations in the plant roots.[11] CBZ is a neutral molecule at all pH values found inside the plant, while a fraction of LTG ($pK_a = 5.7$)[11] is positively charged at typical apoplast and root cell vacuole pH values (pH ~ 5.5).[20] Small differences in pH between species would affect LTG speciation and thus its ability to cross membranes and move through the plant. Differences in the pH of root apolplast fluid, cytoplasm, and vacuole contents among species and how they affect the movement of LTG and other charged is a topic that warrants additional investigation.

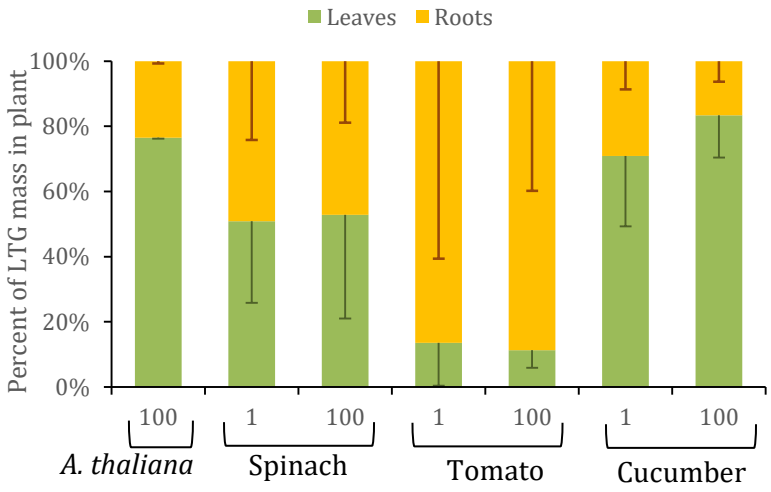

Figure 2. Mass distribution of lamotrigine (LTG) between leaves and roots of the plants. Distribution within the plant varied between species. Numbers on the x-axis represent exposure concentrations of 1 $\mu g \cdot L^{-1}$ and 100 $\mu g \cdot L^{-1}$. Error bars show one standard deviation ($n \geq 3$).

Mixture Effects. Accumulation of CBZ was not affected by the presence of LTG in any of the tested plant species. Additionally, there were no significant differences in LTG concentrations in the roots or leaves of *A. thaliana*, tomato or spinach plants that were also exposed to CBZ. However, for cucumber, exposure to LTG in the presence of CBZ resulted in significantly higher leaf concentrations of LTG in the 100 $\mu g \cdot L^{-1}$ treatment. Leaves exposed to LTG alone contained 2.1 ± 0.2 $\mu g \cdot g_{d.w.}^{-1}$ LTG while leaves exposed to both compounds contained 4.2 ± 0.5 $\mu g \cdot g_{d.w.}^{-1}$ LTG. There were no differences in initial LTG concentration in the nutrient solution, but the concentration of LTG in the nutrient solution at the end of the exposure period was lower for the dual exposure treatment, indicating a difference in LTG uptake from solution as well as accumulation in the plant. Overall, the data suggest that mixture effects on plant uptake of contaminants may be species specific, and that results based on one species may be not be sufficient to make assumptions about the fate of compounds and their interactions in other plants. A full dataset on concentrations in plant leaves and roots for single and dual exposure treatments is provided in Tables S6 and S7.

Metabolite Accumulation. The carbamazepine metabolites 10,11-epoxycarbamazepine (EP-CBZ) and 10,11-dihydro-10,11-trans-dihydroxycarbamazepine (DiOH-CBZ) were quantified in the roots and shoots of each species. Metabolites were quantified in the leaves of all crops (Figure 3). Concentrations of metabolites did not differ between plants exposed to CBZ only and CBZ and LTG together (*t*-tests, $p > 0.05$); hence, the data shown represent all plants together. The parent compound was dominant in all species, comprising over 97% of total carbamazepine in the *A. thaliana* and spinach plants with no significant differences between exposure concentrations. In cucumber plants, the parent compound comprised 80-90% of the total carbamazepine in the plant, also with no significant differences between exposure concentrations. In tomato plants, the 1 $\mu g \cdot L^{-1}$ exposed plants had a significantly lower percentage of the parent compound than the 100 $\mu g \cdot L^{-1}$ exposed plants, with ranges of 82-85% CBZ and 88-92% CBZ respectively. In all species, EP-CBZ was the dominant metabolite detected; while the DIOH-CBZ was found in leaves of all plants at the higher exposure concentrations, it accounted for less than 1% of the total in all samples. For all crops, accumulation of measured CBZ metabolites was minimal in roots.

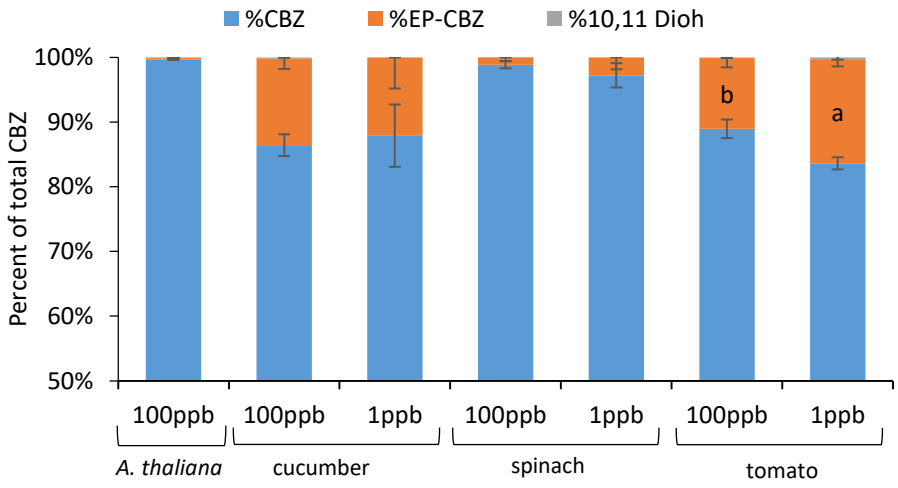

Figure 3. Distribution of carbamazepine and metabolites, 10,11-epoxycarbamazepine (EP-CBZ) and 10,11-dihydroxycarbamazepine (10,11 Dioh), in the leaves of different crops. Metabolite accumulation was very low in *A. thaliana* and spinach. More metabolites accumulated in the 1 $\mu g \cdot L^{-1}$ tomato exposed leaves than the 100 $\mu g \cdot L^{-1}$ exposed leaves. Note that the y-axis begins at 50%. Error bars represent one standard deviation ($n \geq 3$).

A LTG metabolite, lamotrigine *N*-oxide, was measured in the cucumber and tomato samples and was detected in tomato roots that were exposed to 100 $\mu g \cdot L^{-1}$ LTG. The concentration ranged from 10-50 $ng \cdot g^{-1}$ and accounted for less than 1% of the total LTG in tomato roots. Lamotrigine *N*-oxide is very reactive, so the measured concentration may not be representative of the amount formed in the plants. Our data provides preliminary evidence that LTG undergoes metabolism in plant roots.

Mass Balance. Figure 4 shows the distribution of LTG and CBZ within the plant growth set ups for each species and exposure concentration. For tomato, and cucumber, >50% of LTG was not detected in plants or nutrient solution (was "missing") at the end of the exposure period, indicating significant degradation of LTG within the system. For tomato, LTG mass distribution in the low and high exposure treatments did not differ ($p > 0.05$). However, mass distribution differed between exposure concentrations for cucumber plants. At the end of the exposure period, no LTG was detected in the solution containing 1 $\mu g \cdot L^{-1}$, but some LTG remained in the 100 $\mu g \cdot L^{-1}$ solution. This supports the hypothesis that the mechanisms for uptake and degradation of LTG cannot work as efficiently at the higher concentration, perhaps because the plant is not able to produce enough of the relevant transport and/or metabolism enzymes. For the 100 $\mu g \cdot L^{-1}$ exposed cucumber plants, the presence of CBZ affected LTG mass distribution. There was significantly more LTG "missing" and less LTG in solution for the cucumber plants exposed to both compounds than the plants exposed to LTG alone. We also found a higher leaf concentration of LTG in the

plants that were also exposed to CBZ. Together, this data indicates that more LTG is taken up into the cucumber plants and metabolized when CBZ is also present.

We also see significant CBZ loss from the plant growth system for tomato, cucumber, and spinach. For spinach, we only see loss in the low exposure treatment, indicating that the high CBZ exposure may inhibit its own degradation. For cucumber, we see the opposite trend, with more CBZ missing and less in solution in the higher exposure plants, indicating that the high concentration of CBZ may induce its own uptake and metabolism. CBZ loss from the tomato system is not affected by exposure concentration. In mammalian systems CBZ induces the enzymes responsible for metabolizing many pharmaceuticals, including itself and LTG.[21,22] It is possible that CBZ also affects metabolism enzyme activity in plants in a concentration dependent manner.

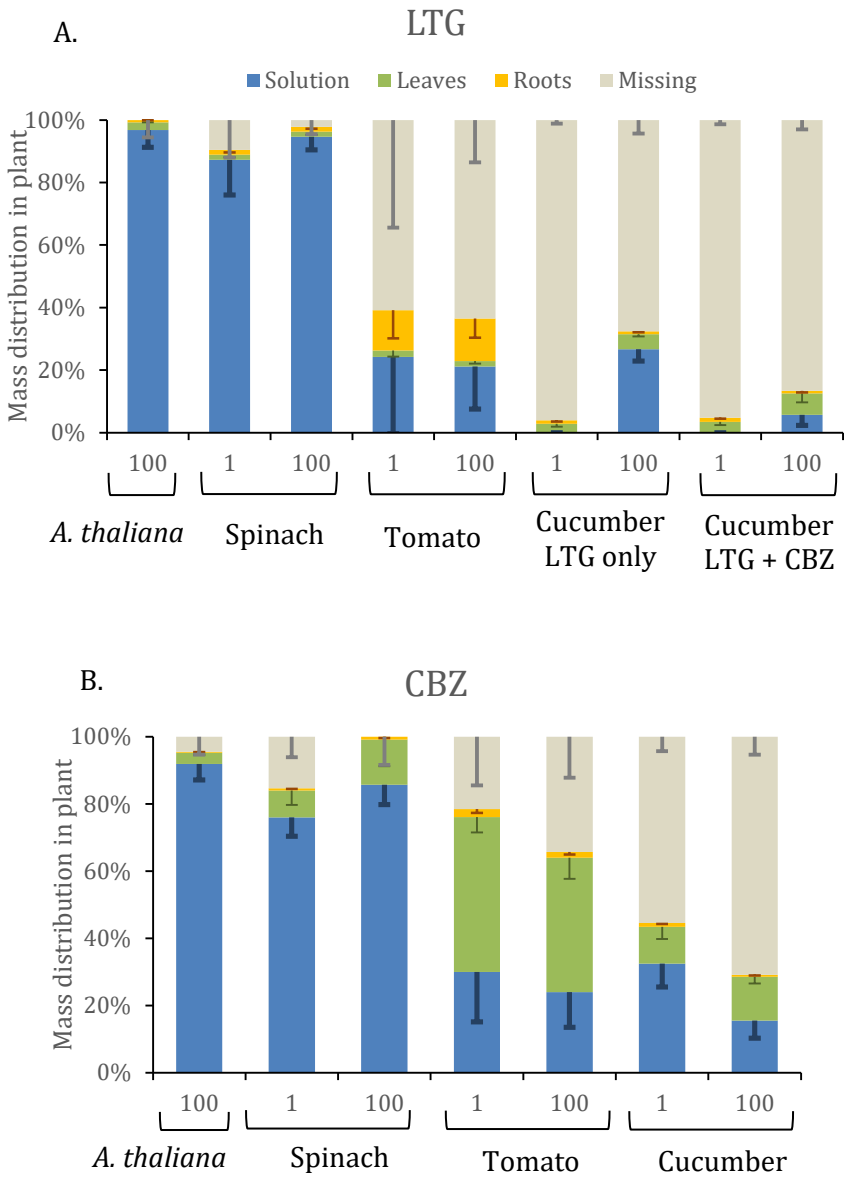

Figure 4. Mass distribution of (A) lamotrigine (LTG) and (B) carbamazepine (CBZ) at the end of the 7 day exposure period. Fraction denoted "missing" was not detected in plants or nutrient solution. Numbers on the x-axis represent exposure concentrations of 1 μg·L^{-1} and 100 μg·L^{-1}. Mixture and single compound exposure data are combined unless otherwise noted. Error bars show one standard deviation ($n \geq 3$).

For *A. thaliana*, all LTG or CBZ added to the initial nutrient solution is accounted for at the end of the exposure period. For spinach, there is no significant loss of LTG for the low or high exposure concentrations. These data tentatively indicates that minimal metabolism is taking place in these treatments. However, the possibility of *in planta* metabolism cannot be eliminated, as the variation in solution measurements is generally high relative to the amount of LTG and CBZ found in the plants.

For *A. thaliana* and spinach, a much larger percentage of LTG and CBZ are found in solution than for tomato and cucumber. This is partially due to variability in transpiration. Tomato and cucumber took up a larger percentage of their initial nutrient solution than spinach or *A. thaliana*. We plotted the percent of each compound removed from solution against the percent of nutrient solution transpired (Figures 5a and 5b). We found significant correlation for both CBZ and LTG (R^2 = 0.78 and 0.76, respectively) indicating that much of the variability in loss from solution is driven by transpiration. It logically follows that plant uptake is responsible for much of the compound loss from solution. Therefore, we hypothesize that most transformation of LTG and CBZ occurs *in planta*. If this is the case, measured amounts of accumulation are likely not representative of the amount taken up by the plant.

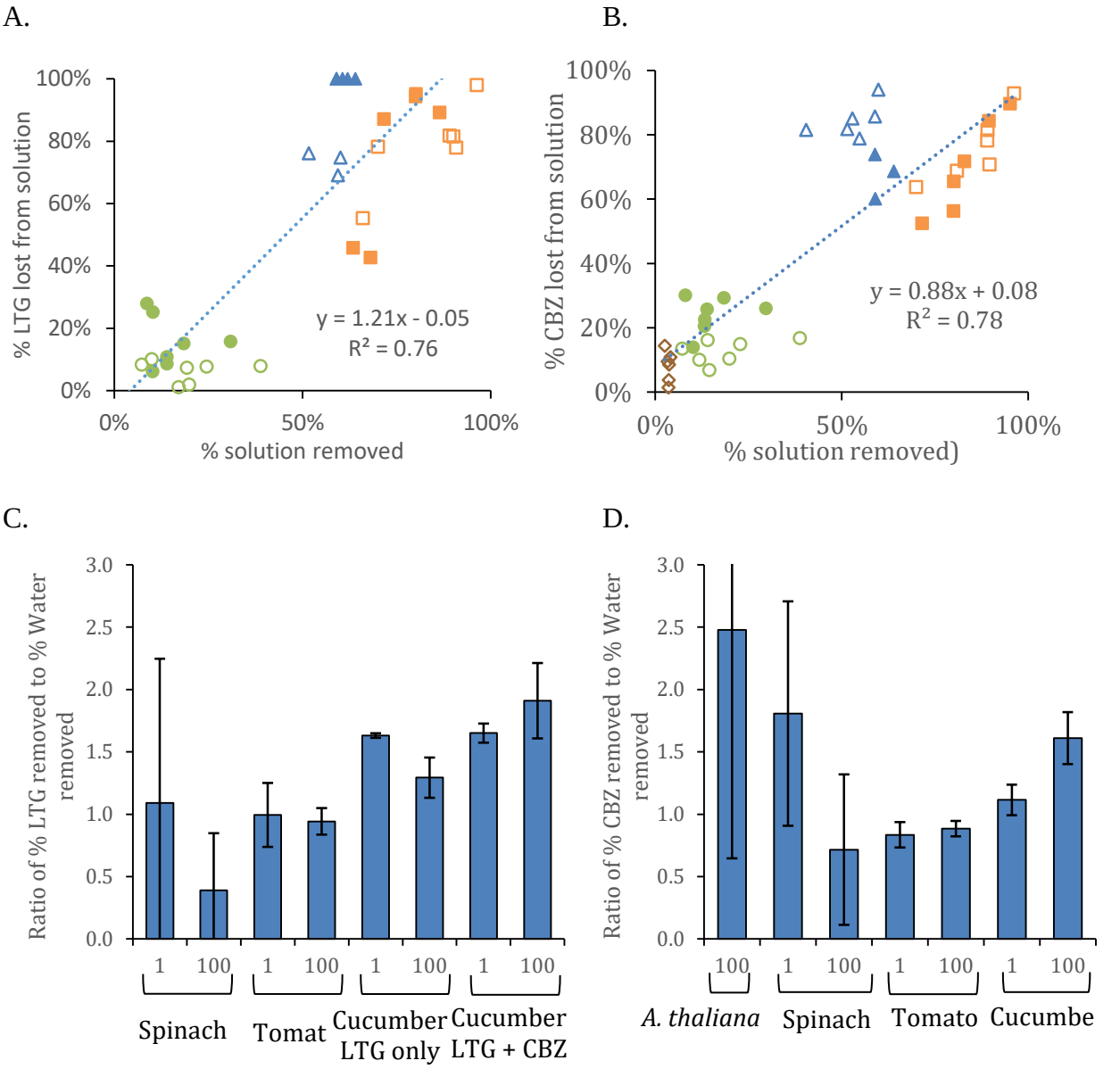

Figure 5. The top panels show correlation between the percent of (A) lamotrigine (LTG) and (B) carbamazepine (CBZ) removed from solution by the end of the exposure period (includes fraction found in plant and "missing" fraction) and the percent of solution taken up by the plant. Orange squares represent tomatoes, blue triangles represent cucumbers, green circles represent spinach, and brown diamonds represent *A. thaliana*. Solid shapes represent the 1 $\mu g \cdot L^{-1}$ exposed plants and open shapes represent 100 $\mu g \cdot L^{-1}$ exposed plants. Each point represents one replicate. Bottom panels show the average ratio of (C) LTG and (D) CBZ removed from solution to solution taken up. Error bars represent 1 standard deviation ($n \geq 3$). *A. thaliana* is not included in the LTG analysis (A. and C.) because there was no measurable loss from solution.

Figures 5c and 5d show the average ratio of compound removal to water removal for each species. For spinach, variability in the data is very high, indicating that transpiration is not responsible for differences in LTG and CBZ loss between replicates. For tomatoes exposed to LTG, the ratio is very close to one, indicating that LTG is lost from solution at the same rate as water. For tomatoes exposed to CBZ, the ratio is less than one, indicating that CBZ uptake into the plant is limited in some way. No differences exist between exposure concentrations for tomatoes exposed to LTG or CBZ. For cucumber, more LTG is lost from solution than water indicating that transpiration is not the sole driver of LTG loss from solution. LTG may also be degraded in solution, sorbed to root tissue, or actively taken up into the plant. For CBZ, the exposure uptake ratio is close to 1 for the low exposure, but the high exposure ratio for the 100 $\mu g \cdot L^{-1}$ indicates an additional driver for CBZ loss from solution.

Comparison of Plant Metabolism Enzymes. Based on Figure 4, we see that much of the CBZ and LTG provided to the plants is degraded *in planta* or in the nutrient solution, and based on Figure 5, we hypothesize that most degradation takes place *in planta*. In humans, CBZ can be metabolized by several cytochrome P450 (CYP450) enzymes, while LTG is metabolized by uridine 5'-diphospho-glucuronosyltransferases (UGTs). We investigated similarities between the human metabolism enzymes that interact with CBZ and LTG and proteins found in *A. thaliana*, spinach, tomato, and cucumber using standard protein BLAST searches using the NCBI database.[23]

In humans, 11 CYP450 enzymes are responsible for most phase 1 drug metabolism. The main metabolism pathway for carbamazepine is formation of EP-CBZ via CYP3A4, though this metabolite is also formed via CYP3A5, CYP2C8, CYP2C19, and CYP3A7.[24] Additionally CBZ can be metabolized to 3-hydroxyCBZ via CYP3A4, CYP2B6, or CYP3A7.[24] CBZ also induces

activity in multiple CYP450 enzymes including CYP1A2, CYP2C9, CYP2C19, CYP3A4, and CYP3A5, to the extent that doses of other drugs must be adjusted in patients who also take CBZ.[21] We hypothesize that CBZ is metabolized by CYP450 enzymes in plants, as the human metabolism products have also been found in plants.[11,18,25]

We searched for proteins in each plant species that corresponded to each of the 11 human CYP450 metabolism enzymes. We recorded all plant proteins with max scores 200 or higher, and homologues of those proteins in other species that had match scores of 160 or higher. A max score of 200 or higher indicates high similarity between proteins, while scores between 80 and 200 indicate proteins from the same family that may not have similar functions. Scores are based on the amount of matches in the amino acid sequence of a protein and the length of the sequence where overlap is found. Results are summarized in Table 1.

Tomato has seven matches with score exceeding 200, while cucumber has three, *A. thaliana* has two, and spinach has zero. Many of the scores above 200 (and all for *A. thaliana*) are for alignment with human CYP1A1, which does not interact with CBZ, but some plant proteins that align with human CYP1A1 also match highly with other human enzymes. Cucumber has one protein with a score higher than 200 for alignment with human CYP3A4, which is the main enzyme for CBZ conversion to EP-CBZ. This predicted CYP450 711A1-like protein is also found in spinach and tomato, though with lower match scores. We hypothesize that this protein may be responsible for CBZ metabolism in these species, as cucumber has both the highest match score and the most CBZ metabolism. We note that we see higher degradation of both CBZ and LTG in cucumber and tomato than in spinach and *A. thaliana* and also higher alignment with human CYP450s for cucumber and tomato than for *A. thaliana* and spinach. The plant proteins identified in this analysis may be good targets for additional research on plant metabolism of xenobiotic

compounds. Additionally, these results indicate that although *A. thaliana* is a valuable genetic resource for plant biology, it may not be a good model organism for investigating drug metabolism enzymes in crop plants.

Plant Protein	Human CYP	Species				CBZ
		A. thaliana	Spinach	Tomato	Cucumber	
CYP1A1	CYP1A1			321		X
	CYP1A2			289		Ind
predicted CYP450 736A12-like	CYP1A1			198	214	X
	CYP1A2			199	199	Ind
CYP450 75B1	CYP1A1	212				X
	CYP1A2	199				Ind
CYP450 81F2	CYP1A1	210				X
	CYP1A2	191				Ind
predicted CYP450 93A2-like	CYP1A1			206	208	X
	CYP1A2			192	197	Ind
CYP450 703A2	CYP1A1	185	196	208	187	X
	CYP1A2	192	184	199	179	Ind
putative flavenoid 3'5' hydroxylase	CYP1A1			207		X
	CYP1A2			194		Ind
predicted CYP450 71A1-like	CYP1A1		176	206	197	X
	CYP2B6			164	161	S
	CYP2C8			179	160	S, Ind
	CYP2C9			176	176	Ind
	CYP2C19			171	172	S, Ind
	CYP2E1			179	168	X
predicted CYP450 83B1-like	CYP1A1	180	167	203		X
	CYP2B6	160		169		Ind
predicted CYP450 711A1-like	CYP3A4	179	196	196	201	S, Ind
	CYP3A5	177	191	185	185	S, Ind
	CYP3A7	171	174	177	178	S

Table 1. Max scores for plant protein alignment with human drug metabolizing CYP450 enzymes found using NCBI BLAST searches. In the CBZ column, S indicates that CBZ is a substrate of the human CYP450, Ind indicates that CBZ is an inducer of the human CYP450, and X indicates no CBZ interaction with the human CYP450. Scores are color coded with darker colors indicating higher scores. Enzyme list was determined by finding all matches to human enzymes with scores above 200, and recording additional homologues with scores above 160.

In humans, LTG is metabolized by UGT enzymes – primarily UGT 1A4, which is also induced by CBZ.[19] We compared this human protein to proteins in the studied plant species using NCBI BLAST searches,[23] and found no closely aligned proteins. Max scores were 89 for spinach, 83.2 for tomato, 73.9 for cucumber, and 70.1 for *A. thaliana*, indicating low similarity. Additional types of comparison such as focusing on the active site of metabolism enzymes may be necessary for determining the LTG metabolism pathway in plants. Metabolism of LTG in plants may differ significantly from metabolism in humans.

Environmental Implications. Carbamazepine and LTG exhibited large differences in accumulation and metabolism among the plant species investigated. We identified species differences in measured compound and metabolite accumulation, LTG distribution within the plant, response to a CBZ and LTG mixture, and the amount of compound transformed during our exposure period. Thus, it is very important to account for species differences when investigating phytoaccumulation of pharmaceuticals on a larger scale, and when estimating potential human exposure to contaminants through consumption of exposed crop plants. Additionally, pharmaceutical metabolism mechanisms and mixture effects across species are topics that warrant future investigation. The large differences between species in our study shows that the use of a model plant such as *A. thaliana* may not provide representative data.

For this publication, spinach and *A. thaliana* experiments were conducted at the University of Wisconsin – Madison, while cucumber and tomato experiments were conducted at the Hebrew University of Jerusalem. Although experimental design, plant growth set up, and analysis methods were similar between the locations, differences may have impacted results in ways that can provide additional hypotheses about the differences in our data. Nutrient solution composition varied between species. Differing ratios of ammonium to nitrate can alter plant accumulation of LTG due

to plant-driven pH changes in the area directly surrounding the roots (see Chapter 2). Though plants for the current study were grown hydroponically and the roots were surrounded with circulating nutrient solution rather than soil, plant-driven pH changes may still have had an effect, as plants may be able to change the rhizosphere pH on a faster time scale than circulation, or produce a strong enough change that the whole container of solution is affected via circulation and diffusion. Additionally, different ionic strength may affect charge-driven interactions between pharmaceuticals and plant roots. Differences in the volume of nutrient solution provided may have also caused differences in uptake and quantification of compound transformation.

Cucumbers and tomatoes were exposed to higher intensity light for a longer period during each day of exposure than were spinach and *A. thaliana*. Though plant growth containers blocked light from reaching the nutrient solution, photodegradation of pharmaceuticals in plant leaves has been hypothesized to occur,[5] and *in planta* photolysis of 2,4-dinitroanisole has been demonstrated.[26] Phototransformation pathways have been determined for both LTG and CBZ.[27,28] Photodegradation results in different transformation products than are formed via metabolic processes, so the transformation products measured in this study do not provide insight to this process. Photodegradation may have been responsible for some of the compound loss observed in our study.

Comparison of plant uptake results across labs and between studies can be facilitated by thorough reporting of methods details that may affect results. Miller et. al., 2016 provides a suggested minimum data set for plant uptake studies that includes general variables such as plant variety and age, solution pH and volume, and limits of detection and quantification.[5] We recommend several additional methods details also be included in all future studies, including light intensity, nutrient solution composition, and parameters for sample storage.

Differences in species and environment can cause substantial variation in plant accumulation and metabolism of pharmaceuticals. Though our data provides some information, there are still many factors to investigate regarding this complex environmental issue. We demonstrated that species differences are important to consider during continuing investigation of pharmaceutical accumulation and metabolism in plants.

ASSOCIATED CONTENT

Supporting information (SI) is available. SI contains additional materials, methods, and results as noted.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL INFORMATION FOR CHAPTER FOUR

PLANT ACCUMULATION AND METABOLISM OF CARBAMAZEPINE AND LAMOTRIGINE: AN INTERSPECIES COMPARISON

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S1. SUPPLEMENTAL METHODS

S1.1. Chemical sources

UW-Madison: Carbamazepine (99% purity) was obtained from ACRŌS Organics. Lamotrigine (98% purity) was obtained from Comb and Blocks. All mass labeled internal standards were obtained from CDN isotopes. Nutrient solution salts were obtained from a variety of sources. We obtained KNO_3 from Fisher, $CaNO_3$ from ACRŌS Organics, KH_2PO_4 from Alfa Aesar, $MgSO_4$ and KOH from dot Scientific, and Murashige and Skoog micronutrient solution from Caisson Laboratories. Ultra-pure water ($\geq$ 18 MΩ·cm) was obtained from a Thermo Scientific GenPure Pro system.

HUJI: (not yet compiled)

S1.2. Plant growth methods

Parameter	*Arabidopsis thaliana*	Spinach	Cucumber	Tomato
Location	UW-Madison	UW-Madison	HUJI	HUJI
variety	*Columbiana*	Tyee Hybrid	Patriot	Vilmorin variety V.409
Source of seeds	Lehle Seeds	Jung Seed	Hazera genetics	Hazera genetics
Seed sterilization	Spread on filter paper, sprayed with 70% ethanol	Soaked in 70% ethanol (60 s) then rinsed 3x then soaked in 4 % sodium hypochlorite (20 min) then rinsed 5x	none	none
Method for sprouting seeds and transferring to hydroponic solution	Planted in agar filled 200 μL pipette tips[17] and grown in tip box for 18-24 days before transfer to solution. Ends of tips cut off and submerged in solution	Placed between folds of wet paper towel in sealed plastic bag for 3 days. Spouts transferred to pipette tips with ends cut off to support the plant with the root	Placed seeds in calcium saturated vermiculite, after 3 days in darkness seeds were moved to the growing chamber. After 4-5 days in the growing	Placed seeds in calcium saturated vermiculite, after 3 days in darkness seeds were moved to the growing chamber. After 4-5 days in the growing

		sprout submerged	chamber seedlings were transferred to the hydroponic system.	chamber seedlings were transferred to the hydroponic system.
Nutrient solution composition	2.375 mM K^+, 0.5 mM Ca^{2+}, 0.5 mM Mg^{2+}, 6.25 μM NH_4^+, 6.25 μM Cl^-, 1.75 mM NO_3^-, 0.5 mM SO_4^-, 0.625 mM $H_2PO_4^-$, 5.7 mL/L Murashige and Skoog micronutrient solution[18]	7.6 mM K^+, 4 mM Ca^{2+}, 2 mM Mg^{2+}, 0.5 mM NH_4^+, 14 mM NO_3^-, 2 mM SO_4^-, 0.5 mM $H_2PO_4^-$, 10 mL/L Murashige and Skoog micronutrient solution	1.6 mM K^+, 2 mM Ca^{2+}, 0.5 mM Mg^{2+}, 4 mM NO_3, mM SO_4^-, 1.2 mM H_2PO_4, 10 μM H_3BO_3, 0.5 μM Mn, 0.5 μM Zn, 0.2 μM Cu, 0.07 μM Mo, 50 μM Fe.	1.6 mM K^+, 2 mM Ca^{2+}, 0.5 mM Mg^{2+}, 4 mM NO_3, mM SO_4^-, 1.2 mM H_2PO_4, 10 μM H_3BO_3, 0.5 μM Mn, 0.5 μM Zn, 0.2 μM Cu, 0.07 μM Mo, 50 μM Fe.
Nutrient solution pH during exposure	7	7	7	7
Growth time in hydroponic solution pre-exposure	5 weeks	47 days	15 days	16 days
Exposure solution initial volume	1.500 L	400. mL	2200 mL	2200 mL
photo period	14 hr	14 hr	16 hr	16 hr
Light intensity	40-80 μmol·m^{-2}·s^{-1}	30-40 μmol·m^{-2}·s^{-1}	200 μmol·m^{-2}·s^-	200 μmol·m^{-2}·s^-
Spiking solution used	40 μL DMSO spike added per tub	40 μL DMSO spike added per tub	10 liter nutrient solution was spiked using 2000 mg/L stock solution in MeOH	10 liter nutrient solution was spiked using 2000 mg/L stock solution in MeOH
Container material	polypropylene	polypropylene	polypropylene	polypropylene
Growth area description	In plant tent with reflective walls in lab with fluorescent lights. Plant tubs in plastic trays with clear plastic domes over plants.	In plant tent with reflective walls in lab with fluorescent lights. Tubs with plants directly on shelves under lights.	Growing room with automatic air temperature control, air circulation, humidity control and lighting. The plants were placed randomly	Growing room with automatic air temperature control, air circulation, humidity control and lighting. The plants were placed randomly

			in individual containers on a shelf under lights.	in individual containers on a shelf under lights.
Temperature and humidity during exposure	~20 °C night, ~26 °C day, relative humidity ~60% night, ~40% day, both measured every 10 s. for first 48 hr of exposure. Sensor placed under plastic dome with plants.	~22 °C night, ~28 °C day, variable relative humidity, 10-27%, both measured every 30 min for exposure duration. Sensor placed on shelf between plants.	24 °C night, ~28 °C day, humidity 40-80% not measured continuously	24 °C night, ~28 °C day, humidity 40-80% not measured continuously

Plant Harvesting

UW-Madison: Plant roots were rinsed with ultra-pure water prior to harvest. Roots and leaves were harvested into separate containers. Plants were frozen at -80 °C, freeze dried, then stored at -80 °C until extraction. Root and leaf mass was measured before and after freeze drying (wet and dry mass). Nutrient solution mass was measured at the beginning and end of the exposure period.

HUJI: Plant roots were rinsed with distilled water prior to harvest. Roots, leaves and stems were harvested into separate plastic bags. Plants were frozen at -20 °C, freeze dried, then stored at -20 °C until extraction. Root, leaf and stem mass was measured at harvest and after freeze drying (wet and dry mass). Nutrient solution mass was measured at the beginning and end of the exposure period.

S.1.3. Extraction and detection of pharmaceuticals and metabolites

Extraction

UW-Madison: Freeze dried plant tissues were ground in a mortar and pestle, then extracted using Accelerated Solvent Extraction (ASE) with 100% methanol. Each ASE cell contained a glass fiber filter and 1.0 g fluorosil at the bottom, over which was placed the tissue sample followed by another 1.0 g fluorosil. We used an ASE 200 with 11 mL stainless steel cells. Samples were spiked with mass labeled internal standards and sat at room temperature overnight prior to extraction. Internal standards used were carbamazepine-d_{10}, 10,11 epoxycarbamazepine-d_{10}, and lamotrigine-$^{13}C_3$. The ASE cycle (completed twice per sample) included a 5 min preheat, 5 min heat, 5 min static extraction, 60% volume flush, and 120 s purge with a pressure of 1500 psi and temperature of 80 °C. Extracts were evaporated to dryness then reconstituted in 80% ultrapure water, 20% acetonitrile, 0.1% acetic acid. All samples were sonicated 10 minutes in reconstitution solvent, centrifuged 20 min. at 17000 g, and filtered through 0.2 μm PTFE filters. Samples were stored at -80 °C until analysis.

HUJI: Freeze dried plant tissues were ground using a "magic bullet" blender and extracted using an accelerated solvent extractor (ASE350, Dionex, Sunnyvale, CA) with 100% methanol. The ASE procedure was identical to method described above at the UW-Madison lab. All extracts were evaporated to dryness and re-dissolved in 990 μL 30% acetonitrile, 70% distilled water and 0.1% acetic acid, spiked with 10 μL of a mixture of stable isotope labeled internal standards in acetonitrile, sonicated (37 kHz, 10 min), centrifuged at 17 000g for 20 min, and filtered (0.22 μm PTFE) prior to LC-MS analysis. Internal standards used were carbamazepine-d_{10}, 10,11 epoxycarbamazepine-d_{10}, lamotrigine-$^{13}C_3$ and Lamotrigine N2-Oxide-$^{13}C_3$.

LC-MS/MS Analysis.

UW-Madison: We measured lamotrigine (LTG), carbamazepine (CBZ), and the CBZ metabolites 10,11-epoxycarbamazepine and 10,11-trans-dihydroxycarbamazepine in plant extracts and nutrient solution samples by LC-MS/MS (Agilent 1260 HPLC, Agilent 6460 triple quadrupole mass spectrometer, ESI+ source). We used a Waters Xterra MS C18 3.5 µm 2.1 × 100 mm column. Mobile phases were 100% acetonitrile (organic phase) and 0.1% formic acid in 10% acetonitrile (aqueous phase) with a gradient of 5% to 95% organic phase. Internal standard-based calibration and measurement was used for plant extracts only. Nutrient solution did not contain internal standards. Column temperature was held at 30° C.

HUJI: Quantitative analysis of pharmaceutical compounds was accomplished using LC-MS/MS system which consisted of 1200 Rapid Resolution LC system coupled to 6410 triple quadrupole mass selective detector (all Agilent Technologies Inc.). Compounds were separated on Acclaim C18 RSLC column (2.1×150 mm, particle size 2.2 µm, Thermo) upon following HPLC conditions:

Time, min	Solvent A,% Water with 1.5% AcOH	Solvent B, % Acetonitrile
0	90	10
1.5	90	10
17	4	96
21	4	96
21.1	90	10
26	90	10
Other Parameters		
Temperature of column, °C		40
Volume of injection, µl		5
Flow rate, ml/min		0.3

Table S.1.1. Mass-to-charge ratios (m/z) for mass spectral measurements (UW-Madison)

Compound	Precursor Ion (m/z)	Quantitative Ion (m/z)	Qualitative Ion(s) (m/z)
lamotrigine	256	43.1	108.9, 58.1
lamotrigine-$^{13}C_3$	259	44.1	59.1
carbamazepine	237.1	194.1	165
carbamazepine-d_{10}	247.2	204.1	173.1
10,11-epoxycarbamazepine	253.1	180.1	210.1
10,11-epoxycarbamazepine-d_{10}	263.3	188.1	220.1
trans 10,11-dihydroxycarbamazepine	271	180.1	210.1

Table S.1.2. Mass-to-charge ratios (m/z) for mass spectral measurements (HUJI)

Compound	Precursor Ion (m/z)	Quantitative Ion (m/z)	Qualitative Ion (m/z)
carbamazepine	237	194	179
carbamazepine $d_2{}^{13}C$	240	197	181
carbamazepine-10,11-epoxide	253	236	180
carbamazepine-10,11-epoxide d_8	261	244	218
cis-10,11-dihydro-10,11- dihydroxy carbamazepine	271	210	180
trans-10,11-dihydro-10,11-dihydroxy carbamazepine	271	210	180
3-hydoxy carbamazepine	253	210	208
2-hydoxy carbamazepine	253	210	208
10,11-dihydro-10-hydroxy carbamazepine	255	237	194
10,11-dihydro-10-hydroxy carbamazepine d_3	258	240	197
lamotrigine	256	211	145
lamotrigine $^{13}C_3$	259	214	145
lamotrigine N-oxide	272	242	185

Extraction Recoveries

Table S.2. Internal standard based recovery for spinach and *A. thaliana*. Root samples contained 400 ng·g^{-1} internal standard and leaf samples contained 100 ng·g^{-1} internal standard (added before extraction). Concentrations reported in this manuscript are recovery corrected based on this table.

Compound	Matrix	Spike level	Recovery	stdev
LTG	spinach roots	80 ng·g^{-1}	0.97	0.04
		10 µg·g^{-1}	1.256	0.006
	spinach leaves	20 ng·g^{-1}	1.3	0.2
		2 µg·g^{-1}	1.19	0.07
	A. thaliana roots	5 µg·g^{-1}	1.13	0.05
	A. thaliana leaves	0.3 µg·g^{-1}	1.15	0.07
CBZ	spinach roots	40 ng·g^{-1}	0.91	0.02
		5 µg·g^{-1}	1.52	0.04
	spinach leaves	125 ng·g^{-1}	1.051	0.006
		15 µg·g^{-1}	1.37	0.09
	A. thaliana roots	6 µg·g^{-1}	1.13	0.02
	A. thaliana leaves	15 µg·g^{-1}	1.17	0.02
epCBZ	spinach roots	0.4 ng·g^{-1}	1.4	0.6
		20 ng·g^{-1}	1.2	0.1
	spinach leaves	3 ng·g^{-1}	0.70	0.03
		150 ng·g^{-1}	0.60	0.07
	A. thaliana roots	5 ng·g^{-1}	1.4	0.1
	A. thaliana leaves	45 ng·g^{-1}	1.057	0.007
hydCBZ	spinach leaves	10 ng·g^{-1}	1.1	0.1
	A. thaliana roots	4 ng·g^{-1}	0.95	0.04
	A. thaliana leaves	15 ng·g^{-1}	0.67	0.02

Recovery experiments are still in progress at HUJI.

Limits of Detection and Quantification

Table S.3. Limits of detection (LOD) and quantification (LOQ) were determined by running a low concentration sample 7 times in a row and taking the standard deviation of the measurements (σ). LOD and LOQ was calculated using the equations:

$LOD = 3\sigma$ (S2) $LOQ = 10\sigma$ (S3)

Compound	Matrix	LOD ($ng \cdot g^{-1}$)	LOQ ($ng \cdot g^{-1}$)
LTG	spinach roots	7	23
	spinach leaves	6	19
	A. thaliana roots	5	15
	A. thaliana leaves		
CBZ	spinach roots	2	5
	spinach leaves	3	9
	A. thaliana roots	4	14
	A. thaliana leaves	3	9
epCBZ	spinach roots	0.6	2.1
	spinach leaves		
	A. thaliana roots		
	A. thaliana leaves	0.6	0.6
hydCBZ	spinach roots		
	spinach leaves	0.3	1
	A. thaliana roots	0.2	0.6
	A. thaliana leaves	1.1	3.7

Equivalent experiments are still in progress at HUJ

S.2. SUPPLEMENTAL RESULTS

Table S5: Plant Mass and Transpiration

Values provided are mean average ± standard deviation. Spinach and *A. thaliana* stem mass was not measured separately because these species grow in a rosette structure.

crop	Treatment	Concen (µg·L-1)	Shoot FW (g)	Roots FW (g)	Stem FW (g)	Leaves FW (g)	Shoot DW (g)	Roots DW (g)	Stem DW (g)	Leaves DW (g)	volume removed
cucumber	**LTG**	**100**	52.30± 9.23	37.80± 5.51	29.44± 3.20	42.18± 5.45	4.65± 0.68	0.86± 0.18	0.81± 0.11	3.84± 0.59	1,172.3± 247.9
cucumber	**CBZ**	**100**	50.22± 9.90	31.04± 10.00	29.12± 4.15	39.78± 4.19	3.93± 1.02	0.74± 0.19	0.70± 0.26	3.23± 0.79	1,148.0± 212.7
cucumber	**LTG + CBZ**	**100**	49.68± 6.35	33.84± 5.65	29.16± 1.54	41.56± 3.41	4.18± 0.95	0.82± 0.21	0.63± 0.18	3.56± 0.80	1,151.1± 159.6
cucumber	**LTG**	**1**	57.44± 4.64	35.18± 1.33	31.18± 2.85	44.90± 2.31	4.83± 0.50	1.01± 0.14	0.82± 0.18	4.01± 0.37	1,310.4± 49.2
cucumber	**CBZ**	**1**	57.14± 5.89	40.62± 9.58	30.98± 2.42	44.82± 3.48	4.55± 0.91	0.84± 0.27	0.65± 0.14	3.90± 0.77	1,271.3± 121.4
cucumber	**LTG + CBZ**	**1**	55.78± 4.18	35.24± 4.56	31.20± 3.50	43.66± .96	4.59± 0.24	1.03± 0.30	0.70± 0.17	3.88± 0.22	1,242.1± 95.4
cucumber	**control**	**0**	56.60± 6.88	27.60± 8.75	34.80± 6.89	41.62± 6.47	5.13± 0.91	1.03± 0.29	0.86± 0.12	4.26± 0.81	1,345.4± 130.5
Tomato	**LTG**	**100**	79.36± 11.40	7.83± 3.40	43.37± 5.47	33.86± 6.39	6.48± 1.90	0.74± 0.39	2.48± 0.73	4.00± 1.18	1,861.9± 261.3
Tomato	**CBZ**	**100**	79.93± 3.15	11.78± 3.35	41.70± 2.25	36.04± 2.12	6.92± 0.16	0.95± 0.20	2.48± 0.17	4.44± 0.19	1,974.0± 191.5
Tomato	**LTG + CBZ**	**100**	81.88± 10.12	9.77± 3.40	43.91± 6.57	35.68± 3.25	7.14± 2.09	1.03± 0.31	2.73± 0.94	4.40± 1.15	1,999.0± 174.0
Tomato	**LTG**	**1**	66.90± 5.65	7.03± 3.33	36.39± 4.48	28.95± 1.79	5.07± 0.78	0.47± 0.04	1.93± 0.53	3.14± 0.30	1,483.1± 257.0
Tomato	**CBZ**	**1**	84.30± 9.85	8.89± 2.68	46.04± 4.87	36.31± 5.47	6.90± 1.82	0.96± 0.45	2.59± 0.72	4.31± 1.14	1,850.5± 130.2
Tomato	**LTG + CBZ**	**1**	72.98± 7.29	8.58± 4.65	38.67± 3.91	32.58± 3.57	5.87± 1.10	0.63± 0.18	2.05± 0.40	3.82± 0.73	1,604.5± 137.1
Tomato	**control**	**0**	71.80± 6.51	10.43± 5.24	38.95± 3.75	31.30± 2.59	5.50± 0.71	0.52± 0.11	1.99± 0.29	3.50± 0.45	1,690.0± 263.9
Spinach	**LTG**	**100**	3.39± 1.34	1.34± 0.38			0.29± 0.13	0.05± 0.02			70.5± 24.1
Spinach	**CBZ**	**100**	3.01± 0.78	1.35± 0.35			0.26± 0.07	0.05± 0.01			63.1± 19.2
Spinach	**CBZ + LTG**	**100**	3.52± 2.59	1.68± 1.05			0.31± 0.24	0.06± 0.04			75.2± 57.3
Spinach	**LTG**	**1**	3.37± 1.99	1.68± 1.13			0.30± 0.19	0.06± 0.04			63.5± 40.7
Spinach	**CBZ**	**1**	3.43± 1.29	1.62± 0.22			0.29± 0.09	0.06± 0.01			64.1± 37.5
Spinach	**CBZ + LTG**	**1**	3.88± 1.15	2.03± 0.58			0.34± 0.10	0.07± 0.02			67.8± 26.1
Spinach	**control**	**0**	2.84± 1.03	1.09± 0.35			0.26± 0.08	0.05± 0.01			56.1± 28.6
Arabadopis	**LTG**	**100**	3.26± 0.17	2.62± 0.17			.057± .004	.258± .015			54.2± 5.4
Arabadopis	**CBZ**	**100**	3.59± 0.48	2.88± 0.45			.067± .010	.314± .042			56.6± 4.1
Arabadopis	**CBZ + LTG**	**100**	2.95± 0.19	2.46± 0.28			.060± .007	.267± .016			48.0± 9.0
Arabadopis	**control**	**0**	2.74± 0.31	2.20± 0.29			.000± .000	.000± .000			43.5± 8.2

Table S.6. Concentrations of carbamazepine, ($ng \cdot g^{-1}$) ± stdev in roots and leaves of four different crops (Arabidopsis, cucumber, spinach, and tomato) exposed to a carbamazepine or carbamazepine + lamotrigine at two different concentrations, 1 $\mu g \cdot L^{-1}$ or 100 $\mu g \cdot L^{-1}$. There were no significant differences between single and dual exposure treatments ($p > 0.05$).

Exposure	Species	1 $\mu g \cdot L^{-1}$ exposure		100 $\mu g \cdot L^{-1}$ exposure	
		Leaf	Root	Leaf	Root
CBZ	spinach	120± 40	43± 4	19,000± 5,000	5,200± 500
	cucumber	50± 30	30± 10	5,000± 600	1,400± 400
	tomato	280± 20	80± 10	21,000± 2,000	6,000± 2,000
	A. thaliana			18,000± 2,000	6,500± 200
CBZ + LTG	spinach	100± 50	43± 4	18,000± 3,000	8,000± 5,000
	cucumber	70± 30	40± 20	9,000± 3,000	1,400± 300
	tomato	250± 70	90± 30	23,000± 9,000	5,000± 3,000
	A. thaliana			17,000± 3,000	5,700± 600

Table S.7. Concentrations of lamotrigine, ($ng \cdot g^{-1}$) ± stdev in roots and shoots of four different crops (Arabidopsis, cucumber, spinach, and tomato) exposed to lamotrigine or lamotrigine + carbamazepine at two different concentrations, 1 $\mu g \cdot L^{-1}$ or 100 $\mu g \cdot L^{-1}$. Cucumbers exposed to lamotrigine only had significantly more accumulation in leaves than cucumbers exposed to both compounds (t-test, $p < 0.05$).

Exposure	Species	1 $\mu g \cdot L^{-1}$ exposure		100 $\mu g \cdot L^{-1}$ exposure	
		Leaf	Root	Leaf	Root
LTG	spinach	21± 3	95± 5	2,200± 300	12,000± 2,000
	cucumber	30± 10	50± 20	2,100± 200	1,900± 300
	tomato	22± 9	610± 90	800± 200	38,000± 5,000
	A. thaliana			280± 30	5,100± 90
LTG + CBZ	spinach	19± 5	100± 10	2,300± 300	12,000± 2,000
	cucumber	50± 20	86± 8	4,200± 500	2,000± 1,000
	tomato	4± 7	500± 100	900± 300	27,000± 5,000
	A. thaliana			300± 60	5,130± 90

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions. The main objectives of this dissertation were to advance knowledge regarding plant accumulation of pharmaceuticals and to investigate processes that are important for mechanisms of uptake. In Chapter 1, we assessed the current state of the literature, attempted compound property based modeling of plant uptake using literature data, and provided recommendations regarding parameters that should be reported in future studies on plants and pharmaceuticals. In Chapter 2, we demonstrated that the form of nitrogen provided influences wheat plant rhizosphere pH, which in turn influences plant accumulation of lamotrigine, an ionizable pharmaceutical. In Chapter 3, we found correlation between transpiration and pharmaceutical accumulation as well as evidence of mixture effects on metabolism of carbamazepine, an antiepileptic drug. In Chapter 4, we conducted experiments in two labs in different parts of the world, and combined the results to look for differences in pharmaceutical accumulation and metabolism between four plant species. Overall, these chapters represent a significant body of research that will help to advance the field and increase knowledge about plant accumulation of pharmaceuticals. Below, I outline some ideas for future research on topics that are addressed in this dissertation.

Rhizosphere Processes. The study presented in Chapter 2 is the first that we are aware of to investigate the effects of plant-driven rhizosphere changes on accumulation of pharmaceuticals. There is still much work left to be done on this topic. While sorption of pharmaceuticals to growth media had minimal effect on our study, this may not be the case for plants grown in field soils, where sorption may limit pharmaceutical bioavailability. Sorption of pharmaceuticals in the rhizosphere may not happen to the same extent as sorption in bulk soil. Previous studies have shown that root exudates can decrease sorption of neutral organic contaminants to soil,[1,2] and that degradation of neutral organic contaminants occurs more quickly

in the rhizosphere than in bulk soil.[3,4] Rhizosphere effects on sorption and degradation of ionizable pharmaceuticals have received little study, but may affect pharmaceutical availability to plants.

While the research in Chapter 2 focused on plant driven rhizosphere conditions, microorganisms are also responsible for many rhizosphere processes. Some species of plants have symbiotic relationships with nitrogen fixing fungi or bacteria,[5] and these relationships may be affected by the presence of pharmaceuticals in the environment. Previous research has shown that antibiotics can change microbial communities and increase the presence of antibiotic resistance genes in soil and field applied biosolids.[6] Additionally, investigation on how different types of rhizosphere microbiota affect the phytoavailability and degradation of pharmaceuticals, including those that contain nitrogen, is warranted. Microorganisms may form different transformation products than are formed *in planta* and may be able to break down nitrogen containing pharmaceuticals into forms of nitrogen that are usable by the plant. Previous research on PAHs and PCBs shows that rhizosphere microorganisms can enhance degradation of contaminants,[3] but there is minimal research on pharmaceuticals.[7]

Characterization of Pharmaceutical Transport and Metabolism in Plants. Much of the discussion presented in this dissertation focused on different ways that molecules can travel through the plant root. However, none of our experiments provide direct evidence about pathways at a subcellular level. So far, we can only hypothesize about potential uptake mechanisms. There are several avenues of research that can provide further insight to how pharmaceuticals move through the root. One is by studying compound interactions with the materials that make up cell walls and membranes to learn more about how pharmaceuticals sorb and diffuse within plant roots. There is already a body of literature studying interactions between

ionizable organic molecules and lipid membranes, but prediction of partitioning constants is still poor, especially for cations.[8,9]

Another approach is to focus on learning more about plant biology and how enzymes within plants affect compound movement and accumulation. Modern environmental toxicology is embracing molecular biology techniques, with studies on how contaminants affect gene and protein expression in humans and animals becoming increasingly important. Plant toxicology lags behind in these types of studies. The proteins responsible for transporting contaminants through plants are largely unknown, and while we are aware of the general classes of enzymes responsible for organic contaminant degradation, specific enzymes have not been identified.[7] Use of genetic mutants with variation in enzymes that are suspected to degrade or transport contaminants (such as those identified in Chapter 4) will be an important next step for learning about pharmaceutical pathways in plants.

There are multiple approaches to studying compound metabolism in plants as well. As described above, a greater understanding of metabolism enzymes is important, but knowledge about what metabolites are produced is important too. Rather than excreting waste like animals do, plants store it in cell vacuoles,[5] so the transformed versions of pharmaceuticals likely stay present in the plant. While the initial steps in pharmaceutical metabolism may be similar to those in mammalian systems (as discussed through Chapters 3 and 4), the end products that are eventually stored in the plant may differ. Characterizing metabolism is important to be able to determine mass balance in systems where pharmaceuticals are taken up, and also to learn about the potential toxicity of transformation products.

Mechanistic Models of Plant Accumulation of Pharmaceuticals. The eventual goal of studying plant uptake of pharmaceuticals is to be able to assess if and when there is human health risk from pharmaceuticals accumulating in crop plants. While most literature has determined that risk to human health is likely to be extremely low,[10–13] this might not always be the case as irrigation water sources change, additional drugs are brought to market, and new classes of organic contaminants are found in treated wastewater. Well tested models for plant uptake of pharmaceuticals and other ionizable organic contaminants will help to determine when additional testing is necessary.

Stefan Trapp has published what is, so far, the most mechanistic model for determining plant accumulation of ionizable organic contaminants.[14] However, his model remains relatively untested, and some parameters he uses could be improved by the addition of newer estimation methods for how ionizable compound interact with membrane lipids.[8,9] Additionally, his model assumes that concentrations of compounds within the plant eventually reach a steady state, which seems unlikely for all systems, given the correlation between compound accumulation and transpiration and the drastically different metabolism rates we observed among species. As models advance, they will need to be more species specific, account for changes in accumulation over time based on transpiration, diffusion rates, and metabolism, and account for how plant driven changes in the rhizosphere may cause compounds to act differently near the roots than they do in bulk soil.

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