Plant Uptake and Accumulation of Polar and Ionizable Organic Contaminants

By

Elizabeth Lianne Miller

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The dissertation is approved by the following members of the Final Oral Committee:

Joel A. Pedersen, Professor, Soil Science
Krishnapuram G. Karthikeyan, Professor, Biological Systems Engineering
Christina K. Remucal, Associate Professor, Civil and Environmental Engineering
Edgar P. Spalding, Professor, Botany
Michael R. Sussman, Professor, Biochemistry

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Abstract

Irrigation with reclaimed wastewater and biosolids-amendment of soils leads to plant exposure to wastewater-derived organic contaminants. Many of these compounds are polar or ionizable, and their fate in the environment, including availability to, uptake by, and accumulation in plants is not yet well understood. We explored current predictive modeling strategies and investigated processes that may play an important role in plant accumulation of polar and ionizable emerging contaminants. We showed that molecular descriptors alone have limited ability to adequately predict ionizable contaminant uptake and accumulation by plants. Exposure to mixtures may alter toxicity and/or metabolism of individual contaminants, which could lead to changes in accumulation. Transpiration of water through plants is a major driver of contaminant uptake, but by itself is also not enough to predict contaminant accumulation. Plants change their soil environment to better suit their needs, and in the process may alter contaminant bioavailability and phytoaccumulation. The form of nitrogen available to plants can influence root exudation and subsequently, rhizosphere pH, which in turn can alter plant accumulation of ionizable contaminants in some soil conditions. Although equilibrium partitioning has historically been how we model contaminant movement through the environment and into biological media, this approach may not be best for ionizable contaminants in plants. Plant processes such as biochemical reactions (e.g. metabolism) and transpiration may be equally or more important than contaminant properties and require further investigation to inform model development for accurate risk assessment.

Chapter 1. Introduction

The vast majority of freshwater withdrawal for human benefit goes towards irrigation, and much of human freshwater use supports global food output. As the global human population continues to grow, water conservation strategies will be increasingly important, especially in the face of global climate change altering our ability to grow crops and threatening access to freshwater resources. Water conservation strategies to decrease demand from water sources that are not replenished as quickly as water is withdrawn are therefore imperative. Reclamation of wastewater is one important strategy for water conservation, and is increasingly common around the world. However, this practice may be harmful to human health without appropriate risk assessment and mitigation.

Reclaimed wastewater may contain many contaminants, even if it has been treated. Many pharmaceuticals, fragrances, dyes, pesticides, plasticizers, flame retardants, and other organic contaminants are not effectively removed during conventional treatment processes, and treatment may also add additional contaminants like disinfection byproducts. Irrigation with reclaimed water can expose crops for human consumption to these contaminants, prompting a need for risk assessment of consumption of contaminated produce. However, there are thousands of structurally diverse emerging organic contaminants, meaning experimental determination of each individual contaminant's fate in agroecosystems is impractical. How many of these emerging organic contaminants move through the environment is poorly understood, as their properties tend to differ significantly from nonpolar legacy contaminants on which the bulk of research has been focused. Many emerging contaminants are polar or ionizable at environmentally relevant pH values, and have limited available toxicity information. In the case of pharmaceuticals, exposure via consumption of contaminated crops is much lower than therapeutic doses; however, the effects of long-term exposure to low levels of mixtures of wastewater-derived contaminants are unknown. Prediction of plant uptake and accumulation of polar and ionizable contaminants is therefore imperative for human health risk assessment,

but a lack of understanding of the mechanisms driving plant uptake and accumulation has thus far hindered development of predictive models.

Here, we explored current predictive modeling strategies and investigated processes that may play an important role in plant accumulation of polar and ionizable emerging contaminants. In Chapter 2, we summarize the current state of the literature, attempt to model lettuce accumulation using compound physicochemical properties and data reported in the literature, and provide recommendations for future study design and reporting. In Chapter 3, we explore the phytotoxicity and more subtle effects of the antiepileptic drug carbamazepine on the model plant *Arabidopsis thaliana*. In Chapter 4, we demonstrate evidence of mixture effects on phytotoxicity in *A. thaliana* and metabolism of carbamazepine in spinach, and explore the use of transpiration measurements as a tool for predicting pharmaceutical accumulation and understanding uptake mechanisms. Finally, in Chapters 5 and 6, we explore the effects of the rhizosphere, the area immediately surrounding plant roots, on phytoavailability of the ionizable antiepileptic drug lamotrigine.

Overall, this thesis represents the first steps in what I hope will be a turning point in the field of polar and ionizable contaminant fate in agricultural systems: a movement from thinking of this problem as only about individual contaminants in specific plant-soil systems to working towards a mechanistic understanding of plant uptake and accumulation. I present this body of work to help in an effort to change the narrative of how we think about plant uptake of emerging organic contaminants; plants are not just a passive compartment in the environment that contaminants may partition into (dependent only physicochemical properties), but living organisms that interact with and change their environments, in ways that can significantly alter contaminant availability, uptake, and accumulation from what we might expect based on partitioning alone.

Chapter 2. Root uptake of pharmaceutical and personal care product ingredients

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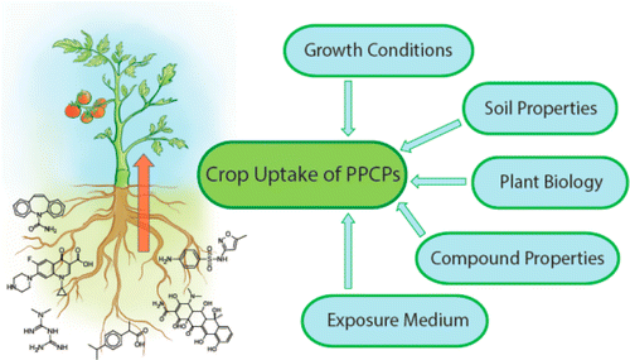

2.1. AUTHOR CONTRIBUTIONS

ELM and SLN compiled the data from the literature. ELM calculated bioconcentration factors and did the modeling. ELM, SLN, KGK, and JAP wrote the manuscript.

2.2. 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.

2.3. 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 (Appendix A).

2.4. PROCESSES IMPACTING AVAILABILITY OF PPCPS TO PLANT ROOTS

2.4.1. 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] Both tetracycline and fluoroquinolone antibiotics form surface complexes with hydrous aluminum and iron oxides.[39,40] The anticonvulsant phenytoin (pK_a = 8.3) appears to interact with iron oxide minerals via weak electrostatic attraction.[41] 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.

2.4.1.1. 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.

2.4.1.2. 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]

2.4.2. 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.

2.4.2.1. 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 are expected to by hydrolyzed *in vivo* or during wastewater treatment.[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]

2.4.2.2. 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]

2.4.2.3. 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.

2.5. ROOT UPTAKE OF PPCPS

2.5.1. Root Physiology and Processes. From the rhizosphere, PPCPs enter the plant through the roots. Figure 2.1 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 2.1). 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]

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.

2.5.2. 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.

2.5.3. 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 Appendix A), 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 2.2a); however, correlations for RCF were strong when data were restricted to neutral compounds (Figure 2.2a).

2.6. ACCUMULATION OF PPCPS IN AERIAL TISSUES

2.6.1. 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.

2.6.2. 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]

2.6.3. 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 2.2b). 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 2.2c-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.

2.7. 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.

2.7.1. 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 I metabolism of herbicides.[226] Understanding mixture toxicity may be important for predicting plant bioaccumulation of PPCPs.

2.8. 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.

2.8.1. 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 A3) to LCF data for lettuce grown under hydroponic conditions by a single research group (see Appendix A 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 A6). 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 A2), 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]

2.8.2. 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]

2.9. 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 2.1 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.

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.

Associated Content. Supporting information (SI) is provided in Appendix A. 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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2.10. FIGURES AND TABLES.

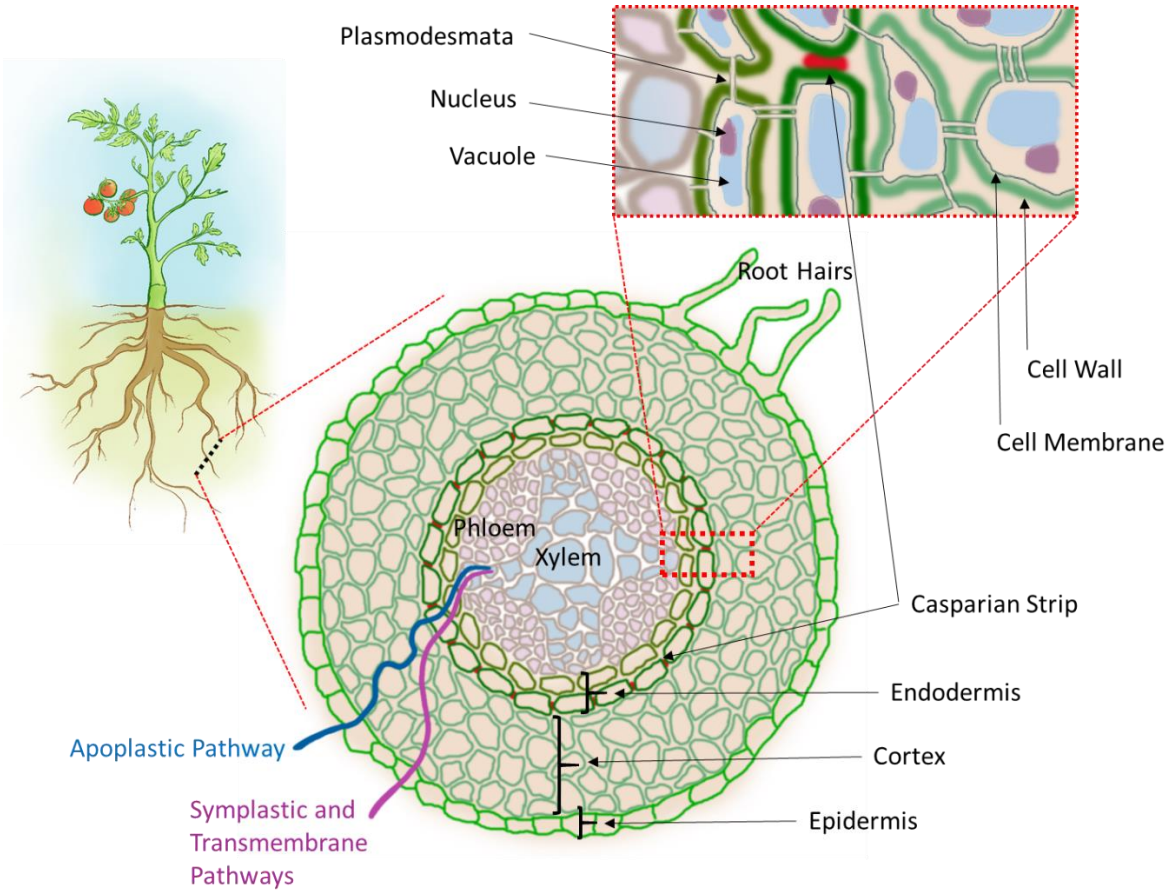

Figure 2.1. Cross-sectional diagram of a young dicot root. Water and solutes may travel from the soil solution to the vascular tissue via apoplastic (between cells along cell walls), symplastic (through cells via plasmodesmata), or transmembrane pathways (through cells via cell membranes). The Casparian 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.

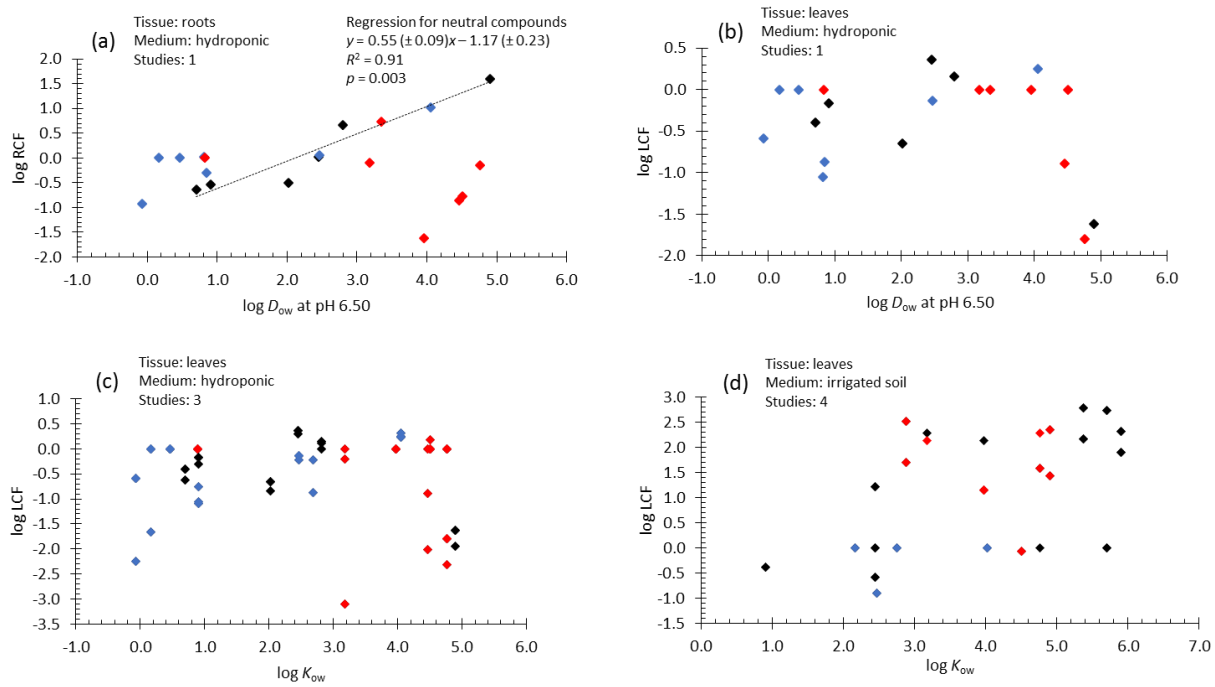

Figure 2.2. Correlation of lettuce uptake of PPCPs with log K_{ow} and log D_{ow} (pH = 6.5).[142,146,153,161,168,179,182,185] Correlations of (a) the logarithm of the root concentration factor (RCF) with D_{ow} (pH 6.5) and (b) log LCF for hydroponically grown lettuce in a single study (adapted from 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.

Table 2.1. 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

2.11. REFERENCES

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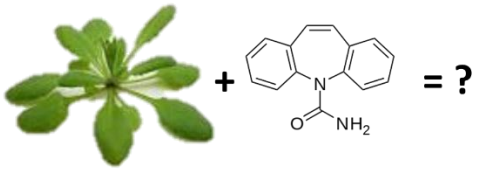
Chapter 3. Root growth and transcriptomic response of *Arabidopsis thaliana* exposed to the anti-epileptic drug carbamazepine

Note: Parts of this chapter are to be worked into a manuscript currently under development.



3.1. AUTHOR CONTRIBUTIONS

ELM performed the root length assays, grew the plants and performed the chemical analysis and RNA extractions. ELM and SLN performed BLAST comparisons. JAP oversaw the design and execution of the experiments and the interpretation of results. ELM and JAP wrote the manuscript.

3.2. ABSTRACT. Irrigation with treated wastewater and soil amendment with biosolids are increasingly common practices in agricultural systems and result in exposure of crop plants to pharmaceuticals. Crop plants may take up and bioaccumulate these contaminants, but prediction of uptake using solely the physico-chemical properties of the compounds can be confounded by metabolism i*n planta* or the influence of one contaminant on the accumulation of another. In humans, some pharmaceuticals interfere with the efficacy of others. An example of this is the anti-seizure drug carbamazepine (CBZ), which up-regulates several important drug-metabolizing enzymes. Carbamazepine is frequently detected in treated wastewater and is known to be taken up by and bioaccumulate in plants. Using root length assays for overt physiological effects and whole transcriptome profiling, we assessed the effects of CBZ exposure on physiology and gene expression in the model plant *Arabidopsis thaliana*. We hypothesized that enzymes homologous to those up-regulated by CBZ in humans are also up-regulated by CBZ in plants. We grew *A. thaliana* hydroponically and exposed roots to 1 $\mu g \cdot L^{-1}$ or 100 $\mu g \cdot L^{-1}$ CBZ for 24 hours. Leaf tissue contained measurable CBZ after this exposure period, and for the high exposure treatment, CBZ metabolites. Whole

transcriptome profiling using extracted RNA and Affymetrix gene expression microarrays was inconclusive due to substantial biological variability in control plant gene expression. Basic Local Alignment Search Tool (BLAST) comparisons of plant genomes and human metabolic enzymes indicate *A. thaliana* may not be a suitable model species for studying crop plant response to pharmaceuticals, as *A. thaliana* has fewer enzymes with high sequence similarity to those responsible for human drug metabolism compared to crop plants such as tomato and cucumber.

3.3. INTRODUCTION

Irrigation with treated wastewater and soil amendment with biosolids are increasingly common practices in agricultural systems. However, these practices result in exposure of crop plants to pharmaceuticals that are not effectively removed by conventional wastewater treatment. A large body of literature demonstrates that crop plants can accumulate pharmaceuticals under field conditions,[1,2] but prediction of uptake using physicochemical properties alone has so far been unsuccessful.[1] Models that rely solely on compound properties may not allow accurate predictions due to metabolism and/or contaminant-contaminant interactions within the plant. Pharmaceuticals are designed to be bioactive molecules, but their effects on plants are not well understood. Most pharmaceuticals are metabolically transformed to some extent in mammals. Mammals and plants share several families of enzymes responsible for pharmaceutical metabolism in humans including cytochromes P450 (CYP450s), glutathione-S-transferases (GSTs), and uridine 5'-diphospho-glucuronosyltransferases (UGTs).[3,4] Although plants have genes from the same enzyme families responsible for xenobiotic metabolism in mammals, many of these enzymes have evolved to fill other essential roles such as production of hormones and other secondary metabolites.

Plant metabolism has been studied for only a few of the hundreds of pharmaceuticals to which plants are exposed due to effluent irrigation and biosolids amendment of agricultural soils. Carbamazepine,[5] tetracycline antibiotics,[6,7] diclofenac,[8–11] diazepam,[12] and ibuprofen[13–15] are transformed in plants to many

of the same phase I and phase II metabolites that are formed in humans, indicating similar enzymes function. In mammals, many drug interactions are caused by effects of pharmaceuticals on metabolic enzymes. The antiepileptic drug carbamazepine (CBZ) is one such pharmaceutical; CBZ induces several CYP450 and UGT enzymes to the extent that doses of other medications must be adjusted for patients concurrently taking CBZ.[16,17] Carbamazepine is frequently detected in treated wastewater and is known to be taken up by and bioaccumulate in plants.[1] However, whether CBZ affects plant metabolism of other contaminants (thereby altering phytoaccumulation) is unknown. In the field, plants are exposed to complex mixtures of wastewater-derived microcontaminants, which may alter compound metabolism and accumulation relative to single compound exposures in controlled studies. This topic has received minimal formal investigation.

The objective of this study was to determine at what concentration exposure to CBZ causes overt physiological changes to plants, whether exposures to concentrations below those that reduce root growth alters gene expression in the model plant *Arabidopsis thaliana*, and if so, to identify what enzymes may have altered levels in CBZ-exposed plants. We hypothesized that enzymes homologous to those up-regulated by CBZ in humans are also up-regulated by CBZ in plants.

3.4. METHODS

3.4.1. Root Length Assays. Overt physiological effects of CBZ was assessed using root length assays on *Arabidopsis thaliana* seedlings. The premise of this method is that contaminants may alter root growth compared to those growing in untreated conditions. Root length assays are fairly common in pharmaceutical plant uptake and phytotoxicity research,[18,19] and can provide an initial indication of whether a contaminant may affect plant biochemical processes. We used this assay to inform our choice of exposure concentrations for subsequent uptake and transcriptional response experiments.

Root length assays were performed in petri dishes containing 25 mL spiked agar (pH 5.7 ± 0.05) containing 500 $mg \cdot L^{-1}$ MES buffer, 2.25 $g \cdot L^{-1}$ Murashige and Skoog phytonutrient mix, and 9 $g \cdot L^{-1}$

phytoblend agar, and various concentrations of carbamazepine (spiked in DMSO; DMSO = 1% total volume). Sterilized (with 70% ethanol) *A. thaliana* seeds were evenly spaced, 12 seeds per petri dish, using a pipette tip. Each CBZ treatment had three petri dishes, with six control petri dishes. Petri dishes were then sealed using parafilm, set upright, and allowed to grow under a 16 hour on / 8 hour off light cycle for one week. On the seventh day after planting, petri dishes were scanned and seedling root length was measured using ImageJ. For all experiments, root lengths were compared using two-way ANOVA (to account for differences in treatments using plates as a blocking variable) with Tukey post-hoc test in R.

3.4.2. *Arabidopsis* Culture. We grew hydroponic *Arabidopsis thaliana* using a system based on Noren et al.[20] and Arteca and Arteca.[21] Briefly, surface-sterilized wild-type (Colombia) seeds were sprouted in agar-filled 200 µL pipette tips. When the seedlings reached the 6-leaf stage (after two and a half weeks), the ends of the tips were cut off and submerged in a dilute nutrient solution (pH 5.7), allowing the roots to grow beyond the agar plug. The plants were grown on liquid nutrient solution for one week before exposure to CBZ. For the CBZ exposure, nine 1.5 mL polypropylene containers were filled with 1.5 mL of nutrient solution gravimetrically measured to within 0.05 g. Each container had nineteen plants. The containers were spiked with CBZ in 150 µL DMSO, for final CBZ concentrations of 0 $\mu g \cdot L^{-1}$, 1 $\mu g \cdot L^{-1}$, and 100 $\mu g \cdot L^{-1}$, with three containers each for each treatment. The exposure period lasted 24 hours and started and ended in the middle of the 14-hour on, 10-hour off light cycle in order to minimize noise from normal light-induced gene expression changes. The light intensity was 35-45 $\mu mol \cdot m^{-2} \cdot s^{-1}$. At the end of the 24-hour exposure, the aerial tissues were harvested. For each treatment, eight replicates of approximately 50 mg of tissue (1-3 plants) were flash frozen in liquid nitrogen for RNA extraction and subsequent transcriptome analysis. The remaining tissue was frozen at -20 °C and then freeze-dried for chemical analysis.

3.4.3. Chemical Analysis. Freeze-dried leaf tissue was ground to a powder with a mortar and pestle, and extracted using an accelerated solvent extractor (ASE350, Dionex, Sunnyvale, CA) using 11-mL extraction cells. Glass-fiber filters (27-mm) were placed at the bottom of the cells and then covered with 1.00 ± 0.005 g florisil. Approximately 0.2 g dry tissue powder was added on top of the florisil, and

spiked with 20 µg carbamazepine-d10 in methanol. An additional 1.00 ± 0.005 g of florisil was added on top, followed by another glass-fiber filter. The packed cells were allowed to sit overnight, and then were extracted in two static cycles (5 min) with 100% methanol at 80 °C under a constant pressure of 10.34 MPa. The extracts were evaporated to dryness under nitrogen in a water bath at approximately 60 °C, then reconstituted in 1 mL 8:2 acetonitrile:water with 0.1% acetic acid. The extracts were sonicated for 10 minutes, then transferred to Eppendorf vials and centrifuged at 17,000 × *g* for 20 minutes and filtered (0.22 µm PTFE syringe filters) prior to analysis.

We measured concentrations of CBZ and the CBZ metabolites 10,11-epoxycarbamazepine (epCBZ) and 10,11-*trans*-dihydroxycarbamazepine (diOH-CBZ) in leaf extracts and nutrient solution using liquid chromatography with tandem mass spectrometry. We used an Agilent 1260 high-performance liquid chromatography (HPLC) with 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). We used a gradient of 5% to 95% organic phase and column temperature was held at 30 °C. For detection of CBZ, we used an Agilent 6460 triple quadrupole mass spectrometer with electrospray ionization in positive mode. Mass-labeled CBZ and ep-CBZ were used for internal standard-based calibration and measurement. Extraction recovery and limits of detection are included in Appendix B.

3.4.4. Transcriptomic Analysis. Sample RNA was extracted from the frozen leaf tissue samples using the Qiagen RNeasy Plant Mini Kit. Total RNA was quantified using a NanoDrop 2000 and mRNA integrity was assessed using an Agilent 2100 BioAnalyzer RNA 6000 Nano Chip. Four replicates from each treatment were chosen based on their RNA quantity and quality. For each replicate, single-stranded complimentary DNA (ss cDNA) was synthesized from 500 ng RNA using the Ambion WT Expression Kit (Pub Part# 4425209 Rev.D Aug2011). All samples were then end-terminus labeled following the guidelines in the GeneChip WT Terminal Labeling and Hybridization User Manual Target (P/N 702808 Rev. 7). Twelve AraGene-1_0_ST arrays were hybridized at 45 °C for 16 hrs O/N following all procedures outlined in the GeneChip WT TerminalLabeling and Hybridization User Manual (P/N 702808 Rev.7) for the

AraGene-1_0_ST Array. Three micrograms of fragmented labeled ss cDNA were applied to each array. GeneChips were post processed on the AFX Fluidics 450 Station according to all AFX protocols and procedures defined for the AraGene-1_0_ST Array (FS450 _0002) as outlined in the GeneChip Expression Wash, Stain and Scan user Manual (P/N 702731 rev.3). GeneChips were scanned on the GC3000 G7 Scanner (Serial #50208130), and data were extracted and processed using Affymetrix Command Console version 4.0.0.1567G. Data Analysis was performed using the Bioconductor affy package for R.[22]

3.5. RESULTS AND DISCUSSION

3.5.1. Phytotoxicity of CBZ. Root length was not affected by CBZ exposure below 30 $mg \cdot L^{-1}$ (Figure 3.1), which is substantially higher than current environmental concentrations of CBZ, which are in the ng to $\mu g \cdot L^{-1}$ range. We therefore chose CBZ concentrations of 1 $\mu g \cdot L^{-1}$ (an environmentally relevant concentration) and 100 $\mu g \cdot L^{-1}$ (100 × the environmental concentration, but still two orders of magnitude below the concentration that caused measurable changes in root growth) for the transcriptomics analysis, as we were interested in transcriptional changes that would be relevant for actual current and future exposure scenarios.

3.5.2. *Arabidopsis* plants take up and metabolize CBZ. Leaf tissues from both the 1 and 100 $\mu g \cdot L^{-1}$ treatments had detectable levels of CBZ after the 24-hour exposure (Figure 3.2). We did not measure root accumulation due to the small size of the plants and previous research indicating CBZ primarily accumulates in leaf tissue.[1,2,5,23] The concentrations of CBZ in the nutrient solution did not change significantly over the course of the exposure time (data not shown), which is unsurprising considering the small volume of liquid transpired by the plants.

In plants, CBZ metabolism has been studied, and the main CBZ metabolites are 10,11-epoxycarbamazepine (epCBZ) and 10,11-*trans*-dihydroxycarbamazepine (diOH-CBZ). Both metabolites of CBZ were found in leaf tissue from the high CBZ concentration treatment. Metabolites were below the

LOQ in exposure solutions, indicating that CBZ metabolism occurred in the *Arabidopsis* plants. This indicates that metabolic enzymes were active within the leaf tissue. However, both metabolites were below the LOQ in the low CBZ concentration treatment. Previous research has shown that the extent of CBZ metabolism varies among species, and the 24 hour exposure period may not have been long enough for the low concentration exposure plants to accumulate and metabolize detectable levels of epCBZ and diOH-CBZ. However, the presence of CBZ metabolites in the high treatment indicated the possibility that CBZ was not only being metabolized, but also inducing the genes for the enzymes responsible for xenobiotic stress response in the leaves.

3.5.3. Biological variability precludes identification of transcriptional changes. We first checked for probe intensity variation among uncorrected ("raw") arrays and evaluated whether the data clustered as expected. Comparison of interquartile ranges indicated excellent uniformity of probe intensities between arrays, and close alignment of each median indicated consistency throughout the nucleic acid preparation procedures, including the hybridization and scanning steps. Background adjustment, inter-array normalization, and summarization was performed using the robust multichip average (RMA) method. After normalization, all arrays exhibited a similar median expression level, indicating successful inter-array RMA normalization (Figure 3.3). There was little evidence of low-intensity probe enrichment. Nevertheless, a 5% quantile of the distribution was used as a threshold to define low-intensity probes. To reduce sample variance, only those probes exhibiting a higher intensity in at least four arrays were retained. After filtering, 1164 probes were rejected leaving 37,244 transcripts available for analysis.

Tests for differential expression of transcripts between treatments were computed with moderated *t*-statistics with shrunken standard deviation for each gene. An empirical Bayes model was used to shrink the variance of each transcript toward a common value for the entire set (37,244) of filtered candidates. The $\log_2$ fold-change (logFC) values were tightly centered on 0. While choosing any fold-change threshold for differential expression, is arbitrary, in general an absolute value of (logFC) > 1 is often considered the cutoff of statistical significance in microarray data. Very few occurrences of an absolute value of (logFC)

> 1 were observed. No transcripts were determined to meet statistical significance at a Benjamini and Hochberg's false discovery rate (FDR) adjustment to correct for multiple testing FDR = 0.10, meaning no definitive differential expression was detected between treatments (Figure 3.4).

A principle component analysis was performed to determine whether the cause of the lack of differential expression was due to variation within treatments, and we found substantial overlap among probe intensity distributions in the three treatments (Figure 3.5). The variation in the transcriptional response, especially for the control samples, suggests substantial biological variability within leaf gene expression. The consistent inter-array median probe intensity and absence of other technical artefacts indicates the RNA extraction and analysis were high quality, and therefore points to biological variability as the most likely cause of the overlap. Each replicate was a pooled sample of leaves from multiple plants, which would have helped to normalize noise. However, our use of whole leaves instead of a single cell type could also have contributed to more variation in gene expression and dampening any signal among the noise.

Despite the lack of definitive evidence for CBZ effects on plant gene expression, we cannot rule out drug-drug interactions as a result of CBZ co-exposure with other contaminants. Even in the absence of biological variability, gene expression changes may not have been detectable. Gene induction/inhibition could take place earlier or later than the tested exposure time, or only with exposure to higher concentrations of CBZ. The high level of biological variability displayed in these samples indicate that changes in gene expression may also be subtle enough that more than four biological replicates per treatment or focus on a single cell type rather than whole leaf tissue would be necessary to detect them. Furthermore, CBZ may cause other biological effects in plants that could lead to effects on uptake and accumulation of other contaminants. For example, mixture exposure may lead to changes in translation of RNA to proteins, competition for enzyme active sites or inhibition of protein activity, or subtle changes in whole organism function such as altered transpiration rates. Due to the high cost of this type of experimental analysis and the many unknowns in experimental design, we decided to take a different approach to investigating the

possible biochemical effects of CBZ on plants rather than continue with transcriptomic analysis, namely, comparison between human and plant enzymes.

3.5.4. Comparison with human metabolizing enzymes. In humans, 11 cytochrome P450 enzymes are responsible for most phase 1 drug metabolism. The main metabolic pathway for CBZ in mammals is formation of epCBZ via CYP3A4, though this metabolite is also formed via CYP3A5, CYP2C8, CYP2C19, and CYP3A7.[24] Additionally, CBZ can be metabolized to 3-hydroxy-CBZ via CYP3A4, CYP2B6, or CYP3A7.[24] We hypothesized that CBZ is also metabolized by CYP450 enzymes in plants, as the human metabolites have also been found in plants.[25–27] In addition to its metabolism, CBZ also induces activity in multiple CYP450 enzymes including CYP1A2, CYP2C9, CYP2C19, CYP3A4, and CYP3A5, to the extent that doses of other drugs metabolized by these enzymes must be adjusted in patients who also take CBZ.[28] In humans, CBZ also induces UGT enzymes (primarily UGT 1A4), which also frequently affect other drug metabolism.[29]

We took the primary amino acid sequence to each of the human CYP450s and UGT1A4 and used the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) to compare these with known *A. thaliana* genes, as well as genes from three common crop plants that have been shown to take up and metabolize CBZ: spinach, tomato, and cucumber. We recorded all plant proteins with max alignment scores of 200 or greater and up to 10 max scores of 190-200 per species per human protein (excluding duplicate entries). We recorded the protein with the highest match if no alignment scores exceeded 190. While the max score of 200 or greater is a somewhat arbitrary cutoff, higher scores indicate higher similarity between proteins, while scores between 80 and 200 indicate proteins from the same family that may not have similar functions. We note that high similarity may not necessarily indicate substrate similarity, and in some cases the active site might not be BLAST searchable. However, high similarity plant proteins may indicate enzymes for which future study of pharmaceutical metabolism is warranted. Results are summarized in Table 3.1, which shows all plant proteins with scores above 200 and instances where the same plant protein was found in multiple plant species or to match with multiple human CYP450s. For

UGT1A4, we did not find any closely aligned proteins. Max scores for UGT1A4 were 89 for spinach, 83.2 for tomato, 73.9 for cucumber, and 70.1 for *A. thaliana*, indicating minimal similarity. A full list of plant proteins, relevant statistics, and accession numbers is included in Appendix D.

Tomato has seven matches with score greater than 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 match with human CYP1A1 also match highly with other human enzymes. Cucumber has one protein with a score greater than 200 for alignment with human CYP3A4, which is the main enzyme for CBZ conversion to 10,11-epCBZ. 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. It is worth noting that we see greater alignment with human CYP450s for cucumber and tomato than for *A. thaliana* and spinach, which may explain reported higher degradation of CBZ in cucumber[23] and tomato[5] compared to our work in *A. thaliana* and spinach (Chapter 4). The plant proteins identified in this analysis may be good targets for additional research on plant metabolism of CBZ and other xenobiotic organic compounds. However, these results also indicate that although *A. thaliana* is a valuable genetic resource for plant biology, it may not be a good model organism for investigating effects of pharmaceuticals on crop plants, as it has fewer drug metabolism enzymes in common with humans compared to tomato and cucumber.

3.5.5. Implications for future research. How wastewater-derived contaminants, including CBZ may affect plant biochemical processes, and therefore metabolism of other contaminants, warrants future study, as little on this topic is currently understood. Future gene expression studies (both transcriptomic and more rapid investigations of in planta transformation) may be more successful in detecting expression changes over the noise of natural biological variation by using a battery of cell cultures approach rather than whole plant tissues that contain multiple cell types, each with their own gene expression profiles. For pharmaceuticals and other contaminants designed to be biologically active, well characterized effects on

mammalian biochemical processes may provide insight for predicting effects on plants. Comparison between mammalian and plant metabolic enzymes and genomes may also help prioritize enzymes, genes, or even species for study by identifying those most likely to be affected by a specific contaminant.

Associated Content. Supporting information (SI) is provided in Appendix B. Details of extraction and detection of CBZ and its metabolites, and full BLAST results comparing *A. thaliana*, spinach, tomato, and cucumber genomes with human CYP450s.

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3.6. TABLES AND FIGURES

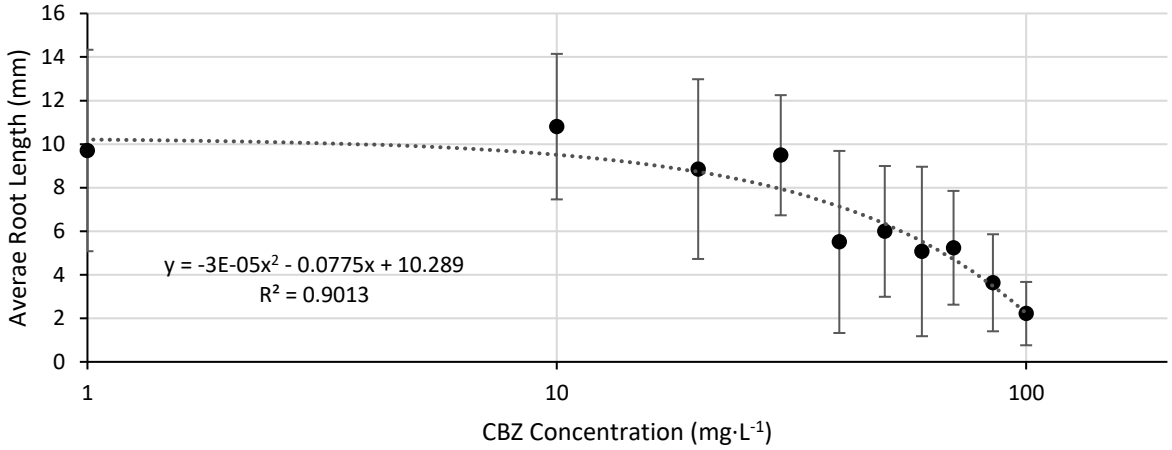

Figure 3.1. *A. thaliana* root length response as a function of carbamazepine (CBZ) exposure concentration. Seedlings were sprouted on agar containing the indicated CBZ concentration and allowed to grow for 7 days prior to root measurement. Error bars indicate standard deviation (n = 36).

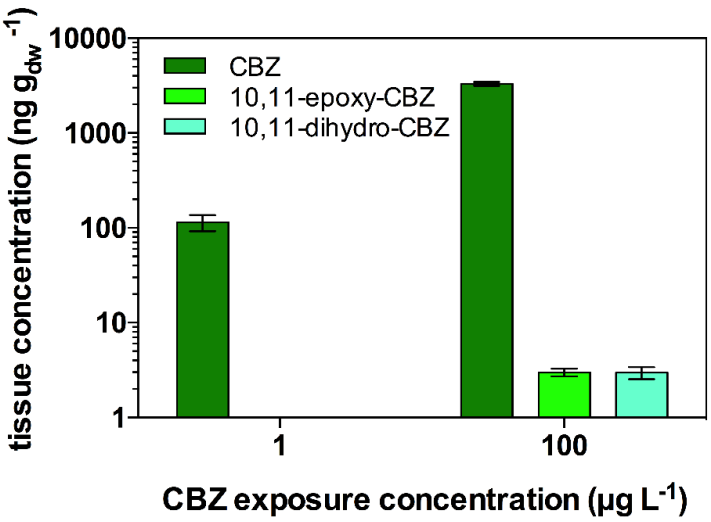

Figure 3.2. Carbamazepine (CBZ) and both its primary metabolites were detectable in *A. thaliana* leaf tissue after 24 hour root exposure. Metabolites were below LOQ in exposure solutions. Error bars indicate standard deviation; dw = dry weight.

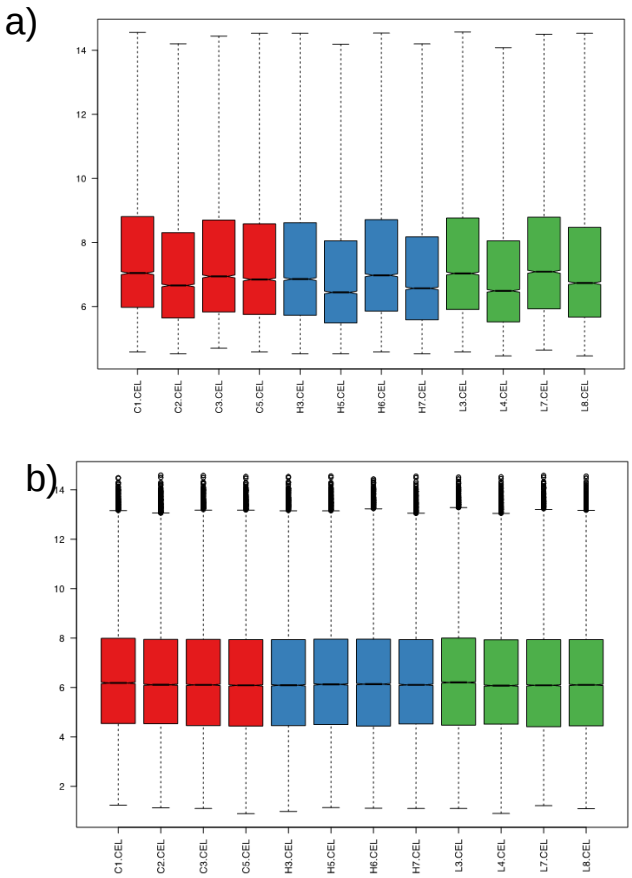

Figure 3.3. a) Raw and b) normalized fluorescence intensities of each sample array. The boxplot of interquartile ranges (IQR) indicates excellent uniformity of probe intensities between arrays. Control leaf RNA are shown in red, 100 $\mu g \cdot L^{-1}$ CBZ exposure treatment leaf RNA in blue, and 1 $\mu g \cdot L^{-1}$ CBZ exposure treatment leaf RNA in green. After normalization, none of the samples stood out from the rest and all arrays exhibited a similar median expression level Close alignment of each median indicates consistency throughout the nucleic acid preparation procedures, including the hybridization and scanning steps.

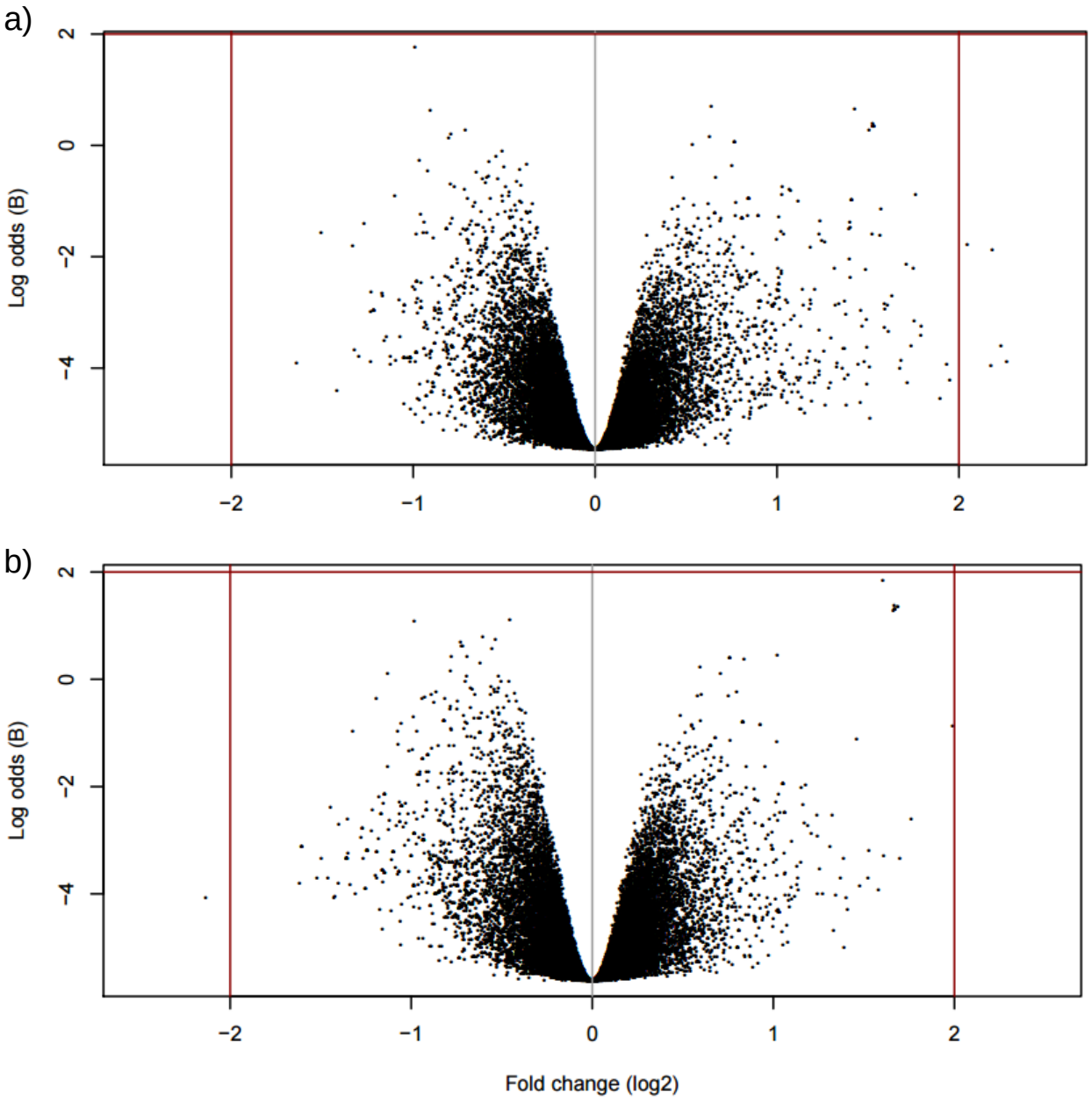

Figure 3.4. Leaf transcript expression difference from control expression for a) 1 µg·L^{-1} CBZ and b) 100 µg·L^{-1} CBZ treated plants. Each black dot represents one gene transcript. Volcano plots show transcriptional response fold change from control vs. odds of differential expression. The x-axis indicates the biological impact of the change (comparison between treatment and control). The y-axis indicates the log odds (statistical evidence) of the fold change. The B-statistic is the log-odds that a transcript is differentially expressed. A B-statistic of 0 corresponds to a 0.5 chance that the gene is differentially expressed (since it's expression can either increase or decrease). By convention, gene expression is not considered significantly different unless the absolute value of the log fold change is greater than one and the B-statistic is greater than two (indicated by the red lines). Differential transcriptional responses do not pass the threshold of adjusted $p < 0.05$.

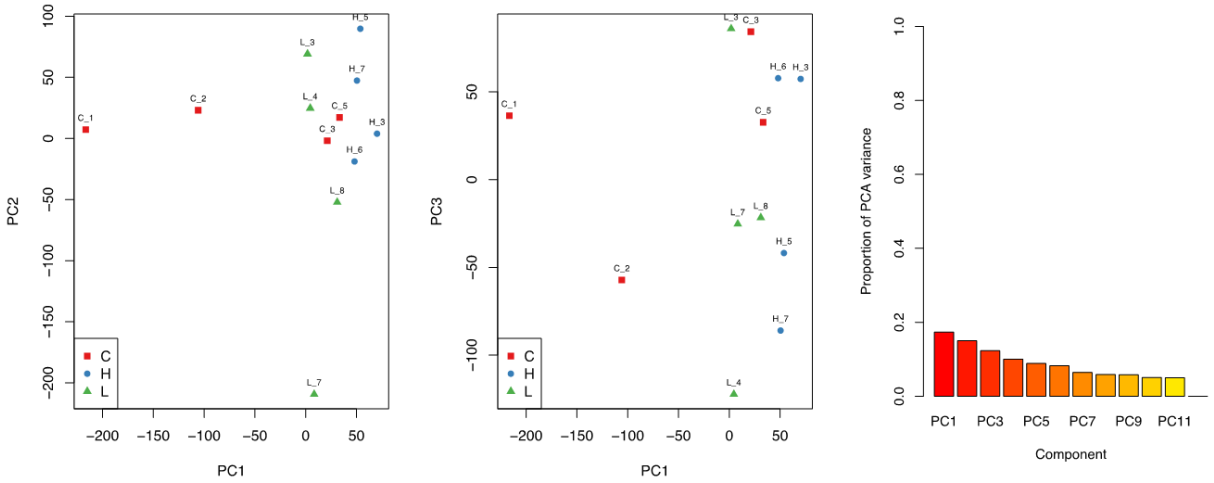

Figure 3.5. Principal Component Analysis (log_2 intensity scale) indicated substantial overlap among probe intensity distributions in the three treatments**.** No treatment groups could be clearly distinguished and numerous strong outliers were identified. Principle component 1 explained less than 20% of variation. Controls are indicated with red boxes, the 100 $\mu g \cdot L^{-1}$ treatment is indicated with blue circles, and the 1 $\mu g \cdot L^{-1}$ treatment is indicated with green triangles.

Table 3.1. Max scores for plant protein alignment with human drug metabolizing CYP450 enzymes.

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

[a] Scores are color coded with darker colors indicating higher scores.

[b] 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.

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Chapter 4. Effects of mixtures on toxicity and phytoaccumulation of pharmaceuticals and personal care product ingredients

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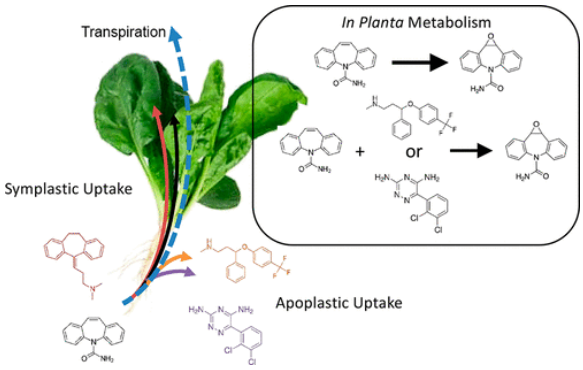

4.1. AUTHOR CONTRIBUTIONS

ELM, SLN, and several undergraduates performed the root length assays. ELM grew the spinach plants and performed the extractions. SLN was responsible for LC-MS/MS analysis. KGK and JAP oversaw the design and execution of the experiments and the interpretation of results. ELM, SLN, KGK, and JAP wrote the manuscript.

4.2. ABSTRACT. Many pharmaceuticals are present in reclaimed wastewater and effluent-dominated water bodies used to irrigate edible crops. Previous research has shown that plants irrigated with reclaimed wastewater can accumulate pharmaceuticals. However, plant-driven processes that contribute to differences in accumulation among compounds are not well understood. Here, we tested the overt effects on physiology of a suite of pharmaceuticals and personal care product ingredients individually and as mixtures using root length assays, and found that seedling root growth in mixtures differed from that of exposures to individual

compounds. We then evaluated the effects of exposures to binary mixtures on spinach accumulation and metabolism of four psychoactive pharmaceuticals found in reclaimed wastewater: carbamazepine, fluoxetine, amitriptyline, and lamotrigine. Co-exposure of plants to carbamazepine and fluoxetine or amitriptyline decreased accumulation of the toxic carbamazepine metabolite 10,11-epoxycarbamazepine. Furthermore, we tested a simple transpiration-based accumulation model and found that transpiration is a strong predictor for accumulation of the studied compounds. Amitriptyline accumulated to a larger extent than predicted from transpiration alone, and we suggest the possibility that a transporter protein may be involved in its uptake. Our findings highlight the need to consider plant physiology and mixture effects in studying accumulation of polar and ionizable organic contaminants and their metabolites.

4.3. INTRODUCTION

Water scarcity is a growing concern as world population expands and climate change makes freshwater availability more unpredictable.[1] Wastewater reuse represents an important strategy to reduce demand on freshwater resources. In arid agricultural areas in both developed and developing countries, irrigation of crops with reclaimed wastewater is already widely practiced.[2,3] However, many contaminants, including pharmaceuticals and personal care product ingredients (PPCPs), are frequently found in treated and untreated wastewater,[4,5] and use of reclaimed wastewater for irrigation can result in human exposure to these contaminants via consumption of irrigated crops.[6]

A large body of literature demonstrates that crop plants can accumulate PPCPs under field conditions,[7,8] but monitoring agricultural produce for all potential wastewater-derived contaminants is impractical. Thus, predicting plant accumulation of PPCPs is an important goal. Most attempts to predict phytoaccumulation in whole plants or in specific tissues have been based on correlations with contaminant physico-chemical properties.[7] For example, plant accumulation of neutral, hydrophobic contaminants can be estimated based on the logarithm of the *n*-octanol-water partition coefficient (log K_{ow}).[9,10] Such approaches assume that plant accumulation is driven by passive processes such as diffusion and

partitioning, and have not proven accurate for polar and ionizable organic compounds.[7] Most PPCPs are nonvolatile, polar or ionizable, and they are designed to have biological effects, yet PPCP effects on and biochemical interactions with plants have only begun to be characterized.[11] Plant uptake of contaminants is partially driven by water flow through the plant via transpiration.[7,12–14] Multiple studies have reported a positive relationship between transpiration and removal of non-polar organic compounds from growth media,[15–17] but the relationship between transpired water and accumulation in plant tissues was not evaluated and would in principle be affected by transport pathways within the plant, *in planta* metabolism, and for some compounds, volatilization from plant leaves.[64]

Most PPCPs are metabolically transformed to some extent in mammals. Mammals and plants share several families of enzymes responsible for pharmaceutical metabolism in humans including cytochromes P450 (CYP450s), glutathione-*S*-transferases (GSTs), and uridine 5'-diphospho-glucuronosyltransferases (UGTs).[7] Plant metabolism has been studied for only a few pharmaceuticals. Carbamazepine, diclofenac, diazepam, and ibuprofen are transformed in plants to many of the same phase I and phase II metabolites that are formed in humans.[18–22] In mammals, many drug interactions are caused by effects of pharmaceuticals on metabolic enzymes. For example, the antiepileptic drug carbamazepine induces several CYP450 and UGT enzymes to the extent that doses of other medications must be adjusted for patients concurrently taking carbamazepine.[23,24] In the field, plants are exposed to complex mixtures of wastewater-derived microcontaminants, which may alter compound metabolism and accumulation relative to single compound exposures in controlled studies.[7] This topic has received minimal formal investigation.[25]

In this study, we used the model plant *Arabidopsis thaliana* to screen 20 structurally diverse PPCPs for mixture effects on plant physiology using root length assays. From these screening experiments, we identified four structurally diverse pharmaceuticals to investigate the effects of binary mixtures on accumulation and metabolism in a crop plant: spinach (*Spinacia oleracea*), which is more relevant for studying plant uptake of wastewater-derived contaminants and is also larger, making it easier to grow hydroponically and measure transpiration and contaminant accumulation in root and leaf tissue from single

plants. We then used a simple transpiration-based approach to evaluate plant uptake mechanisms for the studied compounds. We grew spinach hydroponically to focus on the chemical properties and plant attributes governing accumulation without the complicating factors of sorption to soil components and possible degradation by soil microorganisms.[18]

4.4. MATERIALS AND METHODS

4.4.1. Materials. Chemicals used, suppliers, and purities are described in the Supporting Information (Text C1.1). Structures and selected physico-chemical properties of the 20 PPCPs are displayed in Table 4.1.

4.4.2. Root Length Assays. Overt physiological effects were assessed using root length assays on *Arabidopsis thaliana* seedlings exposed to single or binary mixtures of PPCPs. The premise of this method is that plants will have altered root growth when affected by a contaminant compared to those growing in untreated conditions. Root length assays are fairly common in PPCP uptake and phytotoxicity research.[26,27] We chose to perform root length assays because they provide unambiguous results that can be widely compared with results found in the literature.

Root length assays were performed in petri dishes containing 25 mL spiked agar (pH 5.7 ± 0.05) containing 500 $mg \cdot L^{-1}$ MES buffer, 2.25 $g \cdot L^{-1}$ Murashige and Skoog phytonutrient mix, and 9 $g \cdot L^{-1}$ phytoblend agar, and various concentrations of PPCPs (spiked in DMSO; DMSO = 1% total volume). Sterilized (with 70% ethanol) *A. thaliana* seeds were evenly spaced, 12 seeds per petri dish, using a pipette tip. For each set of experiments described below, each PPCP treatment had 3 petri dishes, with 6 control petri dishes. Petri dishes were then sealed using parafilm, set upright, and allowed to grow under a 16 hour on / 8 hour off light cycle for one week. On the seventh day after planting, petri dishes were scanned and seedling root length was measured using ImageJ.

We first determined dose response relationships for each individual PPCP in Table 4.1. Preliminary root length assay experiments were used to determine the lower bounds of root growth effects. We then tested several different mixtures of the PPCPs, looking for differences in effect thresholds compared to individual compounds: a full mixture containing all 20 compounds, the mixtures containing all but the compounds with the lowest concentrations to alter root growth, and several binary mixtures in which we expected to see interactions based on mammalian drug-drug interaction data. For all experiments, root lengths were compared using two-way ANOVA (to account for differences in treatments using plates as a blocking variable) with Tukey post-hoc test or Dunnett's test for pairwise comparisons. All statistical analysis was performed in R.

4.4.3. Spinach Growth and Exposure Experiments. Tyee Hybrid spinach (*Spinacia oleracea*) seeds were sterilized, germinated in a damp paper towel for 2-3 days, then transferred to a hydroponic setup containing a sterile modified Hoagland's solution at pH 5.7 (Text C1.2). Plants grew hydroponically for 7-8 weeks prior to pharmaceutical exposure, during which period sterile nutrient solution was replenished periodically as needed.

To test the effects of binary mixtures, we exposed spinach plants to CBZ, LTG, AMI, FLX individually, or to mixtures of CBZ with one of the latter three pharmaceuticals for 7 days (Text C1.3). We selected the psychoactive compounds carbamazepine (CBZ), lamotrigine (LTG), amitriptyline (AMI), and fluoxetine (FLX) for study based on their presence in treated wastewater[28,29] demonstrated accumulation in plants,[30–33] and indications of toxicity interactions from our root length assays. Furthermore, CBZ induces the enzymes responsible for metabolizing LTG, AMI, and FLX in mammalian systems,[28] and CBZ affects LTG uptake by cucumber plants.[15] Structures and selected physico-chemical properties of the four pharmaceuticals are displayed in Table 4.2. Starting exposure concentrations were 1 $\mu g \cdot L^{-1}$ (an environmentally relevant concentration) or 100 $\mu g \cdot L^{-1}$ (100× the environmental concentration), and the nutrient solution was not replenished or changed during the exposure period. The concentrations of individual compounds used in mixture exposures were equal. Only the higher exposure concentration was

tested for FLX. We chose CBZ as the basis for mixtures due to its known effects on the metabolism of other pharmaceuticals in mammalian systems[23,24] and our observations of phytotoxicity effects (see below). We also examined CBZ accumulation as a function of time for 14 days. In these experiments, we exposed plants to 100 $\mu g \cdot L^{-1}$ CBZ and sacrificed plants to measure CBZ accumulation after 1, 3, 7, and 14 days (spiked nutrient solution replaced on day 7). Our experiments included control plants not exposed to pharmaceuticals and plant-free controls which contained pharmaceuticals. At least three replicates were used for each treatment.

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 and subtracting estimated evaporation (as distinct from transpiration). Evaporation was estimated by measuring mass loss from control setups without plants situated near each plant-containing setup. Nutrient solution was sampled at the beginning and end of the exposure period and analyzed for pharmaceutical concentrations. After the exposure period, roots and above-ground tissues (leaves) were collected, separately frozen at –80 °C, freeze dried, and stored at –80 °C until extraction. Plant masses were measured before and after lyophilization. Temperature and humidity were monitored throughout the exposure period (Figure C1).

4.4.4. Extraction and Analysis. The extraction and analysis methods were similar to those we previously reported.[35] Briefly, freeze-dried plant tissues were ground, spiked with mass-labeled internal standards, allowed to sit overnight at room temperature, and subjected to accelerated solvent extraction (ASE) with 100% methanol. Extracts were evaporated to dryness, reconstituted in a mixture of water, acetonitrile, and acetic acid, then centrifuged and filtered through 0.2 μm PTFE filters before analysis (Text C1.4). We measured AMI, FLX, LTG, CBZ, and the CBZ metabolites 10,11-epoxycarbamazepine (epCBZ) and 10,11-*trans*-dihydroxycarbamazepine (diOH-CBZ) in leaf and root extracts and starting and ending nutrient solutions using liquid chromatography with tandem mass spectrometry (Text C1.4).

4.5. RESULTS AND DISCUSSION

4.5.1. Root Growth Effects. No individual PPCP altered *A. thaliana* root length at concentrations below 10 $\mu g \cdot L^{-1}$ (Figure 4.1). This result is consistent with the frequent worldwide use of reclaimed wastewater for irrigation, as if wastewater-derived contaminants caused overt physiological changes to plants at their current environmental levels (ng to $\mu g \cdot L^{-1}$ concentrations), farmers would not want to use wastewater for irrigation or biosolids amendment for fertilizer. However, this result also highlights a possible future issue with reuse of treated wastewater for irrigation if contaminant concentrations increase due to increased PPCP use or as new PPCPs are used in commerce and subsequently end up in reclaimed wastewater.

The full mixture of all 20 compounds exhibited root growth effects similar to that of the most individually effective compound, triclosan (Figure 4.1 and Figure 4.2a). However, removing triclosan from the mixture resulted in significant changes to root length at a lower concentration than the full mixture or any individual compounds (Figure 4.2b). Sulfamethoxazole appeared to cause the increased sensitivity of root growth to the mixture (without triclosan), as removing it caused a marked decrease in response (Figure 4.2c). However, this mixture was more effective than sulfamethoxazole alone, indicating an interaction from the combination of sulfamethoxazole and at least one other PPCP in the mixture. We suspected sulfamethoxazole and trimethoprim to interact in plants, as these antibiotics are frequently prescribed in combination. Their combined mechanism of action is inhibition of sequential steps in bacterial synthesis of tetrahydrofolic acid, which is an important intermediate in the synthesis of amino acids and nucleic acids, and plants also have this synthesis pathway.[36] Indeed, sulfamethoxazole and trimethoprim had synergistic effects on root length (Figure 4.2d).

We also tested several binary mixtures in which one pharmaceutical was at a concentration known to decrease root growth and CBZ was present at a concentration much lower than required to cause a decrease in root length by itself. We chose to focus on interactions with CBZ because of its large numbers

of drug-drug interactions; CBZ induces 5 of the 8 CYP450s responsible for drug metabolism in humans, and can therefore often increase metabolism and decrease effectiveness of other drugs, including NSAIDs, anti-depressants, blood pressure medications, and birth control.[23,24] We found that the presence of CBZ sometimes increased root response, as was the case for AMI (Figure 4.3a; Dunnett's test, $p = 0.0131$). This may be due to competition for metabolizing enzymes between the two compounds. Assuming the parent compound, and not a metabolite, is responsible for the effect. We also observed a decrease in root growth for a low concentration of lamotrigine added to a high concentration of carbamazepine (Figure 4.3e; Dunnett's test, $p = 0.0006$). Similar to the drug-drug interactions observed in humans, a small addition of CBZ sometimes lessened the response, as was the case for FLX (Figure 4.3b; Dunnett's test, $p = 0.0505$) and LTG (Figure 4.3c; Dunnett's test, $p = 0.0153$). Alterations in physiology with the addition of a second compound were not observed for all compounds that exhibit drug-drug interactions in humans, as is shown with the example of warfarin with CBZ (Figure 4.3d; Dunnett's test, $p > 0.05$).

Root length effects are a very rudimentary physiological endpoint; subtle effects such as changes in biochemical processes may occur at much lower exposure concentrations. Subtle effects could in turn affect accumulation of these contaminants, but have not previously been reported. We therefore chose four pharmaceuticals for which we observed interactions in root growth (CBZ, AMI, FLX, and LTG) to investigate further in uptake and accumulation studies comparing individual and binary mixture exposures.

4.5.2. Pharmaceutical Accumulation and Metabolism. The studied pharmaceutical compounds differed in their extent of accumulation and tissue distribution in spinach (Figure 4.4). Amitriptyline exhibited the highest overall accumulation with comparable concentrations measured in leaves and roots. Carbamazepine accumulated to a larger degree in leaves than in roots. Lamotrigine and FLX remained mainly in the roots. Our findings for CBZ, LTG, and FLX are consistent with previous literature on the accumulation of these compounds in various plant species.[25,31,32,37–41] Our results for AMI contrast with a previous study that reported accumulation primarily in roots for strawberry plants.[33] Possible explanations

include more rapid degradation in above-ground tissues of strawberry plants or phloem mobility in spinach but not in strawberry plants.

We also measured concentrations of the CBZ metabolites 10,11-epoxycarbamazepine (epCBZ) and 10,11-*trans*-dihydroxycarbamazepine (diOH-CBZ), which are found in both plants and humans[18,42] (Figure 4.5). Leaf concentrations of epCBZ were higher than those in roots by a factor of 12 ± 8 in the 100 $\mu g \cdot L^{-1}$ exposure, indicating that CBZ metabolism primarily occurs in the leaves, consistent with prior reports.[18,25,31,32] Concentrations of epCBZ were below the limit of quantification in the roots of plants exposed to 1 $\mu g \cdot L^{-1}$ CBZ. Concentrations of diOH-CBZ were lower than those of epCBZ by at least an order of magnitude for all plants and exceeded the limit of detection only in the leaves of plants exposed to 100 $\mu g \cdot L^{-1}$ CBZ. In all compartments (leaves, roots, nutrient solution), metabolites accounted for less than 4% of the total CBZ measured.

4.5.3. Pharmaceutical Mixture Effects. Co-exposure to CBZ did not affect accumulation of AMI, FLX, or LTG, nor was CBZ accumulation affected by co-exposure to the other compounds (*t*-tests, $p > 0.05$). This latter result is consistent with a recent report that LTG does not impact CBZ accumulation in cucumber plants.[25] Leaf accumulation of epCBZ was lower in the plants co-exposed to AMI or FLX than in those exposed to CBZ alone (Figure 4.5). Root concentrations of epCBZ were not affected. Lamotrigine did not affect CBZ metabolite accumulation (*t*-tests, $p > 0.05$; data not shown). Concentrations of diOH-CBZ were not affected by the presence of other pharmaceuticals (Dunnett's test, $p > 0.05$).

The interaction observed between CBZ metabolism and exposure to AMI/FLX may occur at the transcriptional (modulation of expression) or enzymatic (competitive or non-competitive inhibition) levels. The compounds used in this study have many inhibitory and inductive effects on the enzymes responsible for drug metabolism in humans, which could occur in spinach plants as well. In humans, FLX inhibits several CYP450 enzymes, including those responsible for metabolizing AMI, CBZ, and itself,[34] while CBZ induces the CYP450s responsible for metabolizing AMI, FLX, and itself.[34] In spinach, AMI and FLX may have similar interactions or induce enzyme(s) that transform(s) epCBZ. Co-exposure to LTG was

previously reported to affect accumulation of diOH-CBZ in cucumber leaves.[25] The absence of such an effect in the present study may reflect differences in exposure time (4 vs. 7 days), concentration (5× lower in the present study), or the identities or expression of metabolic enzymes.

4.5.4. Mole Balance. We conducted a mass (mole) balance to estimate the extent of compound losses in our system. We compared the number of moles initially added to the nutrient solution to the moles in the plant and the nutrient solution at the end of the exposure period, including those of measured CBZ metabolites (Figure 4.6). For FLX and AMI, mole balances were incomplete for the 100 $\mu g \cdot L^{-1}$ exposure, indicating compound loss somewhere within our system. Fluoxetine loss did not differ between treatments containing and lacking plants ($p > 0.05$), suggesting the loss occurred in the nutrient solution reservoir due either to transformation or sorption to container walls. All components of the hydroponic systems were sterilized prior to experiments; however, the plants were not housed in sterile growth chambers and degradation by microorganisms introduced to the system via air cannot be ruled out. No other compounds were degraded in no-plant controls. We therefore hypothesize that AMI losses were due to *in planta* transformation. Summing the moles of CBZ (+ epCBZ and diOH-CBZ) and LTG in the nutrient reservoir, roots and leaves, we were able to account for the amount of these two compounds added to our experimental systems. We note that the mixture effects (*vide infra*) on CBZ metabolism were too small to be detected using a mole balance approach, and that single and dual exposure treatments were combined for Figure 4.6 and all subsequent data analyses. We note that the sizes of the plants and nutrient reservoirs were such that in some cases the amount of compound taken up by the plants was small relative to the total amount of compound in the experimental system. We observed considerable inter-replicate variability in the mass of compounds accumulated in the plants. We hypothesized that at least some of this variability was due to differences in transpiration volumes among plants (Table C4).

4.5.5. Transpiration-Based Accumulation. To our knowledge, three prior studies explicitly addressed correlation between transpiration and plant accumulation of nonvolatile polar and ionizable, organic compounds,[12,13,25] yet none present a compound-level comparison of structurally diverse

contaminants, and only one considers the effects of *in planta* transformation.[25] We used a simple model for transpiration-driven accumulation to compare water and contaminant uptake by the plants. We considered the transport of water from the roots through the xylem (the main vascular tissue that moves water from plant roots to leaves, consisting of hollow tracheary elements connected by perforated plates and walls)[43] to the leaves where the majority of transpiration takes place. If contaminant accumulation was driven solely by water flow, assuming no barriers hindering contaminant mobility, no *in planta* metabolism, and no phytovolatilization, the mass of compound in the whole plant would equal the product of the average contaminant concentration in the external solution and the volume of water drawn in. We term this hypothetical value the transpiration-based accumulation (TBA):

$$\text{TBA} = \bar{C}_{\text{solution}} \times V_{\text{transpired}} \qquad \text{(Eq. 4.1)}$$

where $\bar{C}_{\text{solution}}$ is the average mass concentration of the compound in nutrient solution over the exposure period (computed as the average of the initial and final solution concentrations), and $V_{\text{transpired}}$ is the volume of water transpired by the plant. The TBA is not intended to provide an accurate estimation of contaminant uptake; rather, we used this value primarily to assess the importance of water flow through the plant on contaminant accumulation. Correlation with TBA indicates a direct relationship between contaminant accumulation in the plant and transpiration. Deviations from TBA could suggest the operation of processes limiting or enhancing accumulation. The pathways of contaminant and water movement through the roots to the xylem differ. Uptake of water and its subsequent transport through the xylem is driven by pressure and chemical potential gradients, but uptake into root cells (required to reach the xylem) depends on aquaporins, as water cannot easily diffuse through the lipid bilayer call membrane. Aquaporin channels do not permit substances other than water (with a few specific exceptions) to pass. In contrast, nonionic contaminants are thought to mainly translocate across root-cell membranes by a passive diffusion mechanism. Actual accumulation in leaves that is lower than TBA may indicate *in planta* transformation or that the compound is partially blocked from entering the transpiration stream by the Casparian strip.

Actual accumulation that is higher than TBA suggests the presence of an additional driver for uptake of the contaminant into the plant.

We calculated TBA for each plant in our experiments and compared this value to the actual accumulation in the leaves, roots, and whole plant; for CBZ these analyses included CBZ mass equivalent of measured metabolites. Figures 4.7 and C2 show the correlations for the 100 and 1 $\mu g \cdot L^{-1}$ (AMI, CBZ, LTG) exposures, respectively. Only the higher exposure concentration was used for FLX. Regression slopes did not differ for plants exposed to 1 vs. 100 $\mu g \cdot L^{-1}$ (Table C5).

Leaf accumulation correlated strongly with TBA and explained a large proportion of inter-replicate variability (Table C5). This was expected since as the transpiration stream is the main pathway for root to leaf transport and the majority of transpiration occurs from the leaves. Accumulation in the whole plant (all compounds) was similarly correlated with TBA. Accumulation in the roots correlated with TBA for CBZ, LTG, and FLX, although the amount of inter-replicate variability explained by the correlation was smaller and the slopes of the regression of actual vs. transpiration-based accumulation were shallow (Table C5). The weak correlations for the roots likely reflect sorption to root membranes and negatively charged cell walls (for cationic species)[25] and ion-trapping in root cell vacuoles (for cationic species), which are less related to transpiration than is transport to the leaves via the xylem.

For the compounds exhibiting actual accumulation lower than TBA (viz. CBZ, FLX, and LTG), some of the difference may be attributable to *in planta* transformation, although our mass balance calculations were unable to provide evidence for this (beyond measured CBZ metabolites). For ionic FLX and LTG, the lower accumulation than TBA may also indicate difficulty diffusing across root cell membranes to pass the Casparian strip on their way to the xylem. In the case of AMI, actual accumulation was similar to or slightly exceeded TBA for leaves and whole plants (Figures 4.7 and C2). More AMI was lost from solution than was detected in plant tissue, and AMI was not degraded in no-plant controls. Strong correlations between transpiration and AMI loss from solution and between AMI loss from solution and phytoaccumulation suggest that the missing fraction of AMI was metabolized *in planta* and that total AMI

uptake into the plant is higher than the amount measured at the end of the exposure period (Figure 4.8). Phytovolatilization is unlikely for any of the studied compounds due to their high octanol–air partition coefficients (Table 4.2).[64]

4.5.6. Plant Uptake Processes. The results described above demonstrate that transpiration may be a strong predictor for the accumulation of structurally diverse pharmaceuticals in above-ground tissues. Transpiration is driven principally by water potential differences and is regulated by stomatal openings in leaves and stems and by aquaporins in cell membranes.[65] The pathways taken through the plant root to the vasculature by water molecules and organic contaminants differ, but both must cross a lipid bilayer membrane to enter the symplast (inside of cells) and travel through plasmodesmata (interconnecting channels between cells) to circumvent the waxy barrier of the Casparian strip to get to the xylem.[7,43] When molecules enter the plant root, they first enter the apoplast (space between root cells), where they can remain dissolved, sorb to the cell walls and membranes, or permeate through root cell membranes to enter the symplast.[44] Water movement across root cell membranes is facilitated by aquaporins, as water cannot easily diffuse through lipid bilayers. However, these passive transporters do not accommodate large or charged molecules and are generally specific for water (though some also transport small molecules such as glycerol, urea, or CO_2).[43,45] In contrast, nonionic contaminants are thought to mainly translocate across lipid membranes via passive diffusion. Charged molecules have low membrane permeability due to the high free energies of transfer across the hydrophobic core of lipid bilayers.[46] Furthermore, electrostatic attraction of organic cations to the negatively charged cell walls within the apoplast is expected to lead to retention within the roots. Organic solutes in the apoplast can travel through the root along the pressure gradient toward the xylem, but the Casparian strip blocks entry into the xylem flow to the leaves.

Comparison between actual and transpiration-based accumulation provides insight into the mechanisms driving (or blocking) flux of organic compounds into the xylem. For CBZ, FLX, and LTG, lower accumulation in the plant than would be predicted based on consideration of transpiration alone, especially without clear evidence that this disparity is due to *in planta* metabolism, implies that these

compounds are somehow partially blocked from entering the plant along with water. For LTG and FLX, which are both at least partially positively charged at the apoplastic pH (~5.5),[7,44] the higher accumulation in the roots relative to leaves is consistent with entry to the xylem being hindered by association with cell walls and membrane surfaces and lower permeation across root cell membranes and with previous research.[25,31,32,37–40] Lamotrigine (pK_a = 5.7) may also be subject to ion trapping in root cell vacuoles (pH ~5.5).[28,37,43,50]

As a neutral molecule with a relatively high membrane lipid-water partition coefficient (K_{lipw}; Table 4.1), CBZ is expected to diffuse across cell membranes fairly easily. Both our data and previous studies on other plant species show that CBZ and its metabolites accumulate primarily in leaves, with much lower concentrations present in roots and fruit.[18,25,31,32,37,40] We hypothesize that this is due to high mobility through the symplast and xylem, but minimal movement through phloem, which transports sugars and other molecules from leaves to roots and fruit.[43]

Interestingly, accumulation of AMI was higher than would be predicted based solely on transpiration, despite clear evidence of *in planta* metabolism (Figures 4.6 and 4.8). This implies that an additional mechanism may contribute to AMI uptake. Amitriptyline possesses a pK_a of 9.4 and is present primarily as the cationic species over the pH range found in plants (~5-8). Plant root cells possess negative transmembrane potential, making accumulation of cations in the symplast energetically favorable.[44] The K_{lipw} for neutral AMI is higher than those for the other cationic compounds studied (Table 4.2) allowing more ready permeation of lipid bilayer membranes. We consider ion trapping is unlikely to be responsible for the high uptake of AMI, as the pH of root cell cytoplasm (~7.5) is higher than that of the apoplast (~5.5).[43] Trapping of AMI cations in root cell vacuoles (pH ~5.5)[43] is possible, but would not be expected to lead to the observed high level of AMI accumulation in the leaves. We cannot rule out the possibility that AMI transport into spinach root cells is facilitated by a transporter protein. A previous study found that AMI accumulation in strawberries was primarily in the roots, with minimal translocation.[33] A transporter protein that is present in spinach but not strawberries may account for the observed difference.

Plants have many transporters that are responsible for moving nutrients, hormones, and secondary metabolites through the plant.[44] Passive transporters are important for plant accumulation of positively charged molecules that are essential for plant nutrition such as potassium, calcium, and urea,[44] and are necessary for elongation and growth, maintaining membrane potential, and responses to stress and pathogens.[47] To our knowledge, proteins that transport xenobiotic organic cations in plants have not been specifically identified, but transporters have been implicated in uptake of the cationic antidiabetic drug metformin,[48] phenanthrene uptake into cells (mediated by a proton symporter),[49,50] and antibiotic resistance in plants (connected to membrane transporters such as members of the ATP-binding cassette (ABC) and major facilitator superfamilies of proteins).[51,52] These latter proteins are found in all organisms.[53]

Transport of pharmaceuticals in mammals has been extensively studied; some controversy exists over the relative importance of diffusion through membranes vs. transporter proteins in movement into cells and through tissues.[54] Diffusion is often assumed to be the main mechanism for drug absorption and distribution, but does not explain why drugs can concentrate in specific tissues.[54] For drugs that have been specifically studied, substantial evidence exists for protein-mediated transport.[54] Mammalian systems may provide clues for identifying specific transporter proteins in plant systems. For example, AMI but not FLX appears to be a substrate for the drug efflux ABC transporter Mdr1a P-glycoprotein in the blood-brain barrier, which protects the brain from potentially harmful endogenous and exogenous substances.[55] We note that AMI and FLX otherwise behave similarly to each other in mammalian systems; both are widely distributed throughout the body and exhibit approximately 95% plasma protein binding.[56,57] In plants, similar ABC transporters function in transport of auxins, secondary metabolites, and xenobiotic compounds.[53] Spinach may possess an ABC transporter that functions similarly to the mammalian Mdr1a P-glycoprotein, and recognizes AMI, but not FLX or LTG, as a substrate. The molecular details of ABC transporter substrate recognition are largely unknown and do not seem to correlate well with gene sequence, precluding further identification of a putative transporter in spinach based on data from mammals.[66,67]

4.5.7. Importance of Exposure Time. The results discussed above demonstrate a strong correlation between transpiration and accumulation of the investigated compounds in spinach over a 7-day exposure period. In the field, plants would be exposed to pharmaceuticals intermittently throughout their development for time periods exceeding 7 days. We therefore tested whether the correlations with transpired water would hold for plants harvested at varying exposure time points. A time series experiment was conducted with CBZ to examine accumulation over a 14-day period. We provided each plant with 400 mL of nutrient solution containing ~100 ng·mL^{-1} CBZ at the beginning of the experiment, and harvested three to four plants at each time point (1, 4, 7, and 14 days). Nutrient solution was replaced on day 7 for the plants harvested at day 14. We chose CBZ because as a neutral molecule, it is expected to diffuse most readily through cell membranes and therefore reach a steady-state concentration more rapidly than the other compounds studied, and we could measure two of its metabolites. While still not representative of field conditions, testing multiple time points provides insight to the broader applicability of our results.

Leaf concentrations of CBZ plateaued by day 7 (ANOVA) while that of its primary metabolite continued to increase over 14 days (Figure C3); root concentrations of CBZ appeared to plateau by day 4. The linear correlation between actual accumulation and TBA remained consistent for only the first 7 days of exposure (Figure 4.9). Changes in CBZ accumulation were not due to changes in exposure concentration or transpiration. Initial exposure concentrations for plants harvested on day 14 were 106 ± 2 ng·mL^{-1} and 102 ± 2 ng·mL^{-1} on days 0 and 7 respectively, and transpiration did not differ between the two 7-day periods (paired *t*-test, $p = 0.42$). Cucumber plant accumulation of CBZ (in terms of tissue concentrations) was similarly reported to decrease over time, although this decrease was attributed to metabolism to epCBZ.[25]

These data indicate that accumulation may become decoupled from transpired water volume once a specific level of accumulation has been attained. While the pharmaceutical molecules enter the root with water influx (via convective transport), an opposing diffusive flux out of the root is established as higher concentrations build up in the apoplast, which could reduce accumulation over time. 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, and compounds that travel through the root via different pathways may exhibit different time-dependent accumulation trends. Furthermore, metabolic enzymes may be induced by prolonged compound exposure or may be more/less active at different plant growth stages. This is a topic that merits further investigation, as much existing research focuses on plants exposed for only short time periods.

4.5.8. Broader Implications. We hypothesized that the mechanism of accumulation of some compounds may be altered by co-exposure with other contaminants via altered metabolism. We chose to determine whether binary mixtures may affect individual contaminant accumulation using pharmaceuticals that are known to affect each other's metabolism in mammalian systems, and we show that metabolism of CBZ was altered in plants co-exposed to AMI or FLX, although the measured metabolites comprised a small fraction of total phytoaccumulated CBZ (< 4%). We hypothesize that these effects may be more pronounced at higher exposure concentrations, as we also observed mixture effects on phytotoxicity of these compounds. Other plant species such as tomatoes metabolize CBZ to a larger extent than we found in spinach, and other pharmaceuticals such as ibuprofen and diclofenac are degraded to a larger extent than CBZ.[18,20,21,58] Mixture effects may be more pronounced when metabolism is more extensive, 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. From a mechanistic perspective, this indicates that mixture exposure may alter contaminant accumulation, and mixture effects observed in mammalian systems can provide guidance for investigation of plant metabolism interactions.

Transpiration and accumulation in leaves and whole plants were strongly correlated for each compound in our study, and the relationship between actual and transpiration-based accumulation varied across the compound set we investigated. The correlations we observed were much stronger than those reported by Dodgen et al.;[12] however, those authors reported correlations for all compounds taken together rather than for individual ones. Lamshoeft et al.[13] observed a relatively strong correlation between

transpiration and organic compound uptake (R^2 = 0.80), but did not find clear differences among their tested compounds with molecular masses below 394. This may have been due to the structural similarity of many of the compounds studied. Comparisons between observed phytoaccumulation and accumulation predictions based on transpiration can be used to deduce mechanisms of transport of contaminants through root tissue into the xylem.

Most current literature 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.[31–33,40] Our AMI results suggest that in some cases plant transporter proteins may be important for accumulation of certain pharmaceuticals, as has been previously demonstrated for phenanthrene.[49,50] If this is the case, models of plant accumulation of organic contaminants[59] would require reconceptualization. Literature on plant biochemistry and mammalian uptake and accumulation of xenobiotics may point to possible mechanisms of uptake and accumulation of these contaminants in plants. Focus on the biological aspects of plant accumulation of xenobiotics is important, and in addition to contaminant physico-chemical properties, for increasing understanding of plant accumulation of wastewater-derived organic contaminants.

Associated Content. Supporting information (SI) is available as Appendix C. 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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Hedman for use of instruments and lab space. This work was funded by USDA HATCH (WIS01647 and WIS01936) and the U.S.-Israel Binational Agriculture Research & Development Fund (US-4771-14R).

4.6. TABLES AND FIGURES

Table 4.1. Physico-chemical properties of compounds screened via root length assays.

Compound	Use	Molecular Mass	water solubility ($mg \cdot L^{-1}$)[a]	pK_a [b]	log K_{ow} [b]	log D_{ow}[c] at pH 5.7	log K_{lipw} [d]
Metformin	antidiabetic	165.625	1380	12.4	-2.6	-2.60	-1.64
Caffeine	stimulant	194.191	11000	10.4	-0.07	-0.07	0.02
Cotinine	nicotine metabolite	176.219	117000	4.79 [e]	0.07 [e]	0.02	-0.44
Sulfamethoxazole	antibiotic	253.278	459	1.97; 6.16	0.89	0.76	1.15
Trimethoprim	antibiotic	290.318	615	7.12	0.91	0.89	0.98
Albuterol	bronchodilator	239.311	2150	10.3	1.4	1.40	-0.13
Valsartan	antihypertension	435.519	23.4	4.73	1.499	1.45	3.74
Metoprolol	antihypertension	267.364	402	9.7	2.15	2.15	1.29
Carbamazepine	anticonvulsant	236.269	150	13.9 [e]	2.45	2.45	2.37
Lamotrigine	anticonvulsant	256.091	490	5.7 [f]	2.5	2.20	2.49
Warfarin	blood thinner	308.328	47.2	5.08	2.7	2.61	2.35
Naproxen	NSAID	230.259	51.1	4.15	3.18	3.17	3.47
Diphenhydramine	antihistamine	255.355	75.2	8.98	3.27	3.27	3.25
Gemfibrozil	fibrate	250.333	27.8	4.42	3.4	3.38	4.02
Propranolol	antihypertension	259.343	79.4	9.42	3.48	3.48	3.00
Ibuprofen	NSAID	206.281	68.4	5.3	3.97	3.82	3.38
Fluoxetine	antidepressant	309.326	1.7	9.8	4.05	4.05	3.93
Triclocarban	antibacterial/ antifungal agent	315.58	2.9	12.7 [e]	4.342 [e]	4.34	N/A [g]
Triclosan	antibacterial/ antifungal agent	289.542	6.1	7.9 [e]	4.76 [e]	4.76	4.84
Amitriptyline	antidepressant	277.403	4.5	9.4	4.92	4.92	4.77

[a] Calculated using the ALOGPS 2.1 applet at http://www.vcclab.org, which was developed from a dataset of experimental values for 20−25 °C.[60]

[b] From DrugBank.ca unless otherwise noted. Experimental values used when available.

[c] The octanol–water distribution coefficient for each molecule at pH 5.7 was estimated using logK_{ow} values and assuming D_{ow} is K_{ow} multiplied by the neutral fraction of the compound at the given pH (i.e., that the charged fraction does not partition into octanol). While this is almost certainly an invalid assumption, the resulting K_{ow} and D_{ow} values represent the possible partitioning extremes of the ionized fraction behaving just like the neutral fraction and the ionized fraction being completely lipophobic.

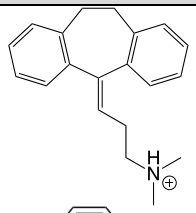
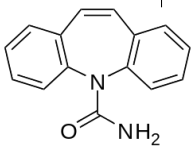
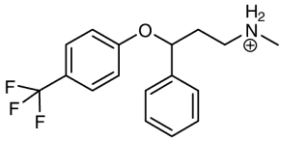
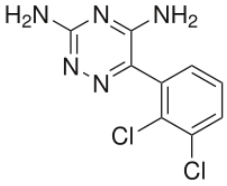
[d] The membrane lipid–water partition coefficient for the neutral form of each molecule was estimated using the poly-parameter linear free energy relationship of Endo et al.[61] using compound descriptors from the LSER Dataset 2017 for CompTox users.[62]

[e] Experimental values from PubChem.ncbi.nlm.nih.gov

[f] From Cheney et al., 2010.[63]

[g] Incomplete chemical descriptors

Table 4.2. Structures and physico-chemical properties of compounds used in uptake studies.

compound	structure	molecular mass	water solubility ($mg \cdot L^{-1}$)[a]	pK_a [b]	log K_{lipw} [c]	log K_{aw} [d]	log K_{oa}
amitriptyline		277.403	4.5	9.4	4.77	–6.78	11.7
carbamazepine		236.269	150	13.9	2.37	–11.0	13.5
fluoxetine		309.326	1.7	9.8	3.93	–6.63	10.7
lamotrigine		256.091	490	5.7 [e]	2.49	–10.0	12.6

[a] Calculated using the ALOGPS 2.1 applet at http://www.vcclab.org, which was developed from a dataset of experimental values for 20−25 °C.[60]

[b] From DrugBank.ca unless otherwise noted. Experimental values used when available.

[c] The membrane lipid–water partition coefficient for the neutral form of each molecule was estimated using the poly-parameter linear free energy relationship of Endo et al.[61] using compound descriptors from the LSER Dataset 2017 for CompTox users.[62]

[d] The air–water partition coefficient for the neutral form of each molecule was estimated using the poly-parameter linear free energy relationship of Goss[64] using compound descriptors from the LSER Dataset 2017 for CompTox users.[62]

[e] From Cheney et al., 2010.[63]

	Concentration ($mg \cdot L^{-1}$)					
Compound	**0.01**	**0.1**	**1**	**10**	**100**	**1000**
Triclosan						
Sulfamethoxazole						
Ibuprofen						
Warfarin						
Naproxen						
Amitriptyline						
Triclocarban						
Fluoxetine						
Trimethoprim						
Gemfibrozil						
Lamotrigine						
Carbamazepine						
Propranolol						
Diphenhydramine						
Caffeine						
Cotinine						
Metoprolol						
Metformin						
Valsartan						
Albuterol						

No statistically significant difference from control
>75% of control root length
50-75% of control root length
25-50% of control root length
<25% of control root length
no root growth
insoluble

Figure 4.1. Relative root lengths of *A. thaliana* seedlings germinated on and then exposed to individual PPCPs for one week compared to control root length. Compounds are ranked in order of plant sensitivity, from lowest to highest concentration required to produce a statistically significant change in root growth compared to unexposed control plans. Statistical significance was assessed using multivariate ANOVA ($p < 0.05$).

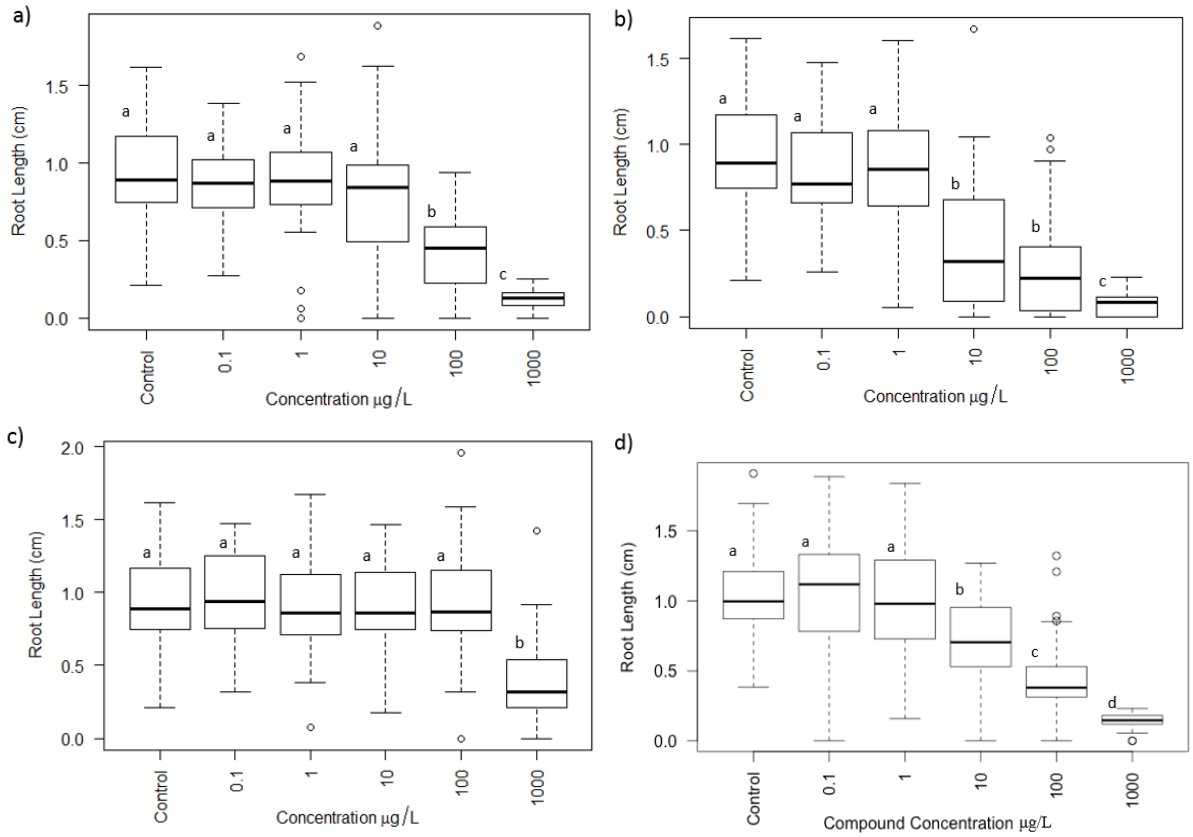


Figure 4.2. *Arabidopsis* root length decreases as a response to PPCP mixtures**:** a) full 20 compound mixture, b) full mixture except for triclosan, c) full mixture except for triclosan and sulfamethoxazole, and d) sulfamethoxazole and trimethoprim. Box plots show median, 1st, and 3rd quartiles; whiskers extend to the most extreme data point which is no more than 1.5 times the inter-quartile range, and points indicate outliers. For all plots, letters denote significant differences between root lengths (2-way ANOVA with TukeyHSD, $p < 0.05$).

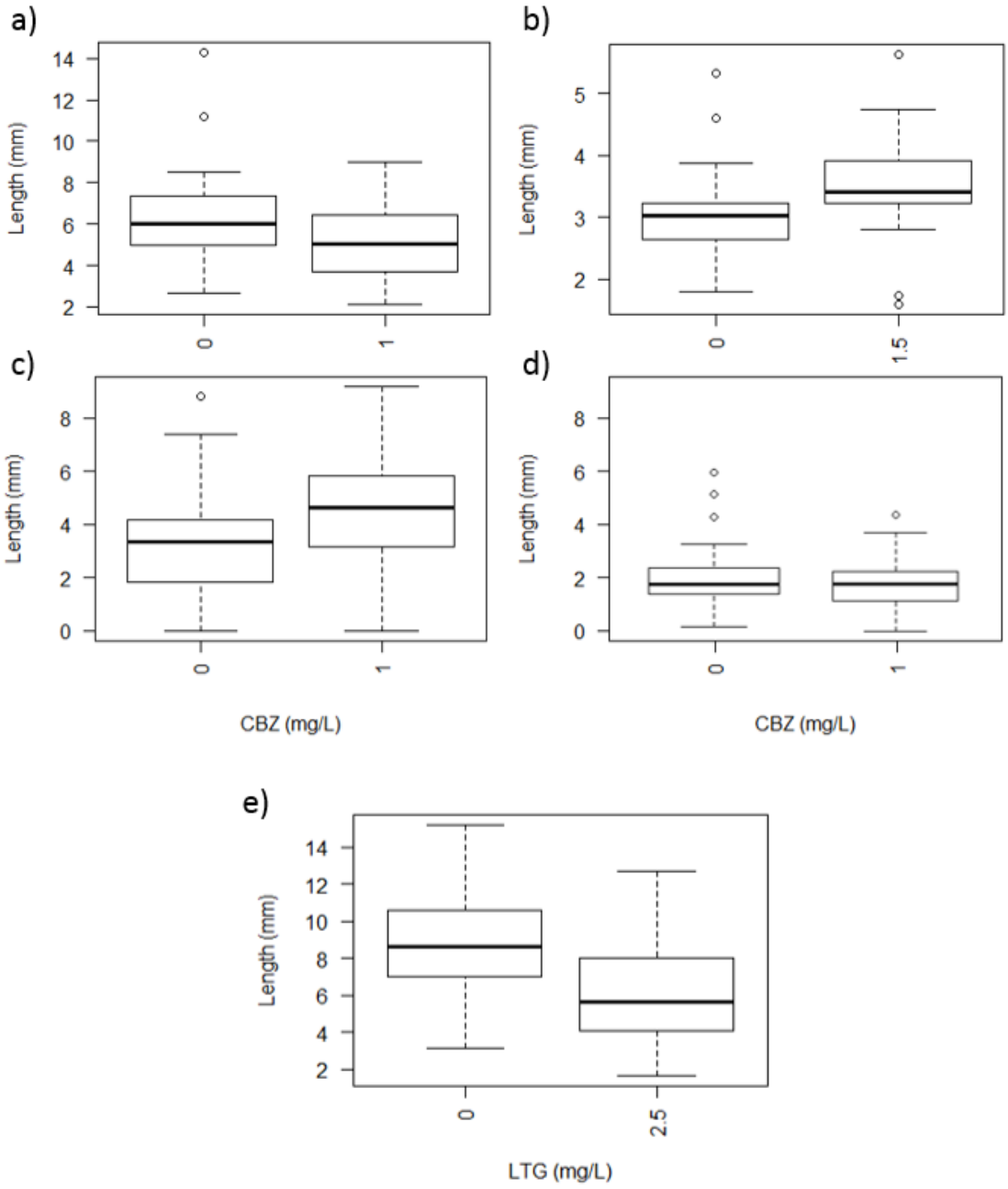


Figure 4.3. *Arabidopsis* root length decreases as a response can be aggravated for a) amitriptyline at 40 mg/L by a small addition of carbamazepine (CBZ) (Dunnett's test $p = 0.0131$), but lessened for b) fluoxetine at 30 mg/L (Dunnett's test $p = 0.0505$) and c) lamotrigine at 80 mg/L (Dunnett's test $p = 0.0153$) by a small addition of carbamazepine. Carbamazepine addition had no effect on d) warfarin (shown at 50 mg/L; Dunnett's test $p = 0.9999$). A similar physiologic effect occurs for a small addition of lamotrigine (LTG) to e) carbamazepine at 50 mg/L (Dunnett's test $p = 0.0006$). Box plots show median, 1st, and 3rd quartiles; whiskers extend to the most extreme data point which is no more than 1.5 times the inter-quartile range, and points indicate outliers.

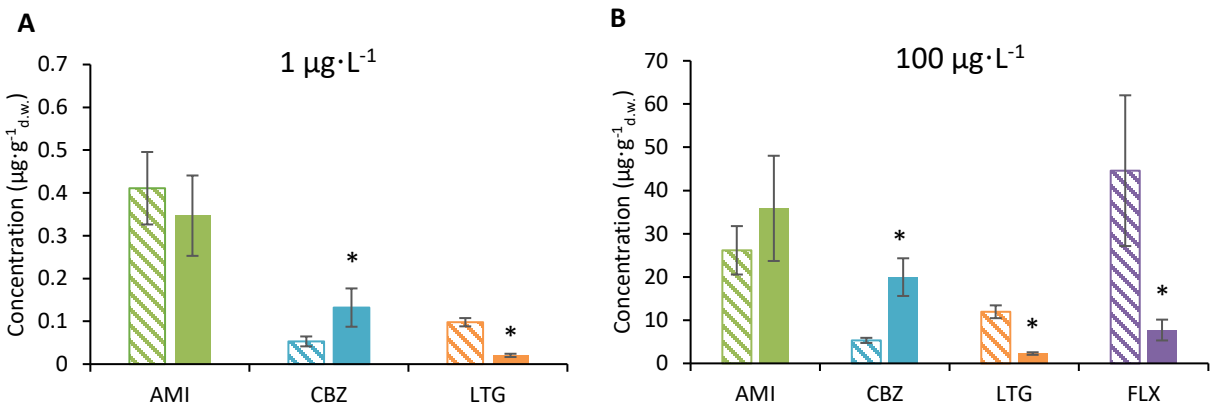


Figure 4.4. Dry weight (d.w.) concentrations of parent compounds in roots (striped bars) and leaves (solid bars) of spinach plants exposed to (A) 1 µg·L^{-1} or (B) 100 µg·L^{-1} of the indicated compounds. Data from mixture and single compound exposures were combined because accumulated amounts did not differ (p > 0.05). Error bars represent one standard deviation ($n \geq 7$). Asterisks denote significant differences between root and leaf concentrations (t-test, $p < 0.05$). Abbreviations: AMI, amitriptyline; CBZ, carbamazepine; LTG, lamotrigine; FLX, fluoxetine.

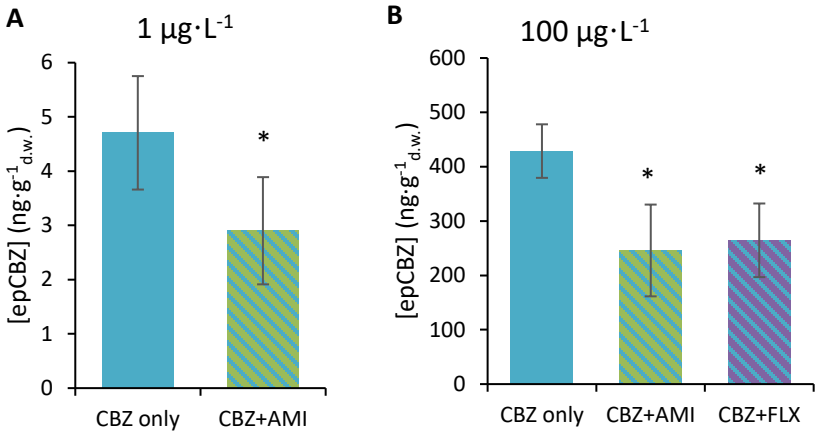


Figure 4.5. Accumulation of 10,11-epoxycarbamazepine in spinach leaves was lower in plants exposed to carbamazepine in combination with amitriptyline or fluoxetine relative to those exposed to CBZ alone at both the (A) 1 µg·L^{-1} and (B) 100 µg·L^{-1} exposure levels ($p < 0.05$, Dunnett's tests, indicated by asterisks). Error bars represent one standard deviation ($n \geq 4$). Abbreviations: AMI, amitriptyline; CBZ, carbamazepine; d.w., dry weight; epCBZ, 10,11-epoxycarbamazepine; FLX, fluoxetine.

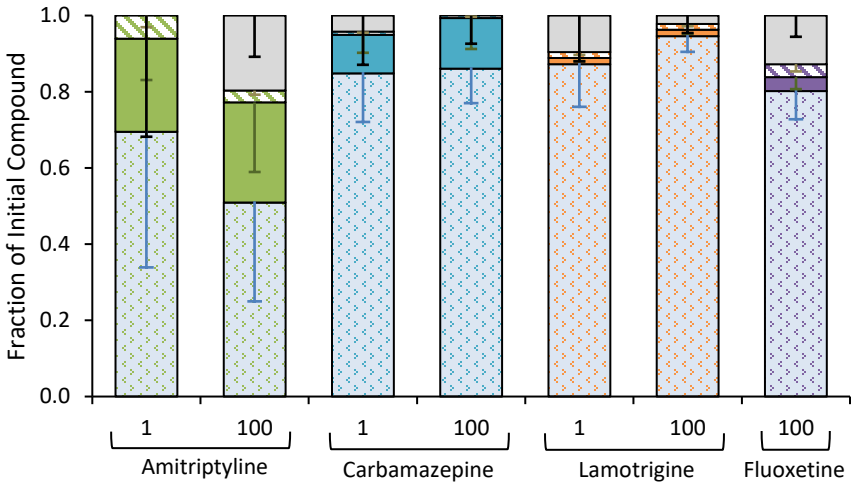


Figure 4.6. Mole balances for pharmaceuticals in experimental systems after 7-day exposure to spinach plants. Fractions shown for each treatment bottom to top of bars: solution (dotted), leaves (solid), roots (stripes), and missing (grey). The missing fraction refers to the difference between the initial amount of compound added to nutrient solution and that detected in nutrient solution and plants at the end of the exposure period. We found measurable loss of amitriptyline and fluoxetine in the 100 $\mu g \cdot L^{-1}$ exposure, but not for carbamazepine or lamotrigine at either exposure concentration or amitriptyline at 1 $\mu g \cdot L^{-1}$. Each bar combines single and dual compound exposures. Carbamazepine data includes measured metabolites. Error bars represent one standard deviation ($n \geq 7$).

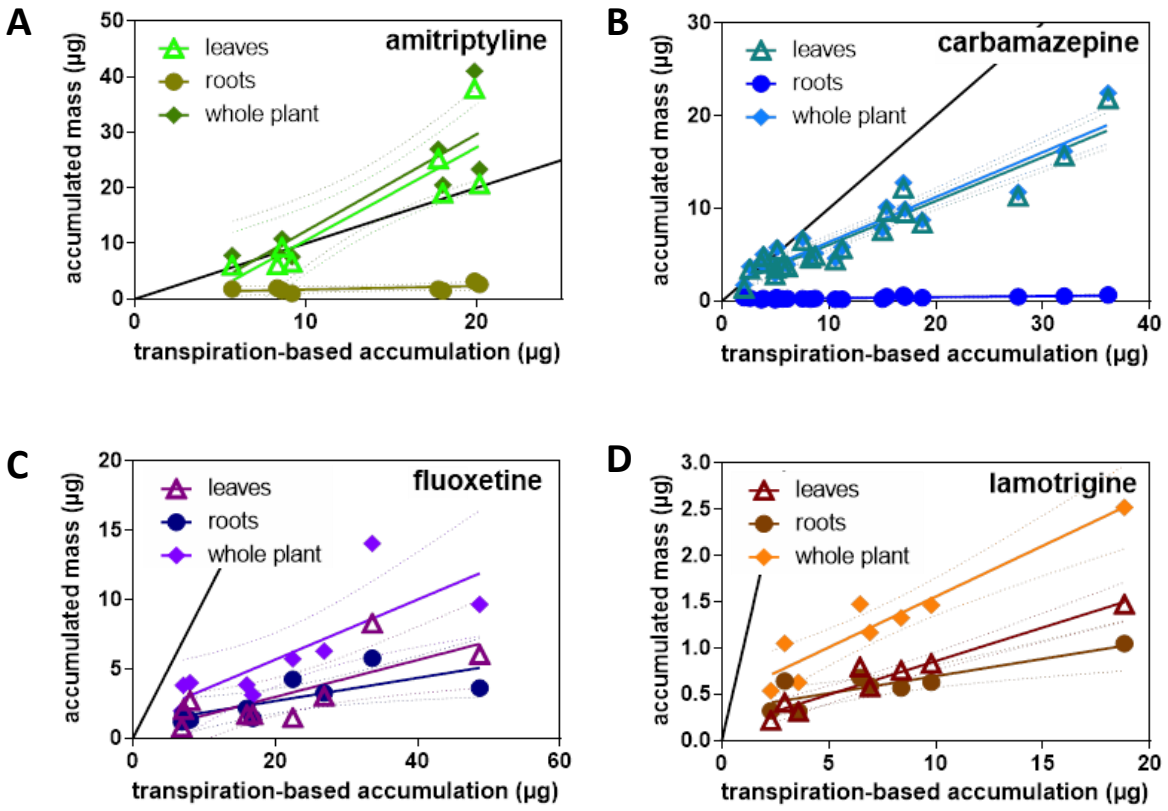


Figure 4.7. Correlation of actual accumulation with transpiration-based accumulation in leaves, roots, and the whole plant for (A) amitriptyline, (B) carbamazepine, (C) fluoxetine, and (D) lamotrigine. Dashed lines represent 95% confidence intervals. All correlations shown are statistically significant ($p < 0.05$) except for amitriptyline in roots ($p = 0.1$). Data shown are for the 100 µg·L^{-1} exposure. The black lines have a slope of 1. Transpiration-based accumulation is calculated using equation 1. Data for the 1 µg·L^{-1} exposure and regression statistics are provided in Figure S2 and Table S5, respectively.

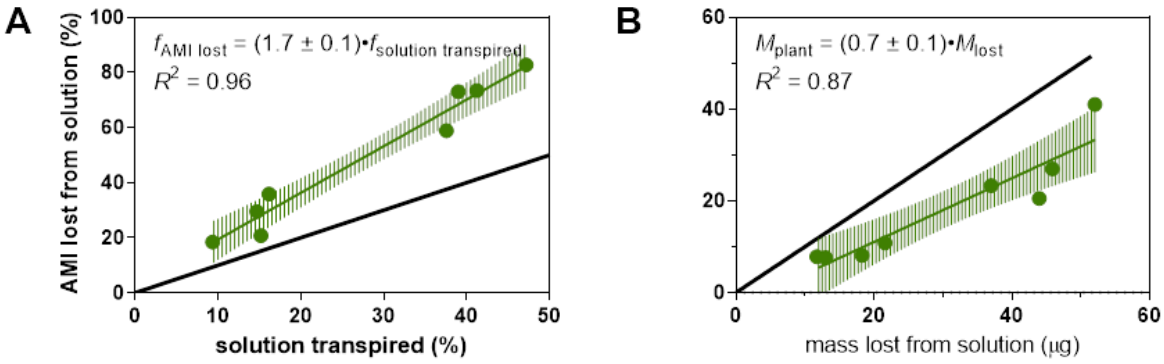


Figure 4.8. Amitriptyline (AMI) loss from solution correlated with (A) transpiration and (B) accumulation in the plant ($p < 0.001$) (green points and lines). Black lines show a slope of one. Strong correlation between transpiration and loss from solution indicates that uptake into the plant is the main mechanism for loss from solution. However, more AMI mass was lost from solution than accumulated in the plant, indicating AMI degradation in the plant. Dashed lines represent 95% confidence intervals. Equations show regression slope ± standard error. The regression *y*-intercepts are not statistically significant ($p > 0.05$).

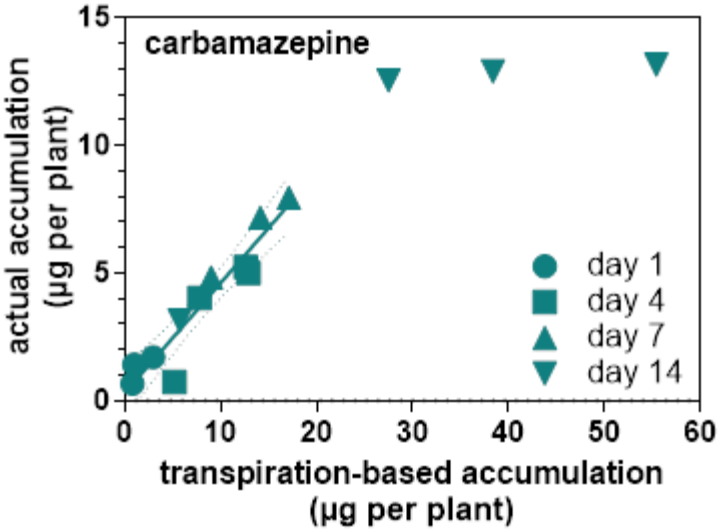


Figure 4.9. Transpiration-based and actual accumulation for whole spinach plants exposed to 100 µg·L-1 carbamazepine and harvested at various time points. The linear correlation shown is for days 1-7: actual accumulation = transpiration-based accumulation × (0.43 ± 0.05), R^2 = 0.90; dashed lines indicate 95% confidence intervals. 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.

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Chapter 5. Rhizosphere impacts on phytoavailability of ionizable contaminants Part A: Sorption

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5.1. AUTHOR CONTRIBUTIONS

SLN performed the quartz sand sorption experiments. ELM performed the soil sorption experiments and data analysis. KGK and JAP oversaw the design and execution of the experiments and the interpretation of results. ELM, SLN, KGK, and JAP wrote the manuscript.

5.2. ABSTRACT. Irrigation with treated wastewater and soil amendment with biosolids are increasingly common practices in agriculture, and result in introduction of a variety of ionizable organic contaminants (IOCs) to agricultural soils. Plants can take up and accumulate IOCs, but our understanding of the variables that control IOC phytoavailability contains many gaps, including limited understanding of processes occurring at the soil-plant root interface. Plants release ions and organic compounds from their roots in response to their environment, sometimes significantly changing the pH and chemistry of the rhizosphere (the 2-3 mm of soil immediately surrounding roots), compared to bulk soil. Root exudates may also alter sorption of pollutants, changing bioavailability and biodegradation potential. However, little is known

about how root exudates may impact sorption of IOCs, thereby impacting IOC availability to plants. We measured sorption of the cationic anti-epileptic drug lamotrigine to quartz sand and three field soils with and without the presence of wheat root exudates. Soil-water distribution coefficients were similar to those reported by previous studies, with point estimates ranging from 0-9.8 $L \cdot kg^{-1}$, and increased with increasing soil organic carbon. Root exudates at environmentally relevant concentrations had little effect on sorption, likely due to their low concentration compared to organic carbon naturally released to the pore water from the soils. Lamotrigine speciation was the major driver of sorption; sorption increased with decreasing pH, corresponding to an increase in the cationic fraction of LTG. Modeling showed neutral lamotrigine contributed more to total sorption than was expected.

5.3. INTRODUCTION

Irrigation with treated wastewater and soil amendment with biosolids are increasingly common practices in agriculture, and result in soil contamination and crop plant exposure to a variety of ionizable organic contaminants (IOCs) that are not completely removed during conventional wastewater treatment. A wide variety of literature has shown that plants can take up and accumulate IOCs, but our understanding of the variables that control IOC phytoavailability still contains many gaps, hindering development of models to predict accumulation of IOCs in edible plant tissue.[1]

One of the important gaps requiring investigation is the behavior of contaminants at the interface between the soil environment and the plant root, known as the rhizosphere. This 2-3 mm region immediately surrounding the root is where plants sense and respond to soil conditions. Roots release ions and organic compounds to maximize nutrient availability, as defense, and to initiate and modulate dialogue with soil microbiota.[2–5] These exudates are complicated mixtures of inorganic ions, organic and amino acids, sugars, chelating agents, and various secondary metabolites, and their release can result in significant changes to the pH and chemistry of the rhizosphere.[6] Consequently, rhizosphere properties can vary significantly from the bulk soil. Exudates may change rhizosphere pH, alter soil chemistry and soil-contaminant interactions,

and ultimately alter contaminant bioavailability and biodegradation potential.[7] Thus, consideration of only bulk soil properties may mischaracterize the speciation and sorption of IOCs in the zone where they are immediately available for transport into plant roots and lead to inaccurate prediction of phytoavailalblilty.

The types of nutrients available to plants can have consequences for the rhizosphere, as plants exude ions and organic acids to maximize nutrient availability to their roots. Plants receiving nitrate as their primary nitrogen source can increase rhizosphere pH up to 2 units higher than plants provided ammonium.[2] Soil pH can also affect contaminants, altering pore water concentrations and thereby changing the contaminant phytoavailability. Plant-driven alteration of rhizosphere pH affects wheat, tomato, and rapeseed accumulation of copper from contaminated soils,[8–10] and arsenic hyperaccumulating ferns mobilize arsenic from soils via release of phytic acid and oxalic acid.[11]

Previous studies have also shown exudation can alter contaminants and contaminant-soil interactions beyond changes in pH. Root exudates can act as natural ligands for and significantly alter the properties of graphene oxide nanoparticles.[12] Root exudates can also alter availability and biodegradation of nonpolar organic contaminants. Root exudation of carboxylic acids is speculated to be one of the predominant factors driving petroleum biodegradation, as these compounds provide an easily degradable energy source and increase phosphorus available to soil microbiota while also enhancing petroleum hydrocarbon bioavailability.[13] Similarly, pyrene degradation by soil bacteria was enhanced by addition of ryegrass root exudates.[14] LeFevre et al.[15] found that soil incubated with root exudates from Cord Grass (*Spartina pectinata*), Porcupine Sedge (*Carex hystricina*), and Purple Prairie Clover (*Dalea purpurea*) had a lower soil-water distribution coefficient (K_d) for naphthalene compared to exudates pre-metabolized by soil bacteria and no-exudate controls ($p > 0.1$). However, to our knowledge, the effects of root exudates on IOCs and their interactions with soil have not been previously addressed.

In this study, we examined the effect of root exudates on sorption of a model IOC, the phenyltriazine anticonvulsant lamotrigine (LTG; conjugate acid $pK_a = 5.7$[16]) to ultrapure quartz sand and three field soils with differing properties over a range of pH values. This pharmaceutical has been detected

in reclaimed wastewater (concentrations up to 488 $ng \cdot L^{-1}$)[17,18] and accumulates in effluent-irrigated plants,[19,20] including in carrots irrigated with reclaimed wastewater to levels exceeding the threshold of toxicological concern (a conservative indicator of when additional study of toxic effects is warranted[21,22]) at normal ingestion rates.[20] We hypothesized increased soil organic carbon and cation-exchange capacity would increase LTG sorption to soil, but that exudates would decrease sorption of LTG through a combination of altering pH and competing with LTG for sorption sites. The results of these studies helped inform our understanding of LTG phytoaccumulation, presented in part B (Chapter 6).

5.4. MATERIALS AND METHODS

5.4.1. Exudate Collection. Durum wheat grass (*Triticum durum)* seeds were sterilized to minimize fungal contamination by soaking in 70 % ethanol for 60 seconds followed by 50 % bleach for 20 minutes, and then were rinsed thoroughly before soaking in sterile ultrapure water in the dark for 6 hours. Soaked seeds were sprouted on sterile moist paper towels, and then transferred to hydroponic nutrient solution after 4 days incubation at room temperature. The hydroponic system consisted of 1 L Erlenmeyer flasks wrapped in aluminum foil to prevent algal growth topped with 1.2 mm polypropylene mesh; the seeds sat on top of the mesh while the roots grew down into the solution. Plants were kept in a plant tent with a 16 hour on/8 hour off light cycle. Plants were grown in two types of nutrient solution (Table 5.1): nitrogen was provided as either nitrate only or a combination of ammonium and nitrate. Other macronutrients were adjusted so that all nutrient solutions had the same overall ionic strength. All nutrient solutions were adjusted to pH 5.7 ± 0.05 using potassium hydroxide (KOH). Nutrient solution was replenished as needed to maintain liquid volume from loss due to evaporation and transpiration. At the end of the 21 day growth period, plants were harvested and weighed, and nutrient solution containing root exudates was collected for analysis and use in sorption experiments. Exudate solutions were filtered through 0.45 µm nylon filters and stored in sterile amber glass bottles at 4 °C after collection. Total exudate concentration was determined by total organic carbon (TOC) analysis using a M5310 C Laboratory TOC Analyzer. Carbohydrate content of the exudates

was measured using an anthrone colorimetric assay.[23] The aromatic fraction of the exudates was estimated from the specific UV absorption at 254 nm using the relationship developed by Weishaar et al.[24] for dissolved organic matter.

5.4.2. Sorption of Lamotrigine to Quartz Sand. We conducted a series of experiments to assess LTG adsorption to ultrapure quartz sand. In each experiment, we equilibrated 8.00 g sand for 24 h with 4.00 mL of solution containing 100 μg·L^{-1} LTG. The pH was controlled via addition of 3 mM acetate, MES, or HEPES buffer. After equilibration, samples were centrifuged (20 min, 4637 ×*g*) and filtered through 0.2 μm PTFE filters. Then pH was measured in each sample and LTG concentrations in the solution phase were measured by HPLC-MS/MS. The amount of adsorbed LTG was determined by the difference between solution concentrations in treatments lacking or including sand. Distribution coefficients (K_d) were determined by dividing adsorbed LTG concentrations by the respective concentrations in the solution that had been equilibrated with sand (Eq. 5.1).

5.4.3. Sorption of Lamotrigine to Field Soils with and Without Exudates. Sorption experiments used three types of soil with varying properties: Richford loamy sand, Elliott silty clay loam, and Bluestem sandy clay loam (Table 5.2), hereafter referred to as Richford soil, Elliott soil, and Bluestem soil, respectively. Experimental conditions for batch sorption tests were chosen to reflect wheat uptake experimental conditions (see Part B). All soils were sterilized by autoclaving twice on gravity cycle at 127 °C for 30 min prior to sorption experiments (soils were not plated out to check for sterility). To measure sorption, soils were equilibrated in the dark at 25 °C for 48 hours with nutrient solution, LTG, and an addition or absence of root exudates. Preliminary experiments were performed to ensure that equilibrium was reached within 48 hours. Each treatment had three replicates containing 1.0 ± 0.01 g soil and 10 mL of solution. Samples without exudates contained only nutrient solution, while samples with exudates contained a mixture of nutrient solution and root exudates added to final concentration of 3 mg/L total organic carbon (TOC). This concentration of root exudates was chosen as an environmentally relevant concentration based on calculations done by LeFevre et al.[15] Lamotrigine concentrations were 200 ng/L (0.00078 mM), added

in methanol (total methanol volume = 10 µL). For Richford and Elliott soils, a LTG concentration an order of magnitude lower (20 ng/mL) was also used. Soil-free controls were performed in triplicate for each nutrient solution, exudate, and LTG treatment.

Sorption was measured in at least three pH values for each soil. Bluestem soil had three pH values: high (~9), natural (~6), low (~4). Richford and Elliott soil had 4 pH values: high (~8), natural (~6.5), medium (~5.7), and low (~4). The pH was adjusted using KOH or HCl. Volumes of acid/base required for equilibration at desired pH values were assessed in preliminary experiments without LTG. Samples were equilibrated in the dark on a shaker plate for 48 hours and then centrifuged at 4637 $\times g$ for 30 minutes. Supernatant was collected and filtered through 0.22 µm PTFE syringe filters before analysis.

5.4.4. Liquid-Chromatography-Tandem-Mass-Spectrometry Analysis. Analytical methods are taken from Nason et al.[25] (additional details in Chapter 6 and Appendix D). For separation of lamotrigine from the soil sorption supernatants, we used an Agilent 1260 high-performance liquid chromatography (HPLC) with 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). We used a gradient of 5% to 95% organic phase and column temperature was held at 30 °C. For detection of lamotrigine, we used an Agilent 6460 triple quadrupole mass spectrometer with electrospray ionization in positive mode. The precursor ion *m*/*z* for lamotrigine is 256.0 and we used 43.1 as our quantitative ion and 108.9 and 58.1 as our qualitative ions.

5.4.5. Sorption Analysis. Solid-water distribution coefficients (K_d) for each soil ± each nutrient solution exudate mixture were calculated using the equation

$$K_d = \frac{C_{soil}}{C_{solution}} = \frac{C_{soil\ free\ controls} - C_{solution}}{C_{solution}} \quad \text{(Eq. 5.1)}$$

where $C_{solution}$ is the concentration of LTG remaining in solution after equilibration with soil and $C_{soil\ free\ controls}$ is the concentration of LTG in solution of the soil free controls after equilibration.

Richford and Elliot medium pH sorption data were fit to the linearized Freundlich (Eq. 5.2) and Langmuir (Eq. 5.3) isotherm models in Origin 2018b using the fitting function builder tool and the Levenberg-Marquardt algorithm to estimate isotherm constants:

$$C_s = 10^{n \log C_w + \log K_F} \quad \text{(Eq. 5.2)}$$

$$\frac{1}{C_s} = \left(\frac{1}{C_{max} K_L}\right)\left(\frac{1}{C_w}\right) + \left(\frac{1}{C_{max}}\right) \quad \text{(Eq. 5.3)}$$

where C_s is the concentration sorbed to the soil, C_w is the concentration in the aqueous phase, K_F is the Freundlich constant or capacity factor, n is the Freundlich exponent (a measure of the cumulative magnitude and distribution of sorption site energies), C_{max} is the maximum achievable surface concentration of the contaminant, and K_L is the Langmuir equilibrium constant. Bluestem data were not fit to these equations because of the limited concentration range tested (small distribution of C_w).

To assess individual sorption coefficients for the cationic and neutral forms of LTG, the entire sorption datasets were fit with an empirical model assuming the overall sorption coefficient at any pH was the sum of contributions from sorption of cationic species and neutral species:

$$K_d = f_{cationic} K_{d+} + f_{neutral} K_{d0} \quad \text{(Eq. 5.4)}$$

where $f_{cationic}$ is the fraction of LTG present in its ionized form, K_{d+} is the solid-water distribution coefficient for cationic lamotrigine, $f_{neutral}$ is the fraction of LTG present in non-ionized form, and K_{d0} is the solid-water distribution coefficient for neutral LTG. Nonlinear curve fitting and model comparison was performed in Origin 2018b using the fitting function builder tool and the Levenberg-Marquardt algorithm to fit Eq. 5.4 to the LTG soil sorption data. Best fits were obtained with both solid-water distribution

coefficients. For comparison, fits assuming only cation interactions and only neutral compound interactions were also calculated by setting K_{d0} and K_{d+} equal to 0 in Eq. 5.4, respectively.

5.5. RESULTS AND DISCUSSION

5.5.1. Root Exudate Properties. Root exudates characterization. Root exudate properties are summarized in Table 5.3. Wheat plants grown on different nutrient solutions did not differ in their exudation rates, and exuded 3 - 6 $mg \cdot g^{-1}$ fresh weight plant. Aromaticity of root exudates did not differ between nutrient solution treatments, but plants provided nitrate only solution exuded fewer carbohydrates than plants provided nitrate and ammonium (*t*-test $p = 0.0199$).

5.5.2. Sorption to Quartz Sand. We first tested LTG sorption to quartz sand at pH values of 4.5 and 8 in a KCl solution equivalent in ionic strength to the initial nutrient solutions (22 mM). Adsorption of LTG to quartz sand exhibited pronounced pH-dependence. We then investigated LTG adsorption over a range of pH values likely to occur in soil using background solutions with the same ionic strength and relative concentrations of K^+, Ca^{2+}, Mg^{2+}, and NH_4^+ as each of the nutrient solutions (Table 5.1). We found that sorption increased with pH up to pH 7, and then declined steeply beyond pH 7.5 (Figure 5.1). This result is consistent with our lab's observations of a steep decline in sorption of LTG to dissolved organic matter as pH increased, although the onset of decline was lower at approximately pH 5 (unpublished work currently in progress). Measured K_d values ranged from 0.047 to 0.24 $L \cdot kg^{-1}$.

Quartz sand possesses a point of zero charge at ~2 and therefore bears 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.[26] Neutral LTG molecules may interact with neutral Si-OH groups via hydrogen bonding and 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.

5.5.3. Sorption to Soils. This study used relatively high concentrations of lamotrigine; we expect the sorption coefficient (the relative proportion of sorbed and aqueous concentrations) at lower concentrations would be higher due to the increased ratio of sorption sites to LTG molecules in the system. The background solutions were chosen to match the plant uptake studies presented in Part B, and we also expect sorption from a solution with lower ionic strength would be higher due to reduced competition between cations for sorption sites and less charge screening of electrostatic interactions. However, soil distribution coefficients (K_d) for LTG sorption to the study soils were similar to those reported by previous studies,[27] with point estimates ranging from 2.5-8.9 $L \cdot kg^{-1}$ for Richford, 4.2-9.8 $L \cdot kg^{-1}$ for Elliott, and 0-8.9 $L \cdot kg^{-1}$ for Bluestem (Figure 5.2). Comparing sorption between soils, K_d appeared to trend with f_{oc} more than other soil properties, as Elliott soil (f_{oc} = 0.029) exhibited higher sorption than Richford (f_{oc} = 0.007) and Bluestem (f_{oc} = 0.0042) soils. This trend is consistent with our lab's observations of high LTG sorption to dissolved organic matter, especially below pH 7 (unpublished work currently in progress). This trend is consistent with previous measurements of LTG sorption to soils.[27]

5.5.3.1. Effect of nutrient solution and root exudates on sorption. The presence or absence of exudates did not affect LTG sorption for any soil at any pH (*t*-tests $p > 0.05$). Nutrient solution composition also did not appear to have much effect on LTG sorption, as K_d values did not differ significantly between nitrate only and ammonium+nitrate treatments for Elliott and Richford soils at any pH value, and for Bluestem soil at medium and low pH values. At high pH, sorption of LTG to Bluestem soil in nitrate only solution was significantly decreased by approximately 2 $L \cdot kg^{-1}$ compared to sorption in ammonium+nitrate

solution (*t*-test $p = 0.0006$), possibly due to ammonia ($pK_a = 9.25$[28]) volatilizing into the headspace and subsequently freeing sorption sites for cationic LTG.

We attribute the lack of differences between treatments to the relatively low exudate concentration compared to the amount of organic matter already present in the soils that was released into the aqueous phase during batch equilibration (3 $mg \cdot L^{-1}$ total organic carbon of exudates compared to 20, 170, and 60 $mg \cdot L^{-1}$ total organic carbon from Richford, Elliott, and Bluestem soils, respectively; Figure 5.3). In soils with lower organic matter or in cases of high root exudation, the presence of root exudates may alter sorption more than we observed.

Due to the apparent lack of effect of nutrient solution composition and root exudate presence on sorption, all sorption data for each soil are combined for all subsequent analysis.

5.5.3.2. Sorption isotherms. The equilibrium distribution between the concentration of sorbate in the aqueous phase and the concentration sorbed at a constant temperature (commonly referred to as a sorption isotherm) can exhibit a variety of shapes, which reflect the affinity of the sorbent (in this case, LTG) to the sorbate (in this case, the soils). Although it is not possible to identify a particular sorption mechanism from isotherm shape, qualitative and quantitative assessment of isotherms can provide clues for sorption mechanisms and allows prediction of sorption in similar systems. We tested the fit of LTG sorption to Richford and Elliott soils at natural pH with linear regression and linearized Freundlich and Langmuir isotherms. The linear and Freundlich isotherms had similar fits (Figure 5.4 b and d), but the linear model had the best fit in terms of R^2 values (Table 5.4). The Langmuir isotherm did not fit ($R^2 < 0$) and was subsequently excluded from the figures and table. For the Freundlich model, n was less than 1 for both soils, indicating concave down isotherms. We can therefore infer that added sorbates are bound with increasingly weaker free energies. Some assumptions of the Freundlich model may not be valid; there may

be limited total sorption sites that become saturated with increasing C_w, which is often better described by the Langmuir model. Bluestem data were unfit for model fitting due to the limited concentration range.

5.5.3.3. Trends with pH. It is well known that IOCs can engage in interactions with soil beyond hydrophobic partitioning to organic matter, including electron donor-acceptor interactions (e.g., hydrogen bonding), cation exchange, protonation, water bridging, cation bridging, and surface complexation.[29–31] Sorption of organic cations is strongly influenced by the density of exchange sites on SOM, phyllosilicate clays, and other mineral surfaces,[32–34] with the sorption of the protonated base generally stronger than that of the neutral species.[34–37] Although we did not identify mechanisms of sorption, our data are consistent with these trends.

For all soils, K_d decreased as pH increased, especially above pH 6 (Figure 5.4 a and c); thus K_d sorption decreased as the neutral fraction of LTG increased (Figure 5.2). Therefore, LTG speciation appeared to at least partially drive sorption. To further explore the effect of pH and LTG speciation on sorption, the data were fit with an empirical model (Eq. 5.4) to assess individual sorption coefficients the cationic and neutral species for each soil (Figure 5.5). Best fits were obtained with the K_d^+ and K_d^0 values listed in Table 5.5. The relative similarity of the the K_d^+ and K_d^0 values indicate that the cationic and neutral species contribute relatively equally to LTG sorption to these soils. This is surprising, as protonated species generally drive sorption.[34–37] Based on the fitted parameters, the contributions of the LTG cation and neutral species to overall sorption are illustrated in Figure 5.5. The cation only fits were obtained by setting K_{d0} equal to 0 in Eq. 5.4 and neutral species only fits were obtained by setting K_{d+} equal to 0 in Eq. 5.4. With the exception of cationic LTG sorption at low pH values (pH < 5), the single species sorption curves clearly do not describe our observations.

Although Eq. 5.4 is empirical and does not indicate the mechanism(s) of LTG interactions with soil, fits of Eq. 5.4 to sorption edges (where 100% of sorption is driven by a single LTG species) do give insight into the relative contributions of LTG species to the overall compound sorption. For both Richford

and Elliott soils, both cationic and non-ionized species appear to play a role in LTG sorption (Figure 5.5). For Bluestem soil, the similarity between best fit and cation only curves indicate cationic LTG was a large contributor to overall LTG sorption even when the dominant solution phase species was the neutral compound (Figure 5.5). This result is consistent with the low amount of organic matter and presence of hematite, an iron (III) oxide mineral with a high cation exchange capacity, in Bluestem soil (Table 5.2).

5.5.4. Environmental Implications. Discussion of how simplifications in our model system may result in differences from what occurs in the field is warranted. Rhizosphere microbiota vary among soils and can affect pH, nutrient availability, and contaminant degradation. Their effects on contaminant sorption to soils and subsequent phytoavailability, and the impact of contaminants on rhizosphere microbial communities have received little study. Furthermore, plant root exudates can vary in amount and composition depending on plant species, growth stage, stress, and microbial community. Further study on varying root exudates effects on sorption is warranted.

The majority of organic contaminant sorption experiments published in the literature have been focused on sorption to single constituents of soil (e.g. clay minerals, humic acid) for elucidation of sorption mechanisms. However, field scenarios are complex, requiring understanding of which constituents control sorption processes. Here, we show that root exudates and background solution composition have little effect on sorption of the ionizable pharmaceutical LTG compared to pH and soil properties. Cationic species appear to drive sorption, especially as soil pH and OC decrease.

Irrigated with reclaimed wastewater exposes agricultural soils and crop plants to a large variety of IOCs; testing all current and future IOCs is impractical. Accurate prediction of IOC phytoavailability and phytoaccumulation may require detailed understanding of rhizosphere processes and how they differ from bulk soil.

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5.6. FIGURES AND TABLES

Table 5.1. Concentrations (mM) of major ions in nutrient solutions [a]

	NO_3^-	NH_4^+	$H_2PO_4^-$	SO_4^{2-}	K^+	Ca^{2+}	Mg^{2+}	Cl^-	Murashige and Skoog Micronutrients ($g \cdot L^{-1}$)	Ionic strength (mM)
Nitrate only	6	0	0.5	3	2.5	2	3	0	9.79	22.5
Nitrate + Ammonium	2	4	0.5	4	2.5	2	1.5	2	9.79	22.5

Solution initial pH = 5.7 ± 0.05. For quartz sand sorption experiments, solutions also included 3 mM acetate (pH 4), MES (pH 5 and 6), or HEPES (pH 7 and 8) buffers.

Table 5.2. Properties of three soil types used for sorption experiments [a]

Analysis Method	Property	Richford	Elliott	Bluestem
Size Fractionation	Textural Class (%)	Loamy Sand	Silty Clay Loam	Sandy Clay Loam
	Sand (0.06 – 2 mm)	87	7	50
	Silt (0.002 – 0.006 mm)	6	62	31
	Clay (<0.002 mm)	7	31	27
Dry Combustion	f_{oc}	0.007	0.029	0.0042
1:1 water	pH	6.2 ± 0.17	6.1 ± 0.15	7.0 ± 0.07
N_2 Adsorption (BET)	Specific Surface Area ($m^2 \cdot g^{-1}$)	1.47	10	10.3
Potassium Chloride	N as NO_3 (ppm)	10.2	4.1	unknown (currently being measured at the UW Soil & Forage Lab)
	N as NH_4 (ppm)	9.7	22.6	
Bray-1	P (ppm)	170	14	
	K (ppm)	109	144	
Ammonium Acetate	Ca (ppm)	752	2309	
	Mg (ppm)	43	530	
	Na (ppm)	5	14	
Summation of P, K, Ca, Mg, and Na	Cation Exchange Capacity (meq/100g)	3.24	15	
X-Ray Diffraction *Mineral Percentages of whole soil*	Quartz	85	71	67
	K-feldspar	5.6	3.6	6.9
	Plagioclase	7.9	8.6	15
	Amphibole	-	2.4	2.5
	Calcite	-	0.3	0.4
	Dolomite	-	1.8	-
	Hematite	-	-	1.2
	Mixed-layer illite/smectite	0	14	4.4
	Illite + Mica	0.4	8.7	1.3
	Kaolinite	0.7	1.7	1.7
	Chlorite	0.4	0.4	0.2

[a]Values of interest for sorption include percent clay, fraction organic carbon, surface area, percent of carbonate (buffer) containing minerals (Calcite, Dolomite), and percent of Hematite (an iron oxide mineral with high cationic exchange).

Table 5.3. Wheat plant root exudate characteristics. [a]

Nutrient Solution	TOC [b] (mg·g^{-1} plant f.w.)	Carbohydrates (% of TOC)	$SUVA_{254}$ [c] (L·m^{-1}·mg^{-1})	Aromaticity [d] (% of TOC)
Nitrate only	3 ± 1	7.7 ± 0.5	6.1 ± 0.9	42 ± 5
Nitrate + Ammonium	6 ± 4	10 ± 1	7.7 ± 1.3	51 ± 8

[a] $n = 4$
[b] TOC: Total Organic Carbon, f.w. fresh weight
[c] $SUVA_{254}$: the specific UV absorbance at 254 nm, normalized to TOC
[d] Calculated using the relationship for dissolved organic matter developed by Weishaar et al.,[24] which we recognize may not be appropriate for root exudates.
Percent aromaticity = 6.54 × $SUVA_{254}$ + 3.63

Table 5.4. Lamotrigine sorption isotherm model parameters and goodness of fit statistics. Bluestem soil data were not fit to the models due to the limited concentration range measured.

Soil		**Linear** [a]	**Freundlich**
Richford mid pH only	model parameter(s)	K_d = 7.7 ± 0.18	K_F = 2 ± 1.4 n = 1.2 ± 0.13
	residual sum of squares	1501867	1366583
	adjusted R^2	0.981	0.905
Elliott mid pH only	model parameter(s)	K_d = 9.6 ± 0.02	K_F = 9.2 ± 0.61 n = 1.0 ± 0.01
	residual sum of squares	27926	27655
	adjusted R^2	0.999	0.998

[a] Model y-intercept was set as 0.

Table 5.5. Solid-water distribution coefficients for cationic (K_d^+) and neutral (K_d^0) species derived from Eq. 5.4, and associated empirical model goodness of fit statistics.

Soil	K_d^+	K_d^0	Residual sum of squares	Adjusted R^2
Richford	8.8 ± 0.37	5.2 ± 0.29	90.710	0.491
Elliott	10.4 ± 0.32	7.5 ± 0.27	65.712	0.446
Bluestem	9.1 ± 0.48	2.0 ± 0.33	85.372	0.797

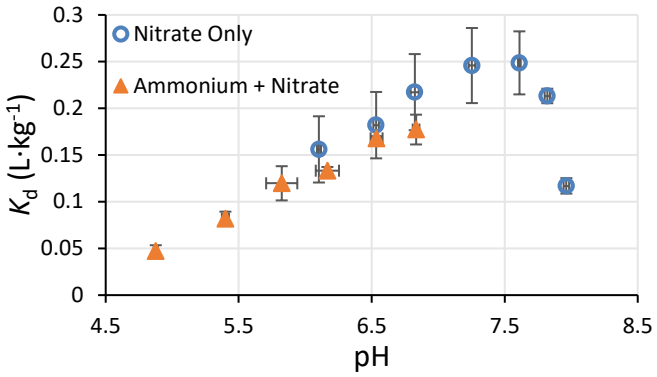

Figure 5.1. Lamotrigine adsorption to quartz sand as a function of pH. Circles represent nitrate only background solution, triangles represent ammonium + nitrate background solution. Error bars represent one standard deviation (n = 3) and do not always extend beyond the data points.

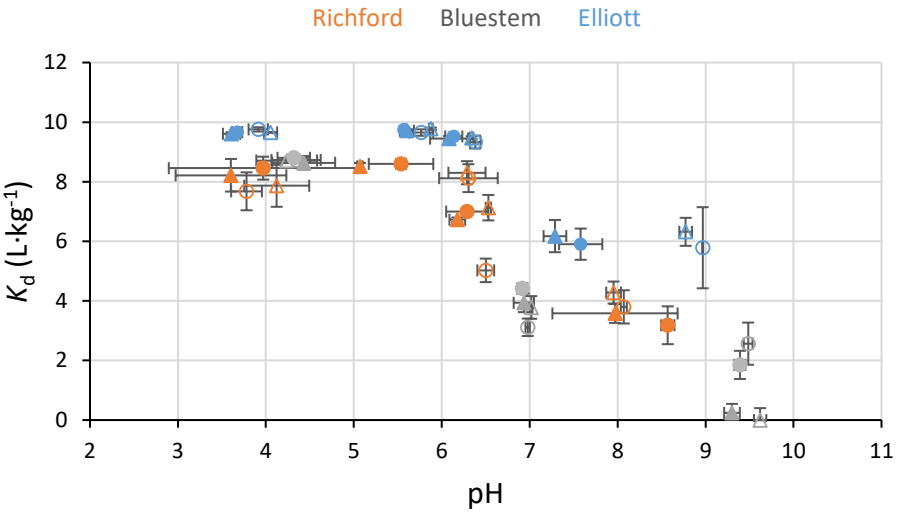

Figure 5.2. Lamotrigine sorption to Richford (orange), Elliott (blue), and Bluestem (grey) soils as a function of pH. Triangles represent nitrate only background solution, circles represent ammonium + nitrate. Filled indicates with exudates. Error bars represent one standard deviation (n = 3) and do not always extend beyond the data points.

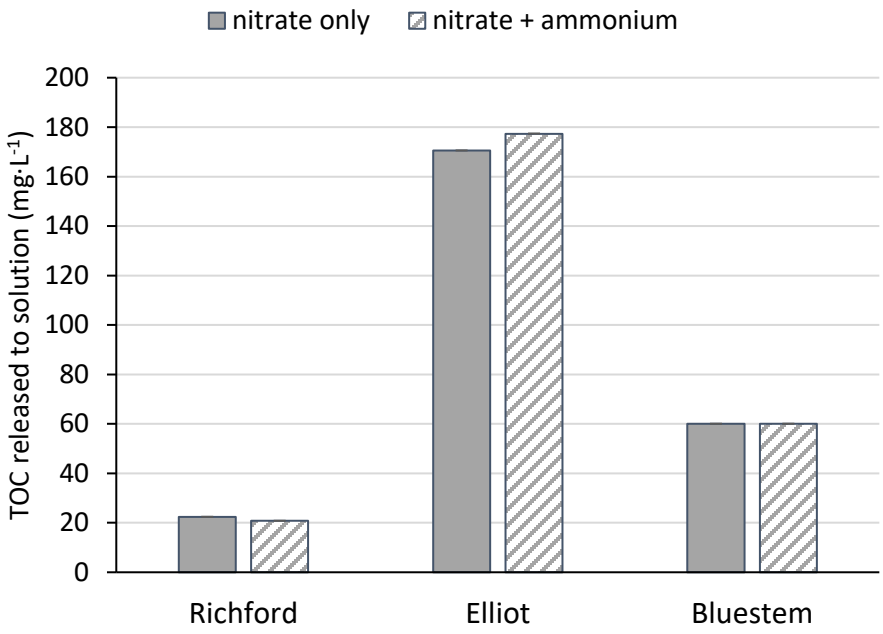

Figure 5.3. Release of organic carbon from soils into aqueous solution after 48 hour equilibration period for both nitrate only (solid) and nitrate + ammonium (striped) background solutions. Error bars represent standard error and do not necessarily extend beyond the bars (n = 3).

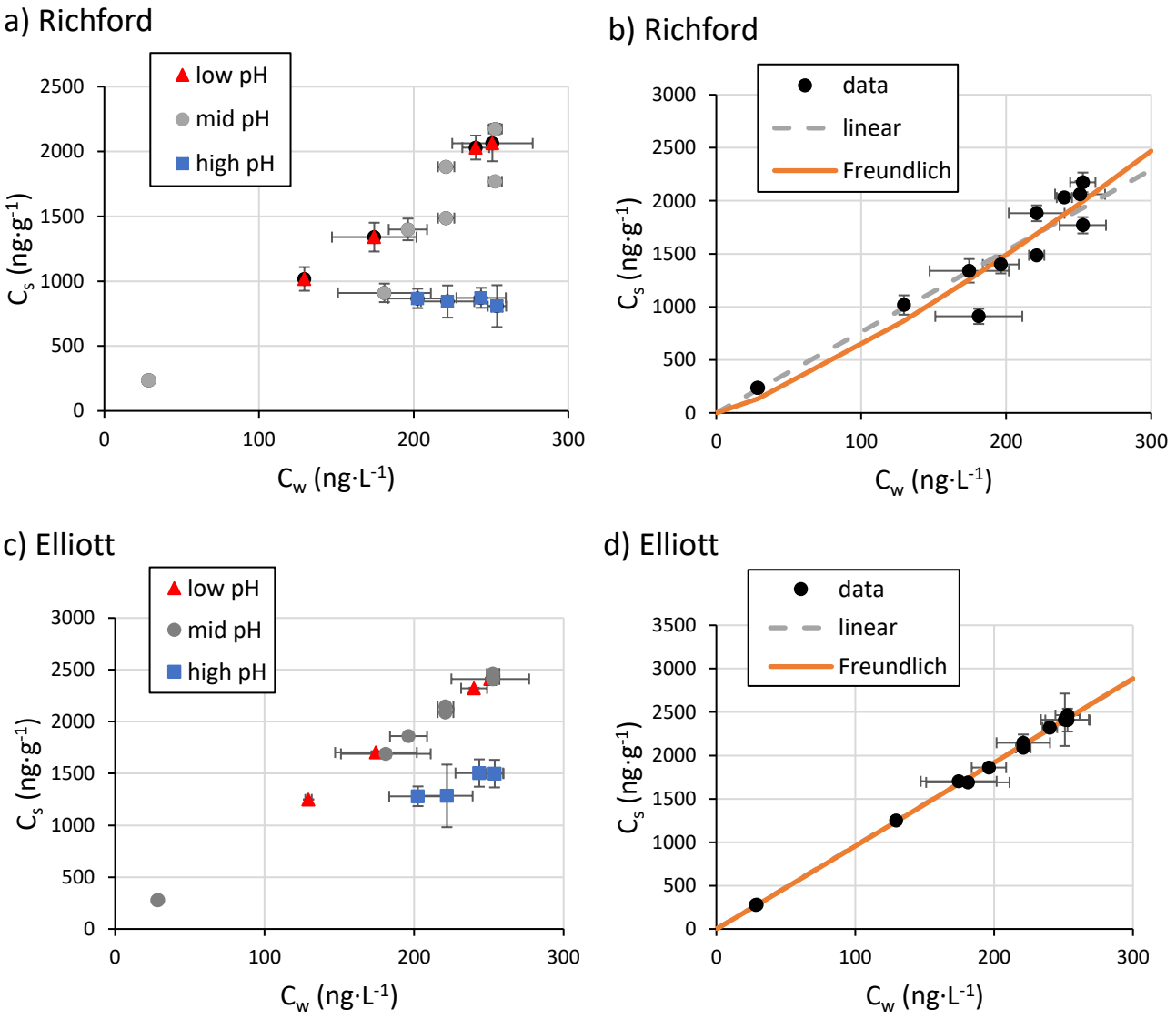

Figure 5.4. Concentration of lamotrigine sorbed to a) Richford and c) Elliot soils as a function of concentration in the aqueous phase varies depending on pH. Sorption at low (red triangles) and medium (grey circles) pH appears to remain constant, but sorption decreases at high pH (blue sqares). Linear (grey dashes) and Freundlich (solid orange) isotherms (at medium pH; data are shown in black) fit lamotrigine sorption to b) Richford and d) Elliott soils well. Fitting parameters and statistics are shown in Table 5.4. Error bars represent one standard deviation (n = 3) and do not always extend beyond the data points.

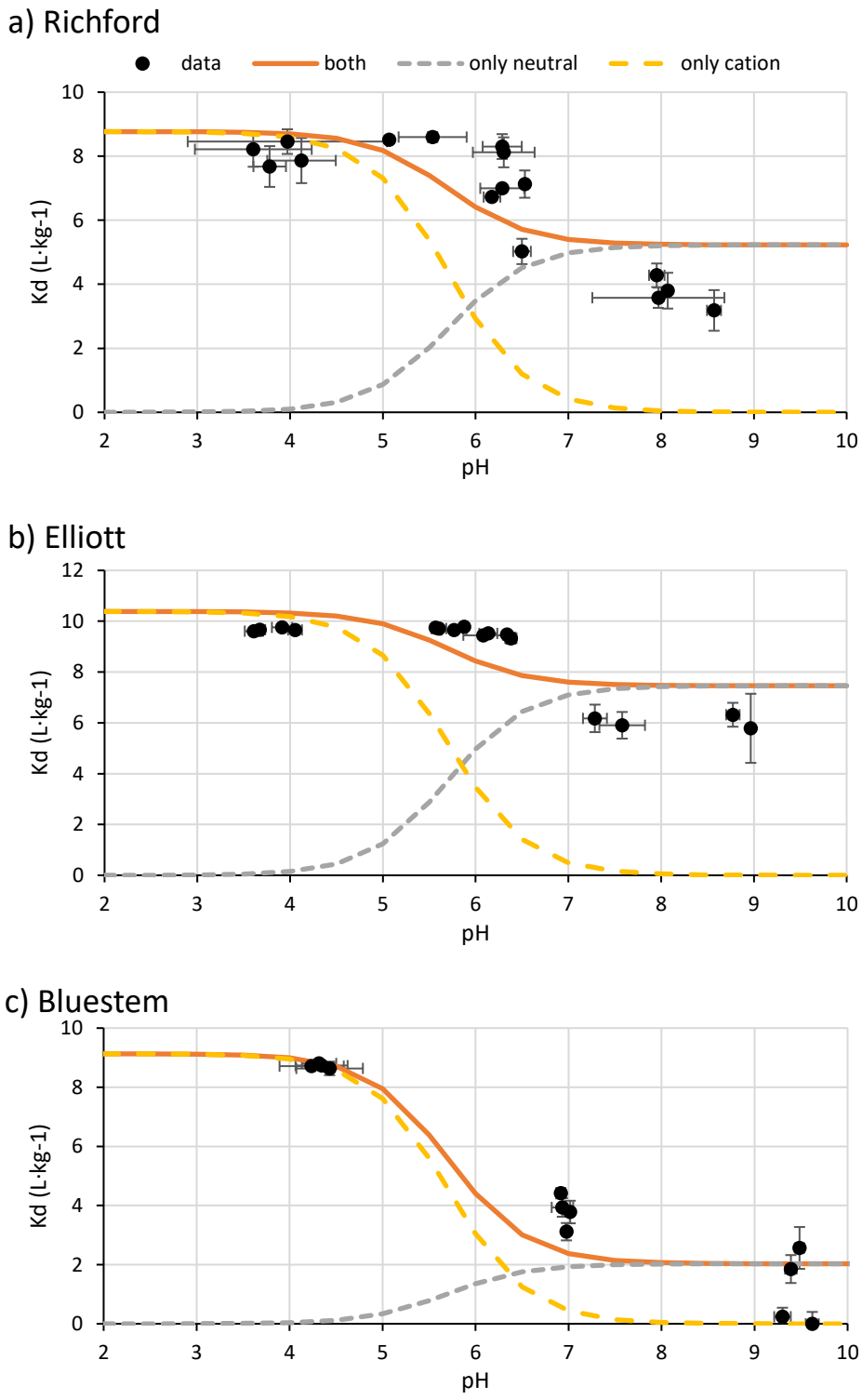

Figure 5.5. Contributions of cationic (dashed yellow) and neutral (dashed grey) species of lamotrigine sorption to a) Richford, b) Elliot, and c) Bluestem soils. The combined empirical model is shown as a solid orange line. Error bars represent one standard deviation (n = 3) and do not always extend beyond the data points.

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Chapter 6: Rhizosphere impacts on phytoavailability of ionizable contaminants Part B: Accumulation by wheat (*Triticum durum*)

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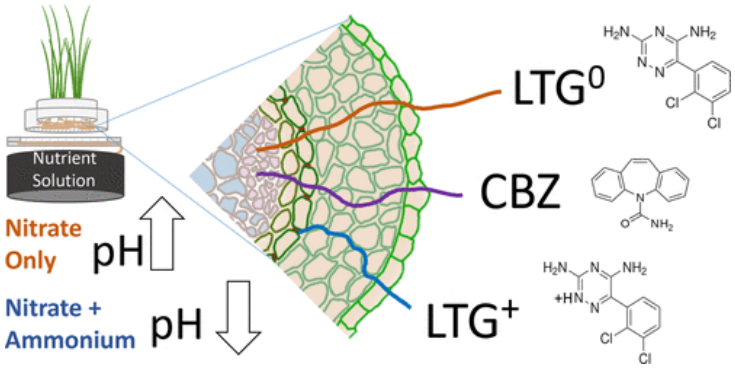

6.1. AUTHOR CONTRIBUTIONS

SLN had the initial idea of focusing a study on rhizosphere processes, designed the initial proof of concept experiments, and was responsible for LC-MS/MS analysis of the quartz sand experiment samples. ELM grew all the plants used for the analysis included in the manuscript, performed all the tissue extractions, and was responsible for the LC-MS/MS analysis of the soil experiment samples. KGK and JAP oversaw the design and execution of the experiments and the interpretation of results. ELM, SLN, KGK, and JAP wrote the manuscript.

6.2. ABSTRACT. Many ionizable organic contaminants (IOCs) are present in treated wastewater used to irrigate edible crops and accumulate in plants under field conditions. 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 plant modulation of rhizosphere pH on IOC accumulation has not been previously reported. Here we provide direct evidence that plant-driven changes in rhizosphere pH impact accumulation of an IOC in plant leaves. Using a modified hydroponic system, we found that rhizosphere pH was higher by 1.5-2.5 units when plants grown on quartz sand received only nitrate rather than a combination of nitrate and ammonium. Plant-driven changes to rhizosphere pH altered accumulation of lamotrigine ($pK_a = 5.7$) but not carbamazepine, a non-ionizable contaminant. Lamotrigine accumulation in leaves correlated strongly with the concentration of the neutral species available in porewater. Plants grown on sterilized field soils were unable to significantly alter rhizosphere pH; lamotrigine phytoaccumulation was therefore unsurprisingly not altered by nutrient availability. However, we expect plant-driven changes in rhizosphere pH to be important in soils with low buffering capacity across a wide range of plant species and IOCs. Consideration of plant modulation of rhizosphere pH may be necessary to accurately predict IOC bioaccumulation.

6.3. INTRODUCTION

Irrigation of food crops with reclaimed wastewater and effluent-dominated water sources is common in arid regions worldwide[1] and is expected to grow as global climate warms, population increases, and demands on freshwater sources rise. However, these practices can result in crop plant exposure to a variety of ionizable organic contaminants (IOCs) that are not completely removed during conventional wastewater treatment. A wide variety of literature demonstrates that crop plants can accumulate pharmaceuticals in edible tissues under field conditions,[2–4] prompting the need to evaluate the potential risks associated with effluent irrigation of food crops. Understanding the controls on IOC availability to

plants may allow identification of situations that lead to unacceptable accumulation in food crops, a task where success of current plant uptake models is limited.[2]

One of the important gaps requiring investigation is the behavior of contaminants at the interface between the soil environment and the plant root, known as the rhizosphere. This 2-3 mm region immediately surrounding the root is where plants sense and respond to soil conditions. Roots release ions and organic compounds to maximize nutrient availability, as defense, and to initiate and modulate dialogue with soil microbiota.[5–8] These exudates are complicated mixtures of inorganic ions, organic and amino acids, sugars, chelating agents, and various secondary metabolites, and their release can result in significant changes to the chemistry of the rhizosphere.[9] Consequently, rhizosphere properties can vary significantly from the bulk soil. Exudates may change rhizosphere pH, alter soil-contaminant interactions, and ultimately alter contaminant bioavailability and biodegradation potential.[10] Thus, consideration of only bulk soil properties may mischaracterize the speciation and sorption of IOCs in the zone where they are immediately available for transport into plant roots and lead to inaccurate prediction of phytoavailability.

The types of nutrients available to plants can have consequences for the rhizosphere, as plants alter rhizosphere pH to maintain electrochemical equilibrium as they take in nutrients. Root cells release H^+ to the rhizosphere as they take in cations (e.g., NH_4^+). Energy for anion (e.g. NO_3^-) uptake is provided via co-transport of H^+ from the rhizosphere into cells.[6] These processes can raise or lower rhizosphere pH by 2-3 units up to 2-3 mm from the root surface, resulting in rhizosphere pH values of 4-8, depending on initial conditions.[6,7,11] Speciation impacts IOC degradability,[12] sorption to soil particles[13,14] and plant roots,[14,15] uptake by roots,[14–16] and movement through plants.[14,15,17] Changes in rhizosphere pH can affect copper, uranium, cadmium, arsenic, and inorganic nutrient phytoaccumulation from soils.[6,18–24] Beyond changes in pH, plant exudates may also alter contaminant-soil interactions,[25–28] thereby altering phytoavailability. Published studies on IOC phytoaccumulation have invoked differences between rhizosphere and bulk soil pH to explain uptake trends;[29–31] however, direct demonstration of the impact of plant-induced changes in rhizosphere chemistry on IOC phytoaccumulation has not been previously reported.

In this study, we examined the effect of nutrient availability on plant-induced rhizosphere changes and subsequent phytoavailability of a model IOC, the phenyltriazine anticonvulsant lamotrigine (LTG; conjugate acid pK_a = 5.7[32]). We used the tricyclic anticonvulsant carbamazepine (CBZ) as a "control" non-ionizable contaminant to discriminate between effects on accumulation due to changes in rhizosphere pH and those due to other potential changes to the plants caused by the different nitrogen sources. Both pharmaceuticals have been detected in reclaimed wastewater (concentrations up to 488 and 1,110 ng·L^{-1}, respectively)[33,34] and accumulate in effluent-irrigated plants.[29,31] Additionally, both LTG and a primary CBZ metabolite, 10,11-epoxycarbamazepine (epCBZ), can accumulate in carrots irrigated with reclaimed wastewater to levels exceeding the threshold of toxicological concern at normal ingestion rates.[29] This threshold serves as a conservative indicator of when additional study of toxic effects is warranted.[35,36] We cultivated wheat with different forms of inorganic nitrogen in a growth system designed to isolate the rhizosphere,[18–20] first with quartz sand and then using four field soils with differing properties. We measured pH and pharmaceutical concentrations in the model rhizosphere and related them to phytoaccumulation. We hypothesized that plants provided ammonia and nitrate would decrease the pH of the rhizosphere (regardless of soil) compared to plants provided only nitrate, thereby increasing the amount of lamotrigine ionized in the soil and decreasing lamotrigine uptake. We further hypothesized increased soil organic carbon and cation-exchange capacity would increase LTG sorption to soil, thereby decreasing LTG pore water concentrations and thus phytoavailability and accumulation.

6.4. MATERIALS AND METHODS

6.4.1. Plant Growth. Durum wheat (*Triticum durum)* seeds were sterilized, soaked, and germinated in a damp paper towel. After 2-5 days, sprouted seeds were transferred to growth cells based on the RHIZOtest (Figure D1).[19,23,37] Four seeds were placed inside each cell, and plants were cultured hydroponically for 21 days, allowing the roots to grow into a mat against a layer of 30-μm nylon mesh (Text D.1.2.3).[19] Plants were cultured hydroponically for 21 days.[19] 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; Table 6.1).

On day 22, each growth cell was transferred to a model rhizosphere setup, placing the mesh containing the root mat against a thin layer of soil (see below). All soils were sterilized by autoclaving twice on gravity cycle at 127 °C for 30 min prior to placement in the rhizosphere setup. A strip of cellulose filter paper wicked nutrient solution to the soil from a reservoir (replenished every 2 days). Soil and paper were saturated with nutrient solution for the experimental duration. All setup components were autoclaved or washed with methanol to minimize microbial growth. Half the rhizosphere setups received nutrient solution with nitrate as the sole nitrogen source; the other half received a solution containing a mixture of ammonium and nitrate (Table 6.1). Nutrient solutions had equivalent ionic strength and were adjusted to pH 5.7. Solution composition was chosen to induce differences in rhizosphere pH while minimizing effects on plant growth. Each treatment (pharmaceutical + solution combination) was replicated nine times; controls lacking pharmaceuticals or plants were conducted in triplicate. One replicate represents a growth cell containing four plants. Transpiration was estimated by mass loss every two days, subtracting average mass lost from nearby no-plant control setups.

6.4.1.1. Experiment A: Quartz Sand. We used ultrapure quartz sand as a control "soil" that would have minimal effects on solution pH and minimal IOC sorption. For this experiment, the rhizosphere setups received 5.00 ± 0.05 g of ultrapure silica sand (between sieve sizes 60-120). Sand and paper were saturated with nutrient solution (containing 100 $\mu g \cdot L^{-1}$ LTG or CBZ) for the 8-day exposure period. During the period between days 8 and 22 (prior to transfer to the rhizosphere setup), all plants received nitrate only nutrient solution. During the rhizosphere exposure period, ammonium + nitrate plants received a 1:2 ammonium-to-nitrate molar ratio solution (Table 6.1). After 8 days, above-ground tissues (hereafter leaves), roots, and 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. Freeze-dried rhizosphere sand (4.000 ± 0.005 g) from each growth cell of plants was rehydrated with 8.00 mL of 10 mM $CaCl_2$ for 3 h and sedimented by

centrifugation (20 min, 4637*g*).[38] We measured the pH of the resulting solution. Additionally, an aliquot (1 mL) of the supernatant was withdrawn and filtered through a 0.2 μm PTFE filter in preparation for LC-MS/MS analysis.

6.4.1.2. Experiments B and C: Field Soils. We chose four field soils with varying properties (Table 6.2) to examine the effect of nitrogen source availability on LTG uptake from real soils. Although these soils have differing nitrogen contents (Table 6.2), the amount of soil in the rhizosphere setups is not enough to sustain the plants on its own, and we expected the nutrient solution nitrogen differences to have a significant impact on what form of nitrogen was available to the plants. For experiment B, (Bluestem, Elliott, and Richford soils), all plants were initially provided nitrate only solution prior to transfer to the rhizosphere setups. On day 22 post-hydration, each growth cell was transferred to a rhizosphere setup, with the model rhizosphere consisting of 4.00 ± 0.05 g soil, and half the plants then received a 2:1 ammonium-to-nitrate molar ratio solution (Table 6.1). For Experiment C (Elliott, Richford, and Plano soils), plants received the same nutrient solution as they did prior to being introduced to the rhizosphere setup; half the plants received nitrate only nutrient solution and half received a 2:1 ammonium-to-nitrate molar ratio solution (Table 6.1). For both experiments, the nutrient solution contained 100 $\mu g \cdot L^{-1}$ LTG. After 8 days of exposure, leaves, roots, and rhizosphere soil were collected, frozen at –80 °C, and stored at –80 °C until extraction and analysis. Plant tissues were freeze dried (masses were measured before and after freeze drying); soil was centrifuged at 4637 ×*g* for 30 minutes. Approximately 1 mL of the pore water was collected and filtered through 0.22 μm PTFE syringe filters before analysis. Soil pore water pH was assessed after LC-MS/MS analysis to prevent LTG carryover contamination.

6.4.2. Extraction and Liquid-Chromatography-Tandem-Mass-Spectrometry (LC-MS/MS). 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 4:1 water:acetonitrile with 0.1% acetic acid. We measured LTG, CBZ, and the primary CBZ metabolites epCBZ and 10,11-*trans*-dihydroxycarbamazepine (diOH-CBZ) in leaf and root extracts,

sand rehydration solution (Experiment A), soil pore water (Experiments B and C), and nutrient solution before and after a two-day replenishment cycle (one per Experiment A, B, and C) using LC-MS/MS (Agilent 1260 HPLC, Waters Xterra MS C18 column, Agilent 6460 triple quadrupole mass spectrometer, ESI+ source) (Text D.1.4.).

6.4.3. Rhizosphere Sorption Corrections. For Experiment A, rhizosphere sand was freeze-dried and then rehydrated to determine CBZ and LTG concentrations in the rhizosphere porewater. We conducted a series of experiments to assess LTG and carbamazepine CBZ adsorption to the rhizosphere substrates (presented in part A). Carbamazepine did not adsorb to quartz sand to a measureable extent; therefore, CBZ pore water concentrations were not corrected. For LTG, we accounted for LTG that remained adsorbed to the sand after rehydration (<11%) using the water-sand distribution coefficients (K_d) for LTG across the experimental pH range. Using the total amount (dissolved and adsorbed) of LTG in the rhizosphere so determined, we estimated the dissolved concentration in the rhizosphere porewater using the appropriate K_d and the rhizosphere solid-to-solution ratio (Table D5). For Experiments B and C, soil pore water was collected immediately upon harvest rather than freeze-drying and reconstituting the soils; measured pore water concentrations were therefore assumed to account for sorption.

6.4.4. Determination of Soil Buffering Capacity. Soil pH buffering capacity (pHBC) is the ability of soil to resist a change in pH when acid-forming or base-forming materials are added, and is typically determined by titration using multiple additions of base to develop a pH buffer curve.[39] We determined pHBC as molar addition of OH^- and H^+. For each soil, 2.5 mL of acid (HCl) or base (KOH) solution was added to 1.00 ± 0.005 g soil in triplicate. The samples were mixed for 30 minutes before determination of the pH of the resulting soil slurries. We plotted mmol OH^- and H^+ added per kg soil was against soil pH, and estimated pHBC as the slope of the regression line.

6.5. RESULTS AND DISCUSSION

6.5.1. Plant Modulation of Rhizosphere pH. Wheat plants altered porewater pH in the sand model rhizospheres in response to the form of inorganic nitrogen provided (Figure 6.1a). Nutrient solution pH in the reservoirs was initially 5.7 ± 0.05 and differed by <0.1 unit at the end of each replenishment cycle. In treatments containing wheat plants, provision of nitrate as the sole nitrogen source resulted in rhizosphere pH increases by 1.5-2.5 units relative to those supplied with ammonium and nitrate. Exposure to CBZ and LTG did not affect rhizosphere pH (Figure D2). The fresh masses of roots and leaves and the transpired water mass did not differ among treatments (Table D5). In contrast, no significant plant-induced changes to rhizosphere pH were observed for any of the field soils (Figure 6.1b-e), which contradicts previous reports of wheat effects on rhizosphere pH in response to variation in the ratio of nitrate to ammonium provided to the plants.[7,19,22,40] This may be due to the higher soil pH of the soils used in this study (> 6) compared to that of previous studies using wheat and the RHIZOtest system (~4), differences in buffering capacity between the soils, or, most likely, the effects of soil microbiota. We used sterile soils in an attempt to isolate the effects of the plants from that of soil microbiota, while no previous reports of plant modulation of rhizosphere pH mention soil sterilization procedures. We therefore assume the previous studies did not use sterile soils and report combined effects of plant and soil microbiota modulation of rhizosphere pH. Bravin et al.[19] were the only previous report of plant-induced rhizosphere pH changes to also include a pHBC curve for their soil, which we compared to the soils in the present study (Figure 6.2); this soil had a higher pHBC (steeper slope) than any of the soils we used, indicating buffering capacity is not the most important variable for modulation of rhizosphere pH. We therefore suggest soil microbiota may be the main driver of plant-induced pH changes in the rhizosphere and recommend further study of plant-microbiota-pH interactions in the rhizosphere. Due to the lack of rhizosphere pH differences, extraction and analysis of LTG concentrations in plant tissues was performed for only Experiments A and B.

6.5.2. Effect of Rhizosphere pH on Plant Accumulation of Pharmaceuticals. We tested the effect of rhizosphere pH on LTG (pK_a = 5.7)[32] and CBZ (non-ionizable) accumulation in wheat roots and

leaves for plants grown on quartz sand and on LTG accumulation for plants grown on field soils. For plants grown on quartz sand, significantly more LTG accumulated in leaves of plants provided nitrate as the sole nitrogen source (rhizosphere pH 7.4 ± 0.6) than in those supplied ammonium and nitrate (rhizosphere pH 5.7 ± 0.4) (Figure 6.3). While 40 ± 3% of LTG mass within plants was in leaves of plants receiving solely nitrate, only 27 ± 4% was in leaves of plants that also received ammonium. Pharmaceutical concentrations in rhizosphere porewater did not differ among nutrient treatments (Figure D3), indicating no differential microbial degradation, if any occurred. Based on the nominal LTG and CBZ concentrations in bulk nutrient solution, we estimate that <1% of total LTG or CBZ mass was in rhizosphere porewater and <4% was in plant tissue at the end of the experiment.

Nitrogen source did not affect average accumulation of LTG in plant roots. Plant tissue LTG concentrations, especially in leaves, correlated with the concentration of uncharged LTG (LTG^0) in porewater (Figure 6.5a). Lamotrigine and CBZ concentrations in plants did not correlate with total concentrations in porewater (p >0.05, Table D6).

Accumulation of CBZ, epCBZ, and diOH-CBZ were also not affected by differences in nitrogen form provided or rhizosphere pH. Of the total CBZ mass measured in the plant, 82 ± 8% was in the leaves regardless of treatment (p >0.05). Concentrations of epCBZ in leaves and roots were 220 ± 90 $ng \cdot g_{d.w.}^{-1}$ and 40 ± 20 $ng \cdot g_{d.w.}^{-1}$. The mass of epCBZ remained below 2% and 0.6% of the total CBZ measured in leaves and roots, respectively. The diOH-CBZ concentration exceeded the limit of quantitation in four root and ten leaf samples (n = 18 for each) and ranged up to 26 $ng \cdot g_{d.w.}^{-1}$ in roots and 23 $ng \cdot g_{d.w.}^{-1}$ in leaves.

For plants grown on field soils, accumulation of LTG did not differ between plants grown on the same soil but nutrient treatments, which is unsurprising given the lack of differences in rhizosphere pH. There was also no correlation with the concentration of neutral LTG in the soil pore water and the concentration of LTG in leaf or root tissue. The amount of LTG in the soil pore water differed significantly between different soils (Figure 6.4a), likely due to sorption differences between the soils (presented in Part A), as the soil with the most organic matter and highest sorption (Elliott) had the lowest concentration of

LTG in the pore water. Accumulation of LTG was much higher in plants grown on quartz sand than in those grown on any of the soils, likely due to higher transpiration rates in these plants (Figure 6.4b and c). There were no significant differences in bioconcentration factors for plants grown on different soils (Figure 6.4b and c).

Quantification of LTG metabolites (and additional CBZ metabolites) was beyond the scope of this study. We therefore cannot exclude that *in planta* metabolism affected our results. However, if the differences in nitrogen source affected compound metabolism, we would expect clear differences in LTG accumulation between nutrient treatments, rather than reasonably strong correlation between LTG^0 concentration and accumulation in leaves of plants grown on quartz sand (Figure 6.5a). Additionally, we would expect to see differences in accumulation of the primary CBZ metabolites.

6.5.3. Effect of Transpiration on Plant Accumulation of Pharmaceuticals. To determine whether transpiration was driving LTG uptake into plants grown on soil, we used our previously described hypothetical transpiration based accumulation (TBA) model[41] (Chapter 4):

$$TBA = C_{solution} \times V_{transpired} \qquad \text{(Eq. 6.1)}$$

where $C_{solution}$ is the concentration of the compound in the soil pore water and $V_{\text{transpired}}$ is the volume of water transpired by the plant. The TBA is not intended to provide an accurate estimation of contaminant uptake, but rather to assess the importance of water flow through the plant on contaminant accumulation. Correlation with TBA indicates a direct relationship between contaminant accumulation in the plant and transpiration. Deviations from TBA suggests the operation of processes limiting (observed accumulation < TBA) or enhancing (observed accumulation > TBA) phytoaccumulation, such as factors controlling contaminant transport through the roots to the xylem or the occurrence of in planta metabolism.

We calculated TBA for each cell of plants in Experiments A and B and compared these predictions to observed LTG accumulation in plant tissues (Figure 6.6). For all treatments, the fit was relatively poor (R^2 varied between 0.1024 and 0.8611) compared to our previous report of LTG accumulation in

hydroponic spinach[41] (Chapter 4). The fits for plants grown in sand (R^2 between 0.455 and 0.6313) were not better than for plants grown in soils, but the soil fits were more variable (R^2 between 0.2924 and 0.8611, 0.2271 and 0.3746, and 0.1024 and 0.6867 for Richford, Elliott, and Bluestem soils, respectively). The poor correlations with transpiration may be due to error in estimating transpiration or changes in LTG soil pore water concentrations over the exposure period, as we were able to measure pore water concentrations only at the end of the experiment. Actual accumulation was significantly below that which would be predicted by transpiration alone, indicating LTG cannot easily enter the transpiration stream, consistent with previous reports.[29,41] Deviations from TBA due to *in planta* metabolism may also be possible. How LTG is metabolized in plants is not yet understood, so we were unable to directly measure LTG metabolites in plant tissue extracts. A mass balance approach to identifying occurrence of *in planta* metabolism is also made difficult in the RHIZOtest system due to the frequent nutrient solution reservoir changes, unknown LTG wick rates, and unknown concentrations of LTG sorbed to filter paper wicks. However, even with these limitations, the R^2 values indicate transpiration appears to explain 10 to 80 % of LTG accumulation, indicating transpiration is a driving force for LTG uptake into the plant.

6.5.4. Physiological Context. Plants must maintain electrochemical equilibrium as they take in nutrients, which can alter rhizosphere pH. 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, although redox conditions and iron and phosphorus availability can also have an impact.[6] Typically, ammonium uptake decreases rhizosphere pH and nitrate uptake increases pH,[7] which is consistent with our results for plants grown on quartz sand, but not on field soils, possibly due to the relatively high natural soil pH.

To be transported from roots to other tissues, a molecule must reach the plant's vascular tissue. Molecules can travel through roots via two pathways. In the symplastic route, molecules cross a cell membrane to enter a root cell, then travel to the vascular tissue via plasmodesmata.[42] In the apoplastic route, molecules move through intercellular space and are blocked from entering the vascular tissue by the Casparian strip.[42] 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.[2,17] Generally, neutral organic molecules have much higher membrane permeability than charged ones.[17] Therefore, absent the action of transporters, we expect neutral IOC species to be transported through plants more readily than their ionized counterparts or similar non-ionizable compounds.

For plants grown on quartz sand, our results agree with this expectation. Rhizosphere pH did not affect CBZ accumulation, and a large fraction of CBZ was transported to leaves; we therefore hypothesize that CBZ travels symplastically through plant roots. In contrast, LTG accumulation was affected by rhizosphere pH; more LTG accumulated when a larger fraction of LTG in the rhizosphere was uncharged. The strong correlation between LTG accumulation in leaves and concentration of LTG^0 in the quartz sand rhizosphere (Figure 6.5a) indicates that LTG speciation is an important variable controlling its access to the vascular tissue. The non-significant *y*-intercept in the correlation is consistent with ionized LTG (LTG^+) not being transported to leaves. The higher *y*-intercept for the correlation between LTG accumulation in roots and LTG^0 in the rhizosphere suggests that LTG^+ adsorbs to or enters root tissue, but to a smaller extent than LTG^0. We hypothesize that LTG^+ did not enter root cells but remained in the apoplastic space and sorbed to the root surface, preventing transport to leaves, whereas LTG^0 was transported symplastically to some extent. However, LTG accumulation in leaves was lower than that of CBZ even when >99% LTG was in the neutral form. This difference has been noted previously[31,43,44] and may be caused by ion trapping in root cell vacuoles.[2,30,31] Lamotrigine speciation is dominated by LTG^0 in root cell cytosol (pH 7-7.4)[14] and by LTG^+ in vacuoles (pH 4-5.5).[14] Thus, if LTG^0 crosses the vacuole membrane, a portion will remain trapped in the vacuole as LTG^+, unable to be translocated through the plant. We also cannot exclude the possibility that CBZ and LTG accumulation differs due at least in part to differential metabolism *in planta*. This topic merits future investigation.

For plants grown on soils, we did not see the expected rhizosphere pH changes as a response to nitrogen form (Figure 6.1b-e). This may be due to a number of factors. Root-associated microbiota may increase the magnitude of rhizosphere pH changes. The plants may also have needed more time to release

enough exudates to overcome the soil pHBC. Ultrapure quartz sand has a low pH buffering capacity and low sorption capacity for CBZ and LTG.[30] Sorption had little impact LTG availability to plants grown on quartz sand, but pH-dependent sorption processes are much more important drivers of contaminant phytoavailability actual soils, as evidenced by the differences in soil pore water LTG concentrations and accumulation in plants grown in differing soils.

Although our results make clear that the magnitude and spatial extent of rhizosphere pH change dependent on the buffering capacity of the soil, we still believe rhizosphere pH changes may be important in field conditions. In contrast with our results, even in a soil with high buffering capacity due to 30% $CaCO_3$ content, chickpeas changed pH by ~2 units within 1 mm of the root surface.[6] However, this report did not mention soil sterilization procedures, so we assume they did not use sterile soils and report combined effects of plant and soil microbiota modulation of rhizosphere pH. 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, although pH-dependent sorption to soil constituents and high soil buffering capacity may impact relative trends in phytoavailability. Soils with different pH and nutrient ratios could result in a higher or lower rhizosphere pH range than observed in our study.

6.5.5. Environmental Implications. We isolated the effect of nitrogen source-induced rhizosphere pH changes on plant accumulation of an IOC, a process that we expect also occurs in the field and may increase in importance if elevated atmospheric CO_2 concentrations promote root exudation of organic acids and shift plant assimilation preferences to reduced nitrogen forms.[45–48] We expect our results to serve as a basis for future experimental studies that incorporate more of the complexity that exists in field scenarios. Discussion is therefore warranted of how simplifications in our model system may result in differences from what occurs in the field.

We used durum wheat, a species used in previous rhizosphere pH studies.[19] Other species known to alter rhizosphere pH in response to inorganic nitrogen availability include maize, sorghum, chickpea, Norway spruce, white lupine, white clover, tomato, and rapeseed.[11,18] 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 most crop species, although the magnitude of plant-driven changes in rhizosphere pH is species-specific and can vary among cultivars.[11]

Rhizosphere microbiota vary among soils and plant species and can affect pH, nutrient availability, and contaminant degradation. Their effect on contaminant phytoavailability and the impact of contaminants on rhizosphere microbial communities have received little study. Although our growth system was not completely sterile (as it was open to non-sterilized air), we expect that any influence of microorganisms on our results was small; we sterilized all components of the system and saw no evidence of microbial growth. However, we observed no pH shifts in any treatment except for the quartz sand rhizospheres, which contradicts previously reported plant-induced rhizosphere pH changes,[19,37] for which soils were not sterilized. Rhizosphere microbiota are a potential factor that could cause differences between our model system and field conditions, even when the same process of nutrient-driven rhizosphere pH change occurs.

We suggest future studies on plant accumulation of IOCs report the main forms of nitrogen and other nutrients supplied to the plants and consider rhizosphere pH. Differences in compound speciation between the rhizosphere and bulk soil may hinder the ability to predict plant uptake of IOCs. State-of-the-art models for organic contaminant uptake allow specification of soil porewater pH.[14] While knowledge of rhizosphere pH is required to account for the differential contaminant uptake demonstrated in the present study, the underlying processes leading to modulation of rhizosphere pH are not currently incorporated into such models. We suggest future models explicitly incorporate rhizosphere processes, as has been done for a model to predict uranium phytoaccumulation.[21] Plants irrigated with reclaimed wastewater are exposed to and accumulate a large variety of IOCs; testing all current and future IOCs is impractical. Accurate prediction of IOC accumulation in plants may require detailed understanding of the impact of rhizosphere processes on IOC availability.

Associated Content. Supporting information (SI) is available as Appendix D. Methodological details and additional results noted in the main text.

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6.6. FIGURES AND TABLES

Table 6.1. Concentrations (mM) of major ions in nutrient solutions provided to wheat plants before and during rhizosphere experiments [a]

	NO_3^-	NH_4^+	$H_2PO_4^-$	SO_4^{2-}	K^+	Ca^{2+}	Mg^{2+}	Cl^-	Murashige and Skoog Micronutrients ($g \cdot L^{-1}$)	Ionic strength (mM)
Nitrate only	6	0	0.5	3	2.5	2	3	0	9.79	22.5
1:2 Nitrate: Ammonium	4	2	0.5	3.25	1.5	2	2.25	0	9.79	22.5
2:1 Nitrate: Ammonium	2	4	0.5	4	2.5	2	1.5	2	9.79	22.5

[a] Solution initial pH = 5.7 ± 0.05.

Table 5.2. Properties of field soils used for model rhizospheres [a]

Analysis Method	Property	Richford	Elliott	Bluestem	Plano
Size Fractionation	Textural Class (%)	Loamy Sand	Silty Clay Loam	Sandy Clay Loam	Silty Clay Loam
	Sand (0.06 – 2 mm)	87	7	50	10
	Silt (0.002 – 0.006 mm)	6	62	31	58
	Clay (<0.002 mm)	7	31	27	32
Dry Combustion	f_{oc}	0.007	0.029	0.0042	0.0014
1:1 water	pH	6.2 ± 0.17	6.1 ± 0.15	7.0 ± 0.07	6.2 ± 0.06
N_2 Adsorption (BET)	Specific Surface Area ($m^2 \cdot g^{-1}$)	1.47	10	10.3	27.5
Potassium Chloride	N as NO_3 (ppm)	10.2	4.1	unknown (currently being measured at the UW Soil & Forage Lab)	10.3
	N as NH_4 (ppm)	9.7	22.6		2.6
Bray-1	P (ppm)	170	14		26
	K (ppm)	109	144		64
Ammonium Acetate	Ca (ppm)	752	2309		1562
	Mg (ppm)	43	530		590
	Na (ppm)	5	14		22
Summation of P, K, Ca, Mg, and Na	Cation Exchange Capacity (meq/100g)	3.24	15		13.9
X-Ray Diffraction *Mineral Percentages of whole soil*	Quartz	85	71	67	58
	K-feldspar	5.6	3.6	6.9	6.5
	Plagioclase	7.9	8.6	15	15.1
	Amphibole	-	2.4	2.5	0.5
	Calcite	-	0.3	0.4	-
	Dolomite	-	1.8	-	-
	Hematite	-	-	1.2	1
	Mixed-layer illite/smectite	0	14	4.4	11
	Illite + Mica	0.4	8.7	1.3	5.5
	Kaolinite	0.7	1.7	1.7	1.7
	Chlorite	0.4	0.4	0.2	0.7

[a]Values of interest for sorption include percent clay, fraction organic carbon, surface area, percent of carbonate (buffer) containing minerals (Calcite, Dolomite), and percent of Hematite (an iron oxide mineral with high cationic exchange).

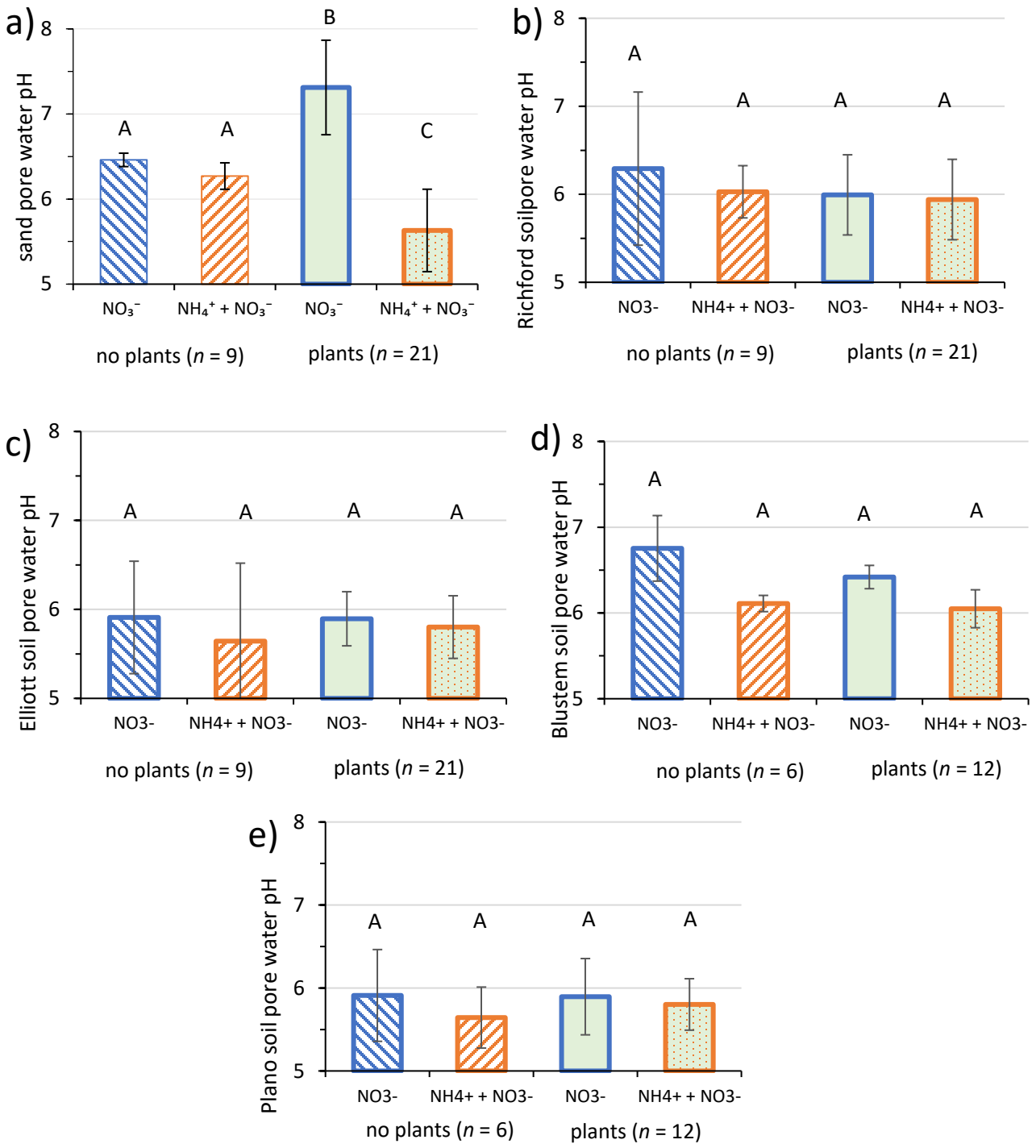
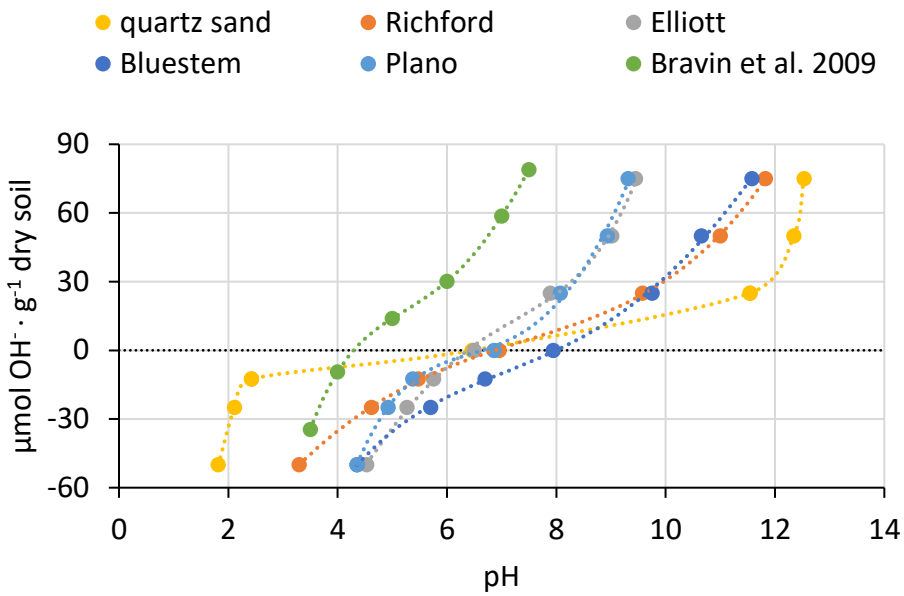


Figure 6.1. Wheat plant effects on soil pore water pH. a) Wheat plants altered porewater pH in model rhizospheres in response to the form of nitrogen provided when grown on sand. Plants raised porewater pH when supplied with nitrate as the sole nitrogen source. Wheat grown on field soils b) Richford, c) Elliott, d) Bluestem, and e) Plano did not significantly alter porewater pH. 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 a Wilcoxon/Kruskal-Wallis test with Steel-Dwass *post hoc* analysis ($p \leq 0.05$). Bars represent mean values; error bars indicate one standard deviation.

a)



b)

	Ax^4 +	Bx^3 +	Cx^2 +	Dx +	E	R^2
quartz sand	0	0	0	4.1461	24.063	0.9847
Richford	0.0147	-0.0562	-3.6544	46.95	-164.74	0.9998
Elliott	0.3438	-7.8247	61.616	-169.81	37.807	0.9985
Bluestem	-0.045	1.911	-26.955	167.8	-411.39	0.9992
Plano	-0.2952	10.686	-132.9	704.93	-1375.8	0.9975
Bravin et al.	-0.7078	18.824	-179.08	748.01	-1159.7	> 0.999***

Figure 6.2. Base addition titration curves as a measure of pH buffering capacity of quartz (yellow), study soils (Richford in orange, Elliott in grey, Bluestem in light blue, Plano in dark blue), and a soil for which wheat plant-driven rhizosphere pH changes were previously reported[19] (green). All curves (a) are shown with best fit 4th order polynomial curves (as was reported by Bravin et al.[19]), except for quartz sand which was estimated as linear between each data point (b). Error bars represent standard deviation and may not extend beyond the points (n = 3).

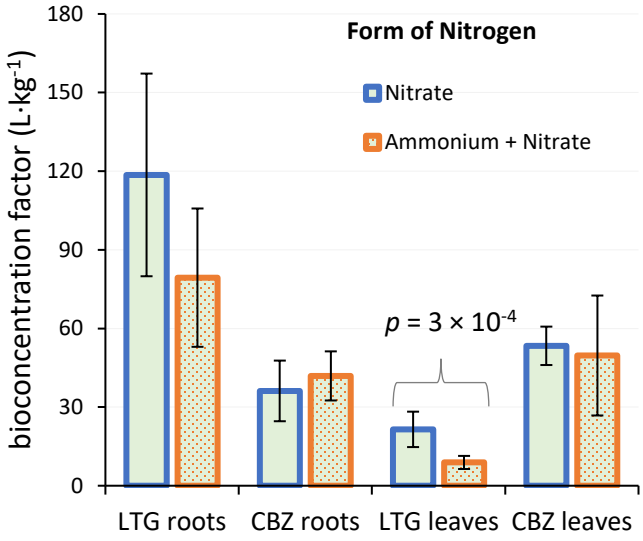


Figure 6.3. Bioconcentration factors for lamotrigine (LTG) and carbamazepine (CBZ) in the roots and leaves of wheat plants grown on quartz sand and supplied with the indicated nitrogen sources. Lamotrigine accumulation in the leaves of plants provided with only nitrate exceeded that in plants receiving 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 porewater at the end of the exposure period. Porewater concentrations of LTG and CBZ did not differ between nitrate and ammonium + nitrate treatments (Figure C3). Concentrations measured in the plant tissue followed the same trends as the shown bioconcentration factors. Welch's *t*-test was used for pairwise comparison between treatments. Error bars indicate one standard deviation.

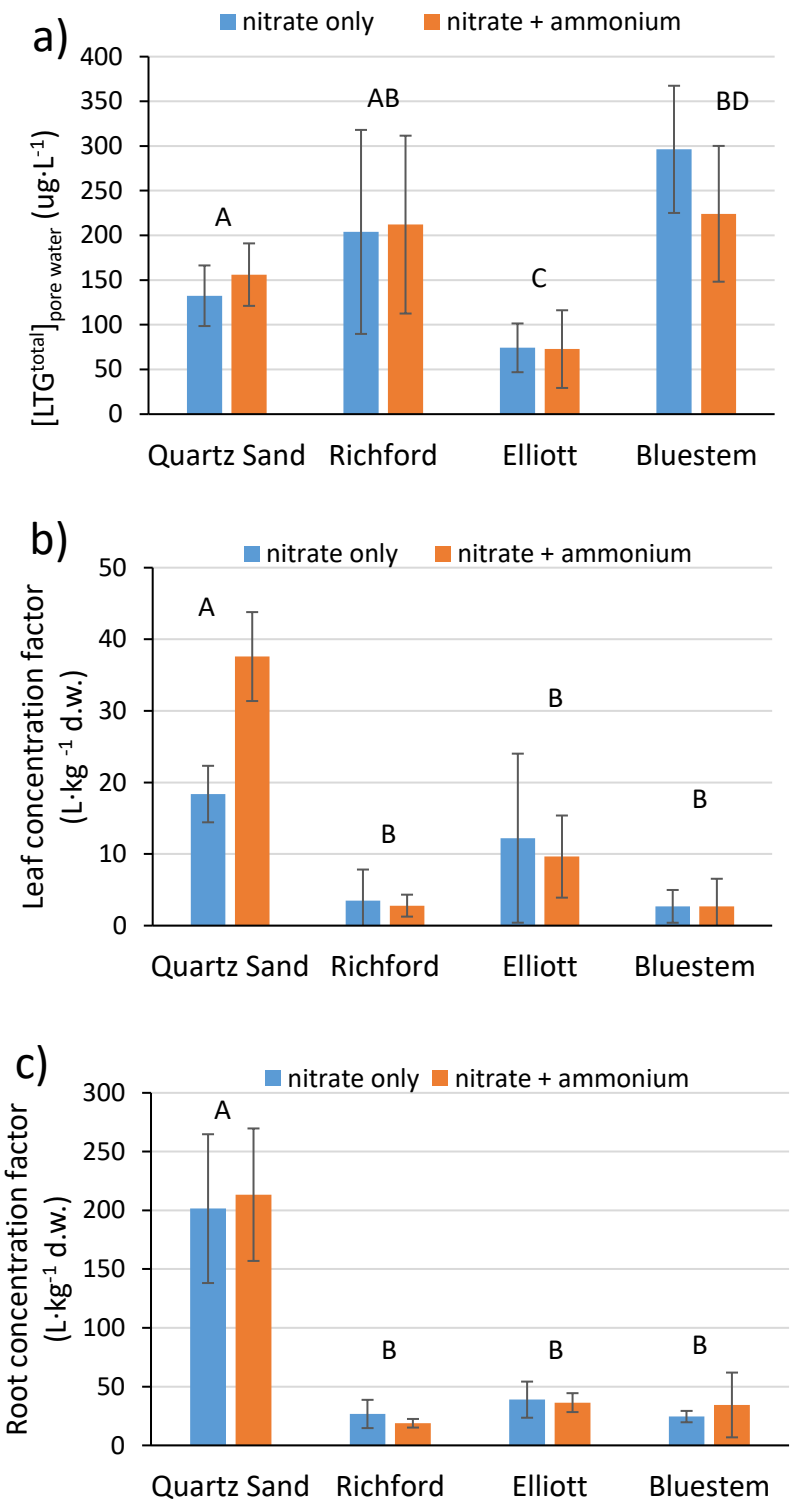
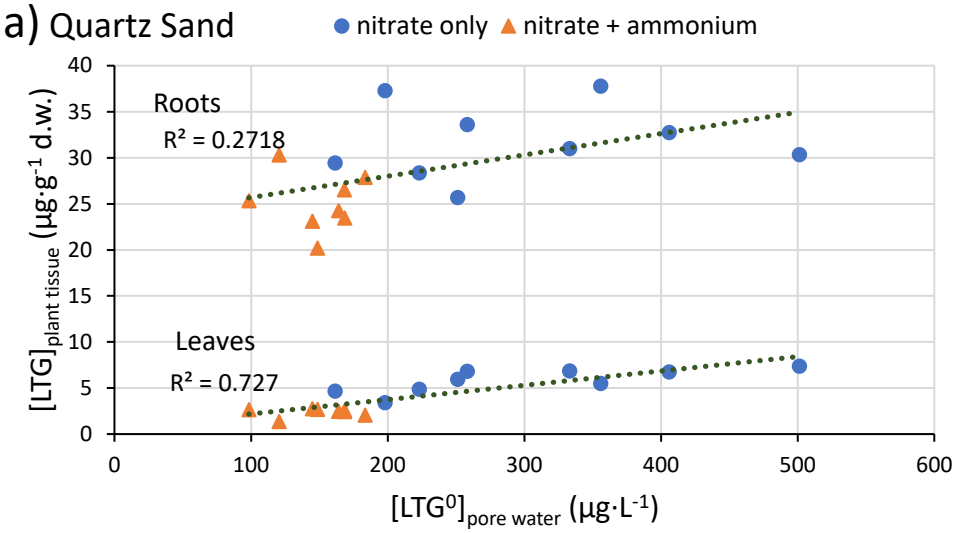


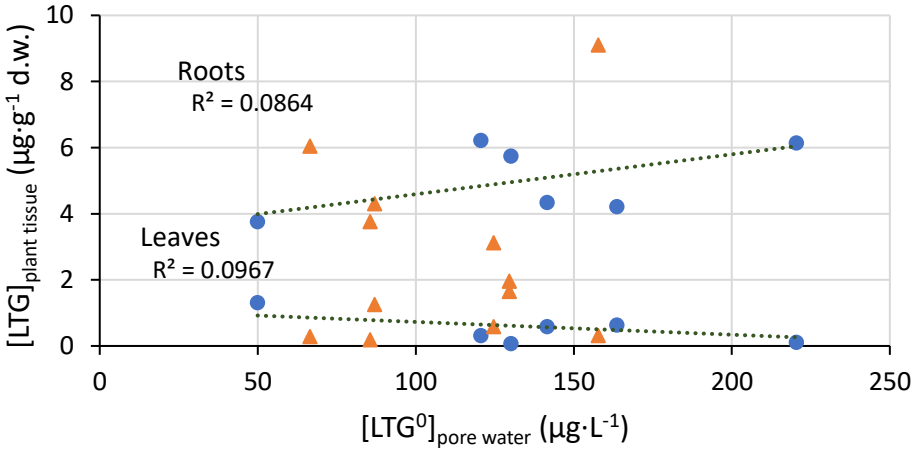
Figure 6.4. Lamotrigine in pore water and wheat plant tissues. a) Soil pore water total lamotrigine (LTG) concentrations were significantly different between soils (letters indicate Kruskal-Wallis One Way Analysis of Variance on Ranks combining data from both nutrient treatments $p < 0.05$; this statistical test was used because data were not normally distributed) but not between nutrient solutions in the same soil

(Welch's *t*-test $p > 0.05$). Concentrations were lowest for Elliott soil, which has the highest percent organic matter (2.9% compared to >1% for all others). Bioconcentration factors for b) leaves and c) roots for LTG accumulation plants grown on quartz sand were significantly higher than those for plants grown on soils (letters indicate Kruskal-Wallis One Way Analysis of Variance on Ranks combining data from both nutrient treatments $p < 0.001$), but there were no differences in bioconcentration factors between plants grown on different soils ($p > 0.05$). The only difference between bioconcentration factors from nutrient solutions in the same growth media was leaf accumulation in plant grown on quartz sand (Welch's *t*-test $p = 3 \times 10^{-4}$, all others $p > 0.05$).

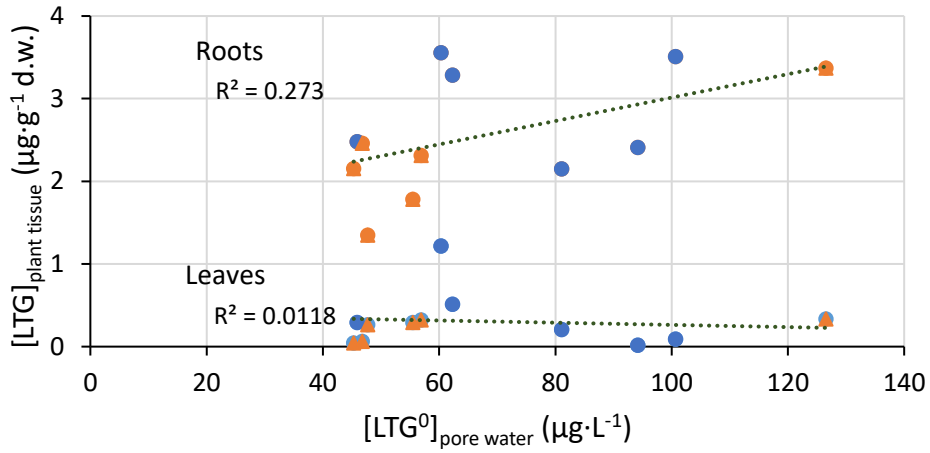
a) Quartz Sand



b) Richford



c) Elliott



d) Bluestem

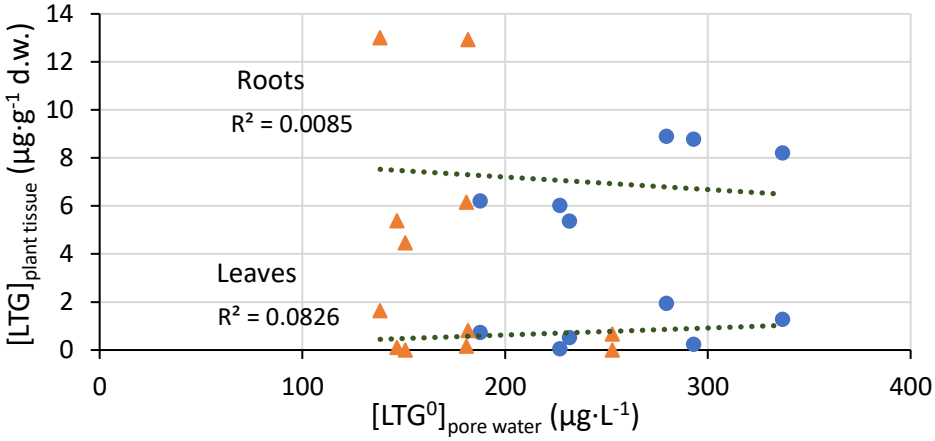
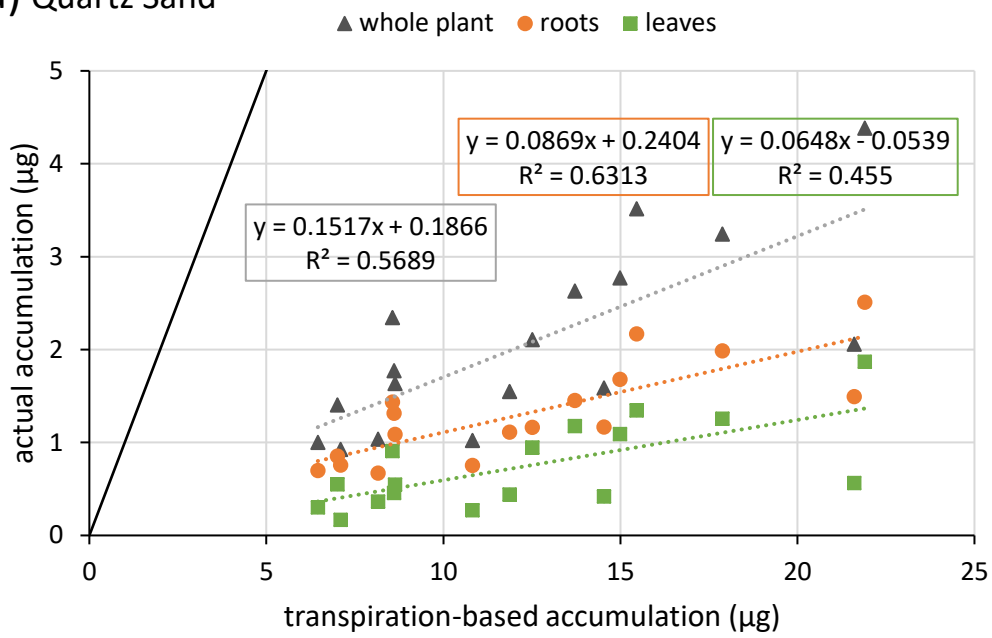
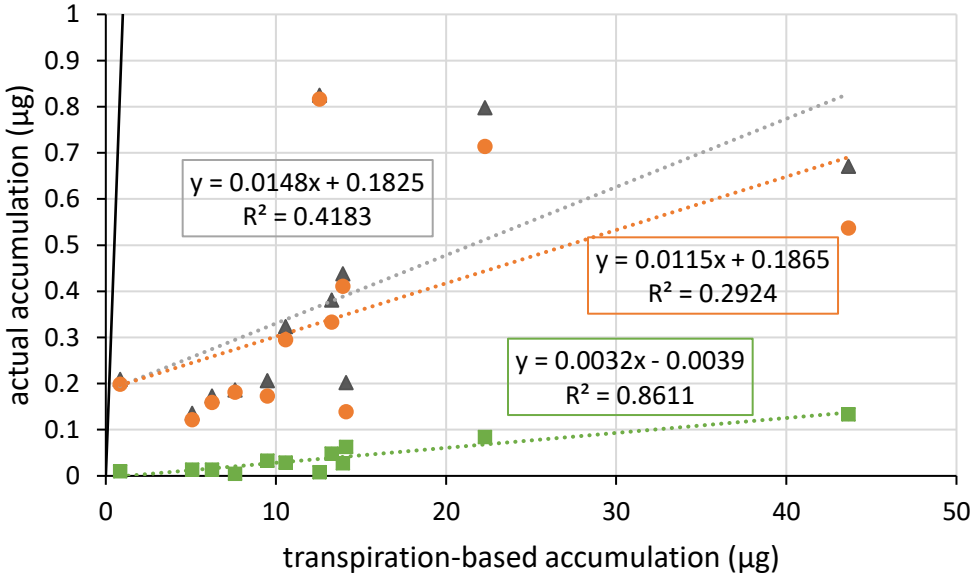


Figure 6.5. Lamotrigine (LTG) accumulation in wheat leaves and roots correlated with the concentration of the neutral LTG species in porewater (calculated via the Henderson-Hasselbalch equation) for plants grown on a) quartz sand, b) Richford soil, c) Elliott soil, and d) Bluestem soil. Blue circles correspond to plants provided with nitrate as the sole nitrogen source. Orange triangles correspond to plants provided with ammonium + nitrate. Only quartz sand showed a strong correlation: The bottom line shows a linear regression of the concentration of LTG in leaves against the concentration of neutral LTG in porewater (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).

a) Quartz Sand



b) Richford



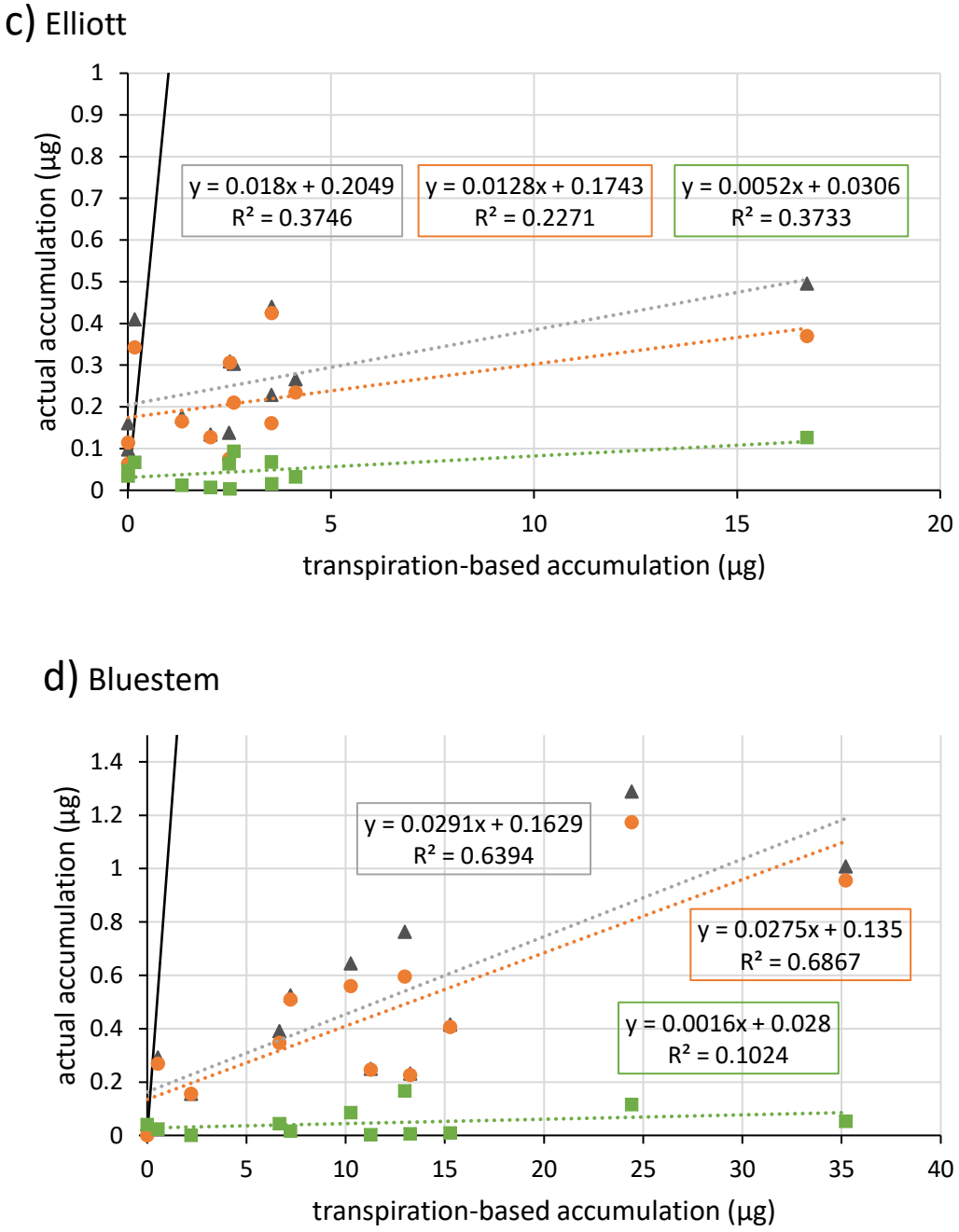


Figure 6.6. Transpiration-based predictions of LTG accumulation in the wheat plants grown compared with measured accumulation in whole plants (grey triangles), roots (orange circles), and leaves (green squares) for plants grown on a) quartz sand, b) Richford, b) Elliott, and d) Bluestem soils. The poor correlations with transpiration may be due to error in estimating transpiration. Actual accumulation was significantly below that which would be predicted by transpiration alone (solid black 1:1 lines); indicating LTG cannot easily enter the transpiration stream, consistent with previous reports. The model also assumes transpiration as the sole driver of accumulation (no active uptake), no in planta metabolism, and no volatilization from leaves.

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Chapter 7. Conclusions

The vast majority of freshwater withdrawal for human benefit goes towards irrigation, much of which goes to support global food output, and is expected to increase as the global human population continues to grow. Water conservation strategies will therefore be increasingly important, especially as global climate change alters weather patterns, changing which land is suitable for crop growth and threatening access to freshwater resources. Reclamation of wastewater is an important strategy for decreasing demand from water sources that are not replenished as quickly as water is withdrawn. Use of reclaimed wastewater for irrigation is increasingly common around the world. However, this practice may be harmful to human health without appropriate risk assessment and mitigation.

Even after treatment, reclaimed wastewater may contain many contaminants that are not effectively removed or are formed during conventional treatment processes, including but not limited to pharmaceuticals, fragrances, dyes, pesticides, plasticizers, flame retardants, and disinfection byproducts. Many of these emerging organic contaminants are polar or ionizable at environmentally relevant pH values, and their fate in the environment is poorly understood. Irrigation with reclaimed water can expose crops for human consumption to these contaminants, prompting a need for risk assessment of consumption of contaminated produce. However, there are thousands of structurally diverse emerging organic contaminants, meaning experimental determination of each individual contaminant's fate in agroecosystems is impractical. Prediction of plant uptake and accumulation of polar and ionizable contaminants is therefore imperative for human health risk assessment. Understanding the mechanisms driving plant uptake and accumulation is key in developing predictive models.

Here, we explored current predictive modeling strategies and investigated processes that may play an important role in plant accumulation of polar and ionizable emerging contaminants. In Chapter 2, we assessed the current state of the literature, attempted modeling plant uptake using compound physicochemical properties and literature data, and provided recommendations regarding future study

design and reporting. We showed that molecular descriptors alone have limited ability to adequately predict ionizable contaminant uptake by plants and phytoaccumulation, and many current studies fail to report information about study design and results that will be essential for more complex modeling efforts.

In Chapter 3, we explored the phytotoxicity and more subtle effects of the antiepileptic drug carbamazepine on the model plant *Arabidopsis thaliana*, but we were unable to identify specific genes or mechanisms of action. In Chapter 4, we demonstrated evidence of mixture effects on phytotoxicity and metabolism of carbamazepine, and explored the use of transpiration measurements as a tool for predicting pharmaceutical accumulation and uptake mechanisms in spinach. Combined, these chapters provide the first evidence that exposure to mixtures may alter toxicity and/or metabolism of individual contaminants, which could lead to changes in accumulation of these contaminants. We also show that transpiration of water through plants is a major driver of contaminant uptake, but by itself is not enough to predict contaminant accumulation, likely because contaminants may take different pathways through roots to the xylem.

Finally, in Chapters 5 and 6, we explored the effects of the rhizosphere on phytoavailability of the ionizable antiepileptic drug lamotrigine. Plants change their soil environment to better suit their needs, and in the process may alter contaminant bioavailability and phytoaccumulation. We demonstrated that the form of nitrogen available to wheat plants can influence root exudation and subsequently, rhizosphere pH, which in turn can alter plant accumulation of ionizable contaminants in some soil conditions. Soil microbiota may play a large role in these processes, as our observations for plants grown in sterile soils differed from previous reports of plant-driven rhizosphere pH changes.

Overall, this thesis represents the first steps in what I hope will be a new trend in the field of polar and ionizable organic contaminant fate in agricultural systems; I believe a different mindset will be necessary for accurate prediction of contaminant accumulation in plants and risk assessment. We may need to rethink the basis of predictive uptake models in order to accurately predict plant accumulation of

ionizable emerging contaminants. Although equilibrium partitioning has historically been how we model contaminant movement through the environment and into biological media, this approach may not be best for ionizable contaminants in plants. Plant processes such as biochemical reactions (e.g. metabolism) and transpiration may be equally or more important than contaminant properties. This thesis provides a basic thought process for designing more relevant and impactful experiments focused on mechanistic understanding of plant uptake and accumulation of emerging organic contaminants. Research that extends beyond this work should be guided by careful consideration of the ways in which plant biology may play an important role, and may perhaps even alter our preconceived notions, regarding processes important for predicting contaminant fate.

I have noted knowledge gaps and provided recommendations for future research throughout the text. Prediction of plant uptake of wastewater-derived contaminants will require accounting for structurally heterogeneous compounds with different uptake patterns, variation in plant species physiology and biochemistry, and the plant-soil environment. That plants can take up and accumulate polar and ionizable emerging organic contaminants is well established, and now is the time to move toward more mechanistic understanding of uptake, translocation, and transformation *in planta*. Wastewater-derived micropollutants also have the potential to affect plant physiology and alter normal biochemical pathways, possibly resulting in altered uptake, metabolism, and accumulation of other contaminants. Root exudates and soil microbiota are important factors governing properties of the soil-plant root interface and may alter bioavailability of contaminants. Tackling these questions will require a multidisciplinary approach and tools from different fields of research. Just as this work would not have been possible without expertise from a wide variety of disciplines, future work on plant uptake of contaminants should find inspiration and approaches from outside of traditional toxicology frameworks.

Appendix A. Root uptake of pharmaceutical and personal care product ingredients – Supplemental Information

A1. Plant Uptake of PPCPs in Relation to Human Exposure Levels of Concern

Table A1 summarizes representative recent literature reports on concentrations of PPCPs detected in environmental media and crops under realistic field conditions. This is not intended to be a comprehensive summary of the literature; we aim only to provide a sense of real environmental concentrations. For more information, we direct readers to recent reviews of PPCPs in plants.[1–3].

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 6 pharmaceuticals from a wide range of classes to be < 15 ng·person^{-1}·d^{-1} and intake of 5 personal care product ingredients to be < 250 ng·person^{-1}·d^{-1}.[8] However, the estimated bioconcentration factors were generally 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.[9,10] Similarly, the potential contribution of vegetable material grown on animal-manure amended soil to the total acceptable daily intake (ADI) of eight veterinary pharmaceuticals was found to be < 10% for all compounds tested,[10] 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 was more than three orders of magnitude smaller than a single therapeutic dose for one compound.[11] 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 $ng \cdot g^{-1}$ concentrations, suggesting limited contamination by target chemicals in realistic food-producing systems.[12] 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.[13]

In some scenarios, however, PPCP uptake may pose a larger risk than the studies summarized above suggest. 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.[14] Although exposure for most individual compounds appears low, little is known about the effects of chronic low-dose exposures, especially to mixtures of PPCPs. In addition, some PPCPs or PPCP metabolites may be of concern if they are highly toxic. For example, to reach the threshold of toxicological concern for carbamazepine and caffeine, an adult would need to consume hundreds of kilograms daily of sweet potatoes or carrots grown in soil irrigated with treated wastewater. In contrast, potentially toxic levels of the carbamazepine metabolite 10,11-epoxycarbamazepine and the anti-convulsant lamotrigine are reached at a much lower daily consumption, because 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).[15] 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;[16,17] the relatively weakly conjugated bonds with sugars 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.

Table A1. Examples of concentrations of personal care product ingredients (PPCPs) in environmental media and crop plants. All studies used realistic field and growing conditions, not spiked growth media.

Compound Class (compound)	Irrigation Water ($\mu g \cdot L^{-1}$)	Soil ($\mu g \cdot kg^{-1}$)	Biosolids ($\mu g \cdot kg^{-1}$)	Crop	Crop ($\mu g \cdot kg^{-1}$)	Ref.
tetracyclines (chlortetracycline)	–	240	4000 – 9000	winter wheat	ND – 874	*4*
macrolides (erythromycin)	0.004 – 0.01	1.1 – 4.4	–	Chinese white cabbage, water spinach, Chinese radish, corn, rice	ND – 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	ND – 487	*4*
NSAIDs	ND – 22.4	–	–	lettuce, carrot	ND – 113	*7*
tricyclic psychoactive drugs (carbamazepine)	0.06 – 0.369	–	–	lettuce, carrot	ND – 52	*7*
miscellaneous basic drugs (caffeine)	0.295– 0.789	–	–	apple tree, alfalfa	0.114 – 110.7	*8*
personal care product ingredients	ND – 0.543	–	–	apple tree, alfalfa	0.024 – 67.6	*8*

Abbreviations: ND, not detected; NSAID, non-steroidal anti-inflammatory drug

A2. Notes on Sabourin et al., 2012[18]

The results of one study stand in contrast to many of those described in the main text.[18] 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. No compounds were detected in both the biosolids and the experimental plants that were not also present at comparable levels in the control plants, though the soil had not previously received biosolids. The authors did 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. 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)**.** The result in this paper may suggest 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.

A3. 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 2.1, along with the fraction of currently published studies that report each parameter. Failure to report critical experimental data limits conclusions that can be drawn when comparing studies. This is demonstrated in both our data compilation (Figure 2.3a-b; Figure A2) and that of Limmer and Burken.[19] 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.

A3.1. Plant Properties. There are many varieties within a given crop species, and these varieties may differ in lipid content, water use efficiency (transpiration rate), or efficiency 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 accumulation, leading to unexplainable differences in reported values when they are not measured. 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.

A3.2. Environmental Conditions. Important environmental parameters to report include temperature, humidity, and exposure duration 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 plant uptake.[20] Temperature and humidity 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 rely on knowing exposure durations in the datasets used in model development and validation. Surprisingly, this factor is not always reported.

A3.3. 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 disappear from solution for reasons other than plant uptake (e.g., sorption to container walls, abiotic degradation, microbial degradation). These factors may 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.

A3.4. Soil Properties. Sorption and desorption processes, degradation, and formation of bound residues control the availability of the contaminant to the plant (see main text). These interactions are governed by contaminant physicochemical properties and soil properties. Therefore, reporting only the textural class of soil used is not sufficient to predict availability from soil. The sorption of PPCPs to soils may depend on the quantity (and quality) of soil organic matter, mineralogy, cation or anion exchange capacity, and pH. 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 disappear from the system for reasons other than plant uptake.

A3.5. Irrigation. For soil studies, irrigation amount and frequency may 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. Therefore irrigation amount and frequency should be reported whenever possible.

A3.6. Analysis. Reporting extraction and detection method parameters such as limits of detection and quantification (LODs/LOQs) and recoveries facilitates 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.

A4. Data Compilation

Figure 2.3 (a) and (b): We found some errors in the pH-dependent *n*-octanol-water partitioning coefficients (D_{ow}) calculated by Wu et al.,[23] 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;[27] 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 to form an anion 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.

Figure 2.3 (c) and (d): We compiled raw data from eight PPCP uptake studies using lettuce, and organized them by growth medium. 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).[21] We used these data to calculate the leaf concentration factor, $LCF = (\frac{\text{leaf concentration}}{\text{exposure concentration}})$ for each compound in each study. In plotting these data, we defined acids as

compounds ionizing to 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 A2. Corrected data from Wu et al.[23] 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[27]	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[23]	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[26]	4.05	0.46	4.05	2	10
Atorvastatin	4.46	4.46[24]	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[28]	4.9	4.9	4.90	0.02	40

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

‡ http://www.drugbank.ca/

Table A3. Hydroponic lettuce data from the literature (used to produce Figure 2.2c). 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	[22]
			0.5	0	0	[23]
			5	0	0	[23]
atenolol	9.6	0.16	0.5	0	0	[22]
			0.5	0	0	[23]
			5	0.108	0.02	[23]
atorvastatin	4.46	4.46[24]	0.5	0.064	0.1	[22]
			0.5	0.064	0.1	[23]
			5	0.048	0.01	[23]
caffeine	10.4	-0.07	0.5	0.128	0.3	[22]
			0.5	0.128	0.3	[23]
			5	0.028	0.01	[23]
carbamazepine	13.94	2.45	0.5	1.148	2	[22]
			0.5	1.16	2	[23]
			5	10	2	[23]
DEET	0.67[23]	2.02	0.5	0.112	0.2	[22]
			0.5	0.112	0.2	[23]
			5	0.72	0.1	[23]
diazepam	3.4	2.8	0.5	0.712	1	[22]
			0.5	0.72	1	[23]
			5	6.4	1	[23]
diclofenac	4.15	4.51	0.5	0	0	[22]
			0.5	0	0	[23]
			5	0	0	[23]
			0.2374	0.362	1.52	[25]
dilantin	8.33	2.47	0.5	0.368	0.7	[22]
			0.5	0.368	0.7	[23]
			5	3.04	0.6	[23]
diuron	8.33	2.68	0.5	0.068	0.1	[23]
			5	3	0.6	[23]
fluoxetine	9.7[26]	4.05	0.5	0.84	2	[22]
			0.5	0.88	2	[23]
			5	10.4	2	[23]
gemfibrozil	4.5	4.77	0.5	0.008	0.02	[22]
			0.5	0.008	0.02	[23]
			5	0.024	0.005	[23]
ibuprofen	4.91	3.97	0.5	0	0	[22]
			0.5	0	0	[23]
			5	0	0	[23]
meprobamate	15.17[‡]	0.7	0.5	0.2	0.4	[22]
			0.5	0.2	0.4	[23]
			5	1.2	0.2	[23]
naproxen	4.15	3.18	0.5	0	0	[22]
			5	0.004	0.0008	[23]
			0.1782	0.1124	0.6308	[23]
primidone	11.5[‡]	0.91	0.5	0.34	0.7	[22]
			0.5	0.34	0.7	[23]

			5	2.44	0.5	[23]
sulfamethoxazole	5.7[27]	0.89	0.5	0	0	[22]
			0.5	0	0	[23]
			5	0	0	[23]
triclocarban	12.7[28]	4.9	0.5	0.012	0.02	[22]
			0.5	0.012	0.02	[23]
			5	0.056	0.01	[23]
triclosan	7.9	4.76	0.5	0	0	[22]
			0.5	0	0	[23]
			5	0	0	[23]
trimethoprim	7.12	0.91	0.5	0.088	0.2	[22]
			0.5	0.044	0.09	[23]
			5	0.4	0.08	[23]

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

‡ http://www.drugbank.ca/

Table A4. Irrigated soil lettuce data from the literature (used to produce Figure 2.2d). 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
ambrettolide		5.37‡	0.497	75	150	7
			0.261	159	609	7
azithromycin	8.74	4.02	1	0	0	29
carbamazepine	13.94	2.45	0.369	0	0	7
			0.061	1	16	7
			0.225	0.058	0.26	11
clindamycin	7.6	2.16	1	0	0	29
clofibric acid	3.2[30]	2.88[30]	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	11
flunixin	5.82[31]	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	11
roxithromycin	9.2[32]	2.75[33]	1	0	0	29
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/

A5. QEPB (Quantitative Estimate of Plant Bioaccumulation) Analysis

We applied the methods of Limmer and Burken[19] 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 transpiration stream concentration factor 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[25] 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.[34] 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 A1):

1. We fixed a number of bins based on the descriptor values. For example, polar surface area (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

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

where $D(x)$ is the desirability function for each molecular descriptor, x, and a–f are fitting parameters. By minimizing the sum of squares $\sum_{i=1}^{m}(h_i - D(x_i))^2$, where 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,[35] 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:

$$QEPB_i = \exp\left(\frac{\sum_j w_j \, log\left(D_j(x_i)\right)}{\sum_j w_j}\right) \qquad \text{(Eq. A.2)}$$

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 A5.

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 A2). We note that the evaluation of the Shannon entropy required to develop the model constrains the output to the closed interval of 0 to 1, meaning that TSCF (or LCF) cannot exceed 1. Although mathematically necessary for the approach taken, this step does not reflect a constraint in uptake because TSCF or LCF can exceed unity,[19] potentially contributing to model inaccuracy and limiting the usefulness of this modeling method. $QEPB_w$ performance may be improved by excluding compounds with LCF >1; however, we did not attempt this because of the small size of our dataset.

Table A5 presents the optimized desirability function weightings for each molecular descriptor with standard errors approximated from a bootstrapping procedure by resampling the data.[35] 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 A4), 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. Limmer and Burken did not report estimated errors for their desirability function weightings.[19]

Table A5. 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)

Table A6. 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)
$QEPT_{max}$	[19]	0.65	0.75	0	0.9	0	0
$QEPT_{100}$	[19]	0.56	0.64	0	0.76	0	0

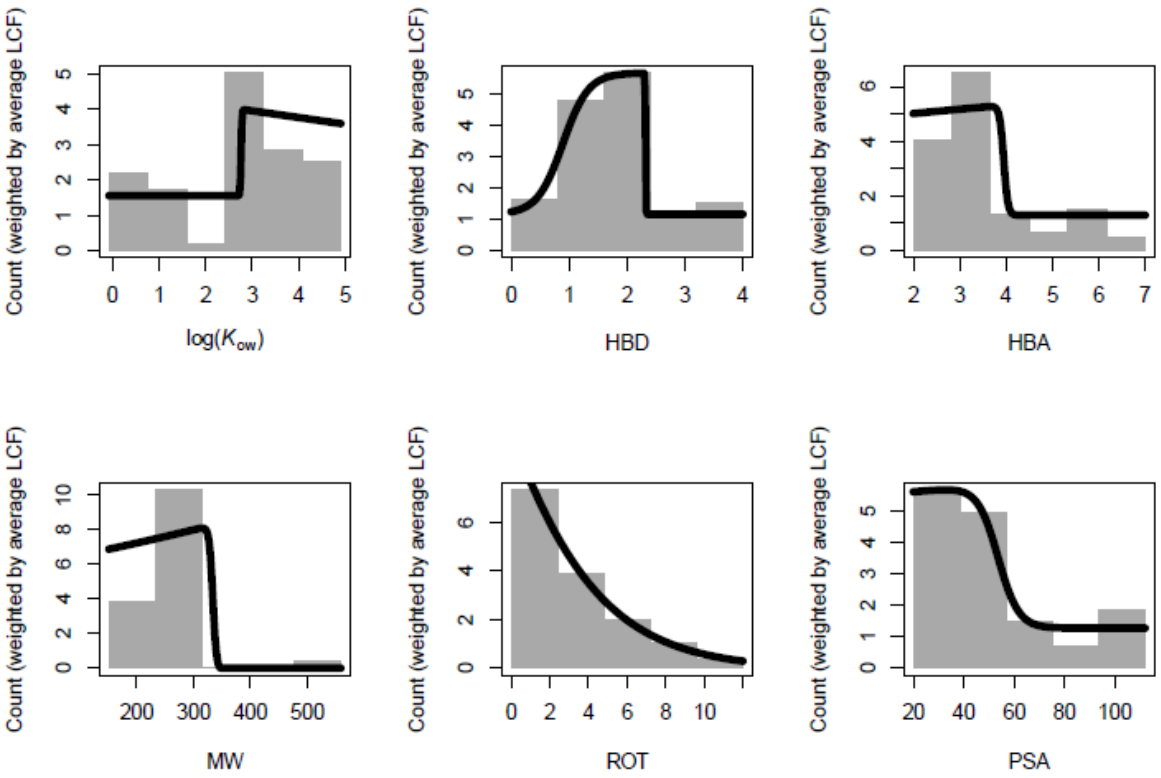

Figure A1. Histograms of LCF values for hydrophobicity (log K_{ow}), number of hydrogen bonding donors (HBD), number of hydrogen bonding acceptors (HBA), molecular mass (MW), number of rotatable bonds (ROT), and polar surface area (PSA). Solid curves are the fitted desirability functions.

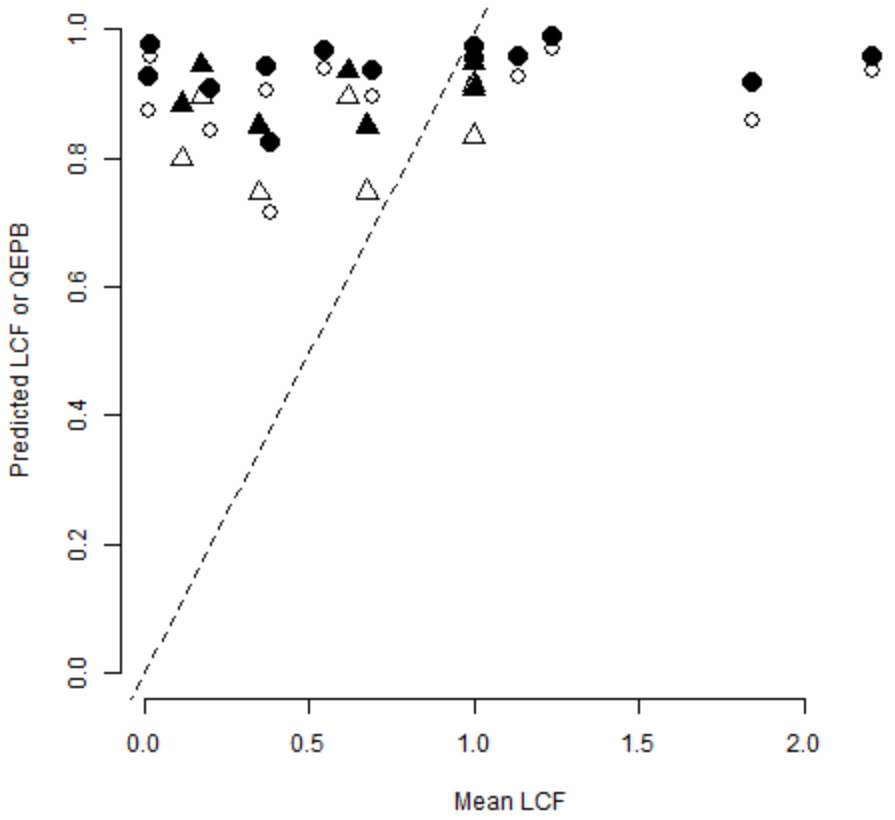

Figure A2. Cross-validation of QEPB LCF predictions compared against LCF measurements. $QEPB_{max}$ calculated values are indicated in black and $QEPB_{100}$ calculated values are indicated in white. Triangles represent hydrophilic compounds with a log K_{ow} of < 1.

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Appendix B. Phytotoxicity and transcriptomic response of Arabidopsis thaliana exposed to the anti-epileptic drug carbamazepine – Supplemental Information

B.1. Supplemental Methods

B.1.2. Extraction Method Recoveries and Limits of Detection. Leaf samples contained 100 $ng \cdot g^{-1}$ internal standard (added before extraction). Concentrations reported in Chapter 3 are recovery corrected based Table B.1. 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:

$$LOD = 3\sigma \quad \text{(Eq. B.1)}$$

$$LOQ = 10\sigma \quad \text{(Eq. B.2)}$$

Table B.1. Internal standard based recovery and limits of detection (LOD) and quantification (LOQ) for carbamazepine (CBZ) and its metabolites 10,11-epoxycarbamazepine (epCBZ) and 10,11-trans-dihydroxycarbamazepine (diOH-CBZ) in *A. thaliana* leaves.

Compound	Spike level	Recovery	stdev	LOD ($ng \cdot g^{-1}$)	LOQ ($ng \cdot g^{-1}$)
CBZ	15 $\mu g \cdot g^{-1}$	1.17	0.02	3	9
epCBZ	45 $ng \cdot g^{-1}$	1.057	0.007	0.6	0.6
diOH-CBZ	15 $ng \cdot g^{-1}$	0.67	0.02	1.1	3.7

B.2. Supplemental Results

Table B.2. All BLAST results for human CYP1A1.

Species	Gene	Max Score	Query Cover	E value	Identity	Accession Number
A. thaliana	CYP450 81F2	212	96	3.00E-62	29	CAR63886.1
A. thaliana	CYP450 75B1	212	95	7.00E-62	31	NP 196416.1
A. thaliana	CYP450, family 76, subfamily C, polypeptide 1	192	96	2.00E-54	29	NP 850439.1
Spinach	CYP450 703A2	196	90	2.00E-56	29	XP 021845179.1
Spinach	hypothetical protein SOVF 073350	196	90	3.00E-56	29	KNA18155.1
Spinach	CYP450 71A4-like	194	89	1.00E-55	28	XP 021863518.1
Spinach	CYP450 71A26-like isoform X1	191	89	1.00E-54	29	XP 021863527.1
Spinach	CYP450 71A26-like	191	89	2.00E-54	28	XP 021863492.1
Spinach	CYP450 76AD1-like	190	90	5.00E-54	28	XP 021856510.1
Spinach	CYP450 84A1-like	190	90	5.00E-54	28	XP 021865288.1
Tomato	CYP450 1A1	321	37	2.00E-108	78	AAD52197.1
Tomato	putative flavenoid 3'5' hydroxylase	207	90	1.00E-60	30	ACF32346.1
Tomato	predicted CYP450 703A2	208	90	1.00E-60	28	XP 004248085.1
Tomato	predicted CYP450 71A1-like	206	90	5.00E-60	30	XP 004237819.1
Tomato	predicted CYP450 93A2-like	206	90	5.00E-60	31	XP 004228848.1
Tomato	predicted CYP450 83B1-like	203	94	4.00E-59	29	XP 004247900.1
Tomato	predicted CYP450 736A12-like	198	89	2.00E-57	27	XP 004237435.1
Tomato	predicted flavenoid 3'-monooxygenase-like	196	92	2.00E-56	30	XP 004250647.1
Tomato	predicted CYP450 71A3	195	90	3.00E-56	29	XP 019068570.1
Tomato	predicted CYP450 84A1	196	89	4.00E-56	28	XP 004232458.1
Cucumber	predicted CYP450 736A12-like	214	90	3.00E-63	30	XP 11658488.1
Cucumber	predicted CYP450 93A2-like	208	90	6.00E-61	31	XP 004150165.1
Cucumber	predicted CYP450 71A1-like	197	92	4.00E-57	28	XP 011660355.1
Cucumber	predicted CYP450 81E8-like	195	89	4.00E-56	28	XP 004141996.2
Cucumber	predicted flavenoid 3'-monooxygenase	196	90	4.00E-56	30	XP 004138192.1

Table B.3. All BLAST results for human CYP1A2**.**

Species	Gene	Max Score	Query Cover	E value	Identity	Accession Number
A. thaliana	CYP450 75B1	199	94	6.00E-58	30	NP 196416.1
A. thaliana	CYP450 family 703 subfamily A polypeptide 2	192	88	1.00E-54	29	NP 171635.1
A. thaliana	CYP450 family 81 subfamily F polypeptide 2	191	94	3.00E-54	28	NP 200532.1
Spinach	CYP450 76AD1-like	186	97	1.00E-52	26	XP 021856511.1
Tomato	CYP450 1A1	289	37	1.00E-99	75	AAD52197.1
Tomato	predicted CYP450 736A12-like	199	91	1.00E-57	28	XP 004237435.1
Tomato	predicted CYP450 703A2	199	89	4.00E-57	28	XP 004248085.1
Tomato	putative flavenoid 3'5' hydroxylase	194	89	7.00E-56	28	ACF32346.1
Tomato	predicted CYP450 93A2-like	192	89	9.00E-55	30	XP 004228848.1
Cucumber	predicted CYP450 736A12-like	199	90	1.00E-57	29	XP 004142333.3
Cucumber	predicted CYP450 93A2-like	197	89	5.00E-57	31	XP 004150165
Cucumber	predicted CYP450 81D1	191	90	2.00E-54	28	XP 004151924.2

Table B.4. All BLAST results for human CYP2B6.

Species	Gene	Max Score	Query Cover	E value	Identity	Accession Number
A. thaliana	cytochrome P450, family 83, subfamily A, polypeptide 1 [*Arabidopsis thaliana*]	183	93%	3.00E-51	28%	NP_193113.1
Spinach	cytochrome P450 71A26-like [*Spinacia oleracea*]	164	88%	1.00E-44	27%	XP_021863492.1
Tomato	PREDICTED: cytochrome P450 83B1-like [*Solanum lycopersicum*]	169	92%	2.00E-46	27%	XP_004247904.1
Cucumber	PREDICTED: cytochrome P450 71A1-like [*Cucumis sativus*]	161	92%	1.00E-43	28%	XP_011651837.1

Table B.5. All BLAST results for human CYP2C8.

Species	Gene	Max Score	Query Cover	E value	Identity	Accession Number
A. thaliana	cytochrome P450, family 83, subfamily A, polypeptide 1 [*Arabidopsis thaliana*]	193	93%	5.00E-55	29%	NP_193113.1
A. thaliana	cytochrome p450 dependent monooxygenase [*Arabidopsis thaliana*]	192	93%	9.00E-55	29%	AAA79982.1
Spinach	cytochrome P450 71A26-like [*Spinacia oleracea*]	170	88%	7.00E-47	28%	XP_021863492.1
Tomato	PREDICTED: cytochrome P450 71A1-like [*Solanum lycopersicum*]	179	94%	3.00E-50	29%	XP_004247902.2
Cucumber	PREDICTED: cytochrome P450 71A1-like [*Cucumis sativus*]	160	90%	2.00E-43	26%	XP_011651857.1

Table B.6. All BLAST results for human CYP2C9.

Species	Gene	Max Score	Query Cover	E value	Identity	Accession Number
A. thaliana	cytochrome P450, family 71, subfamily A, polypeptide 25 [*Arabidopsis thaliana*]	184	95%	5.00E-52	28%	NP_680107.1
A. thaliana	CYP71A25 [*Arabidopsis thaliana*]	184	92%	7.00E-52	28%	OAP01346.1
Spinach	cytochrome P450 76AD1-like [*Spinacia oleracea*]	182	93%	1.00E-51	29%	XP_021856511.1
Tomato	PREDICTED: geraniol 8-hydroxylase-like [*Solanum lycopersicum*]	178	93%	5.00E-50	29%	XP_019069749.1
Cucumber	PREDICTED: cytochrome P450 71A1-like [*Cucumis sativus*]	176	89%	5.00E-49	28%	XP_011651857.1

Table B.7. All BLAST results for human CYP2C19.

Species	Gene	Max Score	Query Cover	E value	Identity	Accession Number
A. thaliana	CYP71A25 [*Arabidopsis thaliana*]	176	95%	9.00E-49	28%	OAP01346.1
A. thaliana	cytochrome P450, family 83, subfamily A, polypeptide 1 [*Arabidopsis thaliana*]	176	96%	1.00E-48	28%	NP_193113.1
A. thaliana	cytochrome P450, family 71, subfamily A, polypeptide 25 [*Arabidopsis thaliana*]	176	95%	1.00E-48	28%	NP_680107.1
Spinach	cytochrome P450 76AD1-like [*Spinacia oleracea*]	176	93%	2.00E-49	29%	XP_021856511.1
Tomato	PREDICTED: cytochrome P450 71A1-like [*Solanum lycopersicum*]	171	90%	2.00E-47	30%	XP_004247902.2
Cucumber	PREDICTED: cytochrome P450 71A1-like [*Cucumis sativus*]	172	91%	1.00E-47	28%	XP_011651857.1

Table B.8. All BLAST results for human CYP2E1.

Species	Gene	Max Score	Query Cover	E value	Identity	Accession Number
A. thaliana	cytochrome P450, family 83, subfamily A, polypeptide 1 [*Arabidopsis thaliana*]	173	93%	1.00E-47	28%	NP_193113.1
Spinach	cytochrome P450 76AD1-like [*Spinacia oleracea*]	158	93%	2.00E-42	26%	XP_021853403.1
Tomato	PREDICTED: cytochrome P450 71A1-like [*Solanum lycopersicum*]	179	94%	2.00E-50	28%	XP_004247902.2
Cucumber	PREDICTED: cytochrome P450 71A1-like [*Cucumis sativus*]	168	89%	2.00E-46	29%	XP_011651857.1

Table B.9. All BLAST results for human CYP3A4.

Species	Gene	Max Score	Query Cover	E value	Identity	Accession Number
A. thaliana	cytochrome P450, family 711, subfamily A, polypeptide 1 [*Arabidopsis thaliana*]	179	96%	1.00E-49	27%	NP_565617.2
A. thaliana	MAX1 [*Arabidopsis thaliana*]	179	96%	1.00E-49	27%	OAP07831.1
Spinach	hypothetical protein SOVF_003710 [*Spinacia oleracea*]	196	95%	3.00E-56	28%	KNA25787.1
Spinach	cytochrome P450 711A1 [*Spinacia oleracea*]	196	95%	4.00E-56	28%	XP_021856628.1
Tomato	PREDICTED: cytochrome P450 711A1 [*Solanum lycopersicum*]	196	95%	3.00E-56	29%	XP_004245085.1
Cucumber	PREDICTED: cytochrome P450 711A1 [*Cucumis sativus*]	201	98%	2.00E-58	30%	XP_004141322.2
Cucumber	PREDICTED: cytokinin hydroxylase-like [*Cucumis sativus*]	185	85%	3.00E-52	28%	XP_004145660.1

Table B.10. All BLAST results for human CYP3A5.

Species	Gene	Max Score	Query Cover	E value	Identity	Accession Number
A. thaliana	cytochrome P450 711A1 [*Arabidopsis lyrata subsp. lyrata*]	177	97%	1.00E-48	27%	XP_020884785.1
Spinach	hypothetical protein SOVF_003710 [*Spinacia oleracea*]	192	95%	2.00E-54	28%	KNA25787.1
Spinach	cytochrome P450 711A1 [*Spinacia oleracea*]	191	95%	3.00E-54	28%	XP_021856628.1
Tomato	PREDICTED: cytochrome P450 711A1 [*Solanum lycopersicum*]	185	90%	3.00E-52	28%	XP_004245085.1
Cucumber	PREDICTED: cytochrome P450 711A1 [*Cucumis sativus*]	185	97%	4.00E-52	28%	XP_004141322.2
Cucumber	PREDICTED: cytochrome P450 CYP749A22-like [*Cucumis sativus*]	184	78%	4.00E-52	29%	XP_004135430.2
Cucumber	PREDICTED: cytokinin hydroxylase-like [*Cucumis sativus*]	184	87%	7.00E-52	28%	XP_004145660.1

Table B.11. All BLAST results for human CYP3A7.

Species	Gene	Max Score	Query Cover	E value	Identity	Accession Number
A. thaliana	cytochrome P450, family 711, subfamily A, polypeptide 1 [*Arabidopsis thaliana*]	171	97%	2.00E-46	26%	NP_565617.2
Spinach	hypothetical protein SOVF_003710 [*Spinacia oleracea*]	174	95%	7.00E-48	25%	KNA25787.1
Spinach	cytochrome P450 711A1 [*Spinacia oleracea*]	174	95%	9.00E-48	25%	XP_021856628.1
Tomato	PREDICTED: cytochrome P450 711A1 [*Solanum lycopersicum*]	177	91%	3.00E-49	28%	XP_004245085.1
Cucumber	PREDICTED: cytochrome P450 CYP749A22-like [*Cucumis sativus*]	180	77%	2.00E-50	30%	XP_004135430.2

Appendix C. Effects of mixtures on toxicity and phytoaccumulation of pharmaceuticals and personal care product ingredients – Supplemental Information

Note: The portion of this work describing results in spinach is reproduced with permission from Nason, S.L.; Miller, E.L.; Karthikeyan. K.G.; Pedersen. J.A. "Effects of Binary Mixtures and Transpiration on Accumulation of Pharmaceuticals by Spinach" *Environ. Sci. Technol.* **2019**. DOI: 10.1021/acs.est.8b05515.

C1. Supplemental Methods

C1.1. Materials. Tyee Hybrid spinach (*Spinacia oleracea*) seeds were obtained from Jung Garden Center, Madison, WI. 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-d_{10}, 10,11-epoxycarbamazepine-d_{10}, lamotrigine $^{13}C_3$, amitriptyline-d_6, and fluoxetine-d_6 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).

B1.2. Plant Germination and Growth. Tyee Hybrid Spinach seeds 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 sterile 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 container holding ~400 mL sterile nutrient solution. Plants grew hydroponically in this solution for 7-8 weeks prior to exposure to pharmaceuticals.

Nutrient solution was replenished periodically throughout the growth period. Light was provided by Verilux natural spectrum 48-inch T12 fluorescent bulbs; the photon flux density was 35 to 80 $\mu mol_{photons} \cdot m^{-2} \cdot s^{-1}$ throughout the growth area. The light cycle was 16 h light/8 h dark.

C1.3. Exposure Experiments. We conducted three separate sets of spinach uptake experiments. In Set A, 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 Set B, 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 as the neutral species. We did not observe any plant health effects due to the pH change. Each treatment in Sets A and B included 4-5 plants (each plant considered as one replicate). Carbamazepine uptake did not differ between Sets A and B, so data was combined for the analysis in Figures 4.4, 4.7, 4.8, and C2. In Set C, we conducted a time series experiment with CBZ to test accumulation over time for 14 days with nutrient solution pH at 5.7. Three or four plants were harvested at each time point. A full list of treatments across the three sets of experiments and the number of plants in each treatment are provided with plant mass and transpiration data in Table B4. Temperature and humidity varied to some degree among the sets of experiments (Figure C1), which caused some variation in plant size and transpiration (Table C4).

During the exposure experiments, individual plants were placed in polypropylene tubs holding 400 mL of sterile nutrient solution. Pharmaceuticals were added in DMSO resulting in DMSO concentrations of 0.01% in mixture experiments and 0.005% in the CBZ time series experiments. Pure DMSO was added to pharmaceutical-free control solutions to achieve the same concentration. Each tub of solution was mixed and sampled before the plant was added and sampled again at the end of the exposure period. 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.

C1.4. Analytical Methods Details. Plant samples were freeze dried, ground with a mortar and pestle, and subjected to Accelerated Solvent Extraction (ASE) with 100% methanol. Each ASE cell

contained a glass fiber filter and 1.0 g Florosil at the bottom, over which was placed the tissue sample (0.2 ± 0.05 g for leaves and 0.05 ± 0.005 g for roots) followed by another 1.0 g Florosil. We used an ASE 200 (Dionex) 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, reconstituted to 1 mL, sonicated (10 min), centrifuged (20 min, 17000 ×*g*), and filtered through 0.2 μm PTFE filters.

The extraction method differed somewhat among the three sets of experiments. For Sets A and C, the reconstitution solvent was 20% ultrapure water, 80% acetonitrile, 0.1% acetic acid; for Set B, we used 80% ultrapure water, 20% acetonitrile, 0.1% acetic acid. Extraction recoveries and limits of detection are provided for both reconstitution solvents (Tables C1 and C2). The lower percentage of acetonitrile was used for Set B to improve chromatographic peak shape for LTG analysis.

Additionally, the timing of the solvent gradient on the HPLC differed slightly among sets of samples although the same mobile phases were used. Mass-to-charge ratios used were the same for both methods (Table B3).

We measured AMI, FLX, LTG, CBZ, and the CBZ metabolites 10,11-epoxycarbamazepine (epCBZ) and 10,11-*trans*-dihydroxycarbamazepine (diOH-CBZ) in leaf and root extracts and starting and ending nutrient solutions using 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). Mobile phases were (A) 10% acetonitrile in water with 0.1% formic acid and (B) 100% acetonitrile. We used a gradient of 5% to 95% (B), and column temperature was held at 30 °C. Internal standard-based calibration and measurement was used for plant extracts. Nutrient solution samples did not contain internal standards. Extraction recoveries, limits of quantification and detection, and *m*/*z* values used for measurement are provided in Tables C1-C3.

C1.5. Statistical Analyses. We used Microsoft Excel Professional Plus 2013 to conduct *t*-tests (assuming unequal variance). We used Graph Pad Prism to perform linear regression statistics. We used JMP Pro 12.2.0 (SAS) for ANOVA tests with Tukey's *post hoc* analysis and Dunnett's tests.

Table C1. Extraction Recoveries [a]

		80% Acetonitrile Reconstitution		20% Acetonitrile Reconstitution	
Compound	Matrix	Spike level ($ng \cdot g^{-1}$)	Recovery (%)	Spike level ($ng \cdot g^{-1}$)	Recovery (%)
AMI	roots	400	108 ± 4		
		20,000	88 ± 4		
	leaves	5,000	97 ± 4		
		20,000	96 ± 1		
FLX	roots	33,000	100 ± 20		
	leaves	7,500	95 ± 3		
CBZ	roots	40	120 ± 20	40	91 ± 2
		5,000	114 ± 2	5,000	152 ± 4
	leaves	63	140 ± 20	125	105 ± 0.6
		20,000	103 ± 4	15,000	137 ± 9
10,11-epoxycarbamazepine	roots	0.4	144 [b]	0.4	140 ± 6
		30	104 ± 3	20	120 ± 10
	leaves	3	110 ± 10	3	70 ± 3
		250	97 ± 4	150	60 ± 7
10,11-*trans*-hydroxycarbamazepine	leaves			10	110 ± 10
LTG	roots			80	97 ± 4
				10,000	126 ± 0.6
	leaves			20	130 ± 20
				2,000	119 ± 7

[a] Table shows mean values ± standard deviation. Compound recoveries are based on a calibration using internal standard (IS) ratios. 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.

[b] Replicate measurements not available.

Table C2. Limits of Detection and Quantification (ng·g^{-1} dry weight) [a]

	80% Acetonitrile reconstitution				20% Acetonitrile reconstitution			
Compound	Root LOD	Root LOQ	Leaf LOD	Leaf LOQ	Root LOD	Root LOQ	Leaf LOD	Leaf LOQ
Amitriptyline	3	9	6	20.				
Fluoxetine	2	8	1.3	4.3				
Carbamazepine	2	6	2.3	7.9	1.5	5.1	3	9
10,11-epoxycarbamazepine	0.8	2.6	0.08	0.26	0.6	2.1	1.3	4.6
10,11-*trans*-hydroxycarbamazepine					0.3	1.0	0.16	0.56
Lamotrigine					7	23	6	19

[a] 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 were calculated using the equations:[1]

$LOD = 3\sigma$ (Eq. C.2)

$LOQ = 10\sigma$ (Eq. C.3)

Table C3. 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

C2. Supplemental Results

C2.1. Plant Mass and Transpiration. Plant mass and transpiration data are shown in Table C4. Plant mass did not differ within each batch of plants (ANOVA, $p > 0.3$, batches outlined in section C1.1). Transpiration and water loss did not differ within sets A and B (ANOVA, $p > 0.4$). As expected, water loss and transpiration increased over time in the CBZ time series experiment (set C), with significant differences between days 1 and 14 (ANOVA, Tukey's post hoc, $p < 0.05$). Set B had less water loss and transpiration than the others ($p < 0.0002$), and lower leaf wet mass ($p < 0.05$). Set C had higher wet and dry root and leaf masses than the other experiments ($p < 0.0005$). We attribute the differences in experiments to variation in environmental conditions (Figure C1). Spinach grows best in cool, humid air. Set B had the lowest humidity and highest temperatures, while Set C had the lowest temperatures. We note that our total plant mass was low relative to spinach plants grown on soil under ideal growth conditions. Plants exhibited no signs of malnutrition, disease, or toxicity such as misshapen leaves, leaf discoloration or wilting. We do not think the relatively small plant size impacted the relationships between transpired water and accumulated pharmaceuticals.

Accumulation and metabolism of CBZ were similar in Sets A and B. Data reported in Figures 4.6, 4.7, and 4.8 include plants from both sets of experiments.

Table C4. Plant Mass and Transpiration [a]

				Mass (g)					
						Roots		Leaves	
Set [b]	Treatment	Conc. (μg L^{-1})	# Plants	Water Loss	Transpiration	Wet Mass	Dry Mass	Wet Mass	Dry Mass
	Control		5	93 ± 28	80 ± 23	1.7 ± 0.7	0.07 ± 0.03	4.3 ± 1.6	0.33 ± 0.15
	CBZ	1	5	101 ± 24	91 ± 29	1.8 ± 0.4	0.07 ± 0.01	4.6 ± 2.1	0.36 ± 0.13
	AMI	1	4	123 ± 69	117 ± 67	2.0 ± 0.8	0.08 ± 0.03	5.3 ± 2.7	0.40 ± 0.20
	CBZ-AMI	1	4	88 ± 23	81 ± 22	1.8 ± 0.5	0.07 ± 0.02	4.4 ± 0.8	0.36 ± 0.09
A	CBZ	100	5	90 ± 36	79 ± 31	1.5 ± 0.5	0.06 ± 0.02	4.4 ± 1.7	0.35 ± 0.16
	AMI	100	4	117 ± 60	110 ± 56	1.9 ± 0.8	0.08 ± 0.02	5.1 ± 2.6	0.41 ± 0.21
	FLX	100	4	110 ± 43	100 ± 34	1.8 ± 1.0	0.07 ± 0.04	5.3 ± 3.2	0.46 ± 0.30
	CBZ-AMI	100	4	117 ± 75	111 ± 73	1.8 ± 1.0	0.08 ± 0.04	5.6 ± 3.6	0.46 ± 0.31
	CBZ-FLX	100	4	94 ± 61	86 ± 64	1.8 ± 0.8	0.06 ± 0.03	4.1 ± 2.8	0.33 ± 0.21
	Control		4	56 ± 29	50 ± 28	1.1 ± 0.4	0.05 ± 0.0	2.8 ± 1.0	0.26 ± 0.08
	CBZ	1	4	64 ± 37	58 ± 40	1.6 ± 0.2	0.06 ± 0.01	3.4 ± 1.3	0.29 ± 0.09
	LTG	1	4	64 ± 41	57 ± 37	1.7 ± 1.1	0.06 ± 0.04	3.4 ± 2.0	0.30 ± 0.19
B	CBZ-LTG	1	4	68 ± 26	60 ± 28	2.0 ± 0.6	0.07 ± 0.02	3.9 ± 1.2	0.34 ± 0.10
	CBZ	100	5	54 ± 27	45 ± 28	1.2 ± 0.5	0.04 ± 0.02	2.5 ± 1.2	0.22 ± 0.11
	LTG	100	4	71 ± 24	63 ± 22	1.3 ± 0.4	0.05 ± 0.02	3.4 ± 1.3	0.29 ± 0.13
	CBZ-LTG	100	4	75 ± 57	68 ± 60	1.7 ± 1.1	0.06 ± 0.04	3.5 ± 2.6	0.31 ± 0.24
	Control		3	132 ± 60	123 ± 57	2.7 ± 1.1	0.10 ± 0.03	7.5 ± 3.1	0.68 ± 0.29
	Day 1	100	3	16 ± 11	15 ± 11	2.4 ± 0.8	0.08 ± 0.03	6.7 ± 2.9	0.50 ± 0.17
C	Day 4	100	4	94 ± 31	89 ± 34	2.4 ± 0.9	0.08 ± 0.03	6.7 ± 3.5	0.52 ± 0.22
	Day 7	100	3	134 ± 29	123 ± 35	3.4 ± 0.7	0.13 ± 0.03	8.0 ± 2.5	0.85 ± 0.30
	Day 14	100	4	256 ± 141	239 ± 141	2.8 ± 1.1	0.11 ± 0.04	7.3 ± 3.2	0.85 ± 0.46

[a] Table shows mean values ± one standard deviation.

[b] As described in section C1.1, plants for the experiments were grown in three sets (A: AMI, FLX, CBZ mixtures; B: LTG, CBZ mixture; C: CBZ times series). Plant mass and transpiration data are reported by treatment. The compounds to which the plants in each treatment were exposed are indicated (abbreviations: AMI, amitriptyline; CBZ, carbamazepine; FLX, fluoxetine; LTG, lamotrigine).

Table C5. Linear correlations between transpiration-based and actual accumulation in leaves, roots, and whole plants [a]

	Exposure Concentration ($\mu g \cdot L^{-1}$)	Compartment	R^2	Slope		Intercept	
				Coefficient [b]	*p*-value	Coefficient [b]	*p*-value
Amitriptyline	1	Leaves	0.83	1.4 ± 0.3	0.002	10 ± 30	0.7
		Roots	0.009	0.01 ± 0.07	0.8	27 ± 7	0.008
		Whole Plant	0.82	1.4 ± 0.3	0.002	20 ± 30	0.6
	100	Leaves	0.77	1.7 ± 0.4	0.003	6 ± 5	0.3
		Roots	0.33	0.06 ± 0.04	0.1	1 ± 0.5	0.1
		Whole Plant	0.77	1.7 ± 0.4	0.004	5 ± 6	0.4
Carbamazepine	1	Leaves	0.81	0.43 ± 0.05	9.E-07	7 ± 5	0.2
		Roots	0.36	0.018 ± 0.006	0.01	2.1 ± 0.6	0.002
		Whole Plant	0.83	0.46 ± 0.05	3.E-07	10 ± 5	0.07
	100	Leaves	0.89	0.47 ± 0.03	4.E-11	1.3 ± 0.6	0.03
		Roots	0.51	0.011 ± 0.002	1.E-04	0.2 ± 0.04	2.E-05
		Whole Plant	0.89	0.48 ± 0.04	5.E-11	1.5 ± 0.58	0.02
Lamotrigine	1	Leaves	0.68	0.07 ± 0.02	0.01	2 ± 1	0.2
		Roots	0.52	0.06 ± 0.02	0.04	2 ± 1	0.1
		Whole Plant	0.66	0.13 ± 0.04	0.01	5 ± 3	0.1
	100	Leaves	0.95	0.072 ± 0.007	5.E-05	0.13 ± 0.06	0.08
		Roots	0.74	0.037 ± 0.008	0.01	0.3 ± 0.08	0.006
		Whole Plant	0.90	0.11 ± 0.01	3.E-04	0.4 ± 0.1	0.01
Fluoxetine	100	Leaves	0.57	0.13 ± 0.04	0.02	0.3 ± 1	0.8
		Roots	0.56	0.08 ± 0.02	0.02	1 ± 0.7	0.2
		Whole Plant	0.63	0.22 ± 0.06	0.01	1.3 ± 1.5	0.4

[a] Correlations graphed in Figures 4.7 and C2.
[b] Standard error is provided for each value.

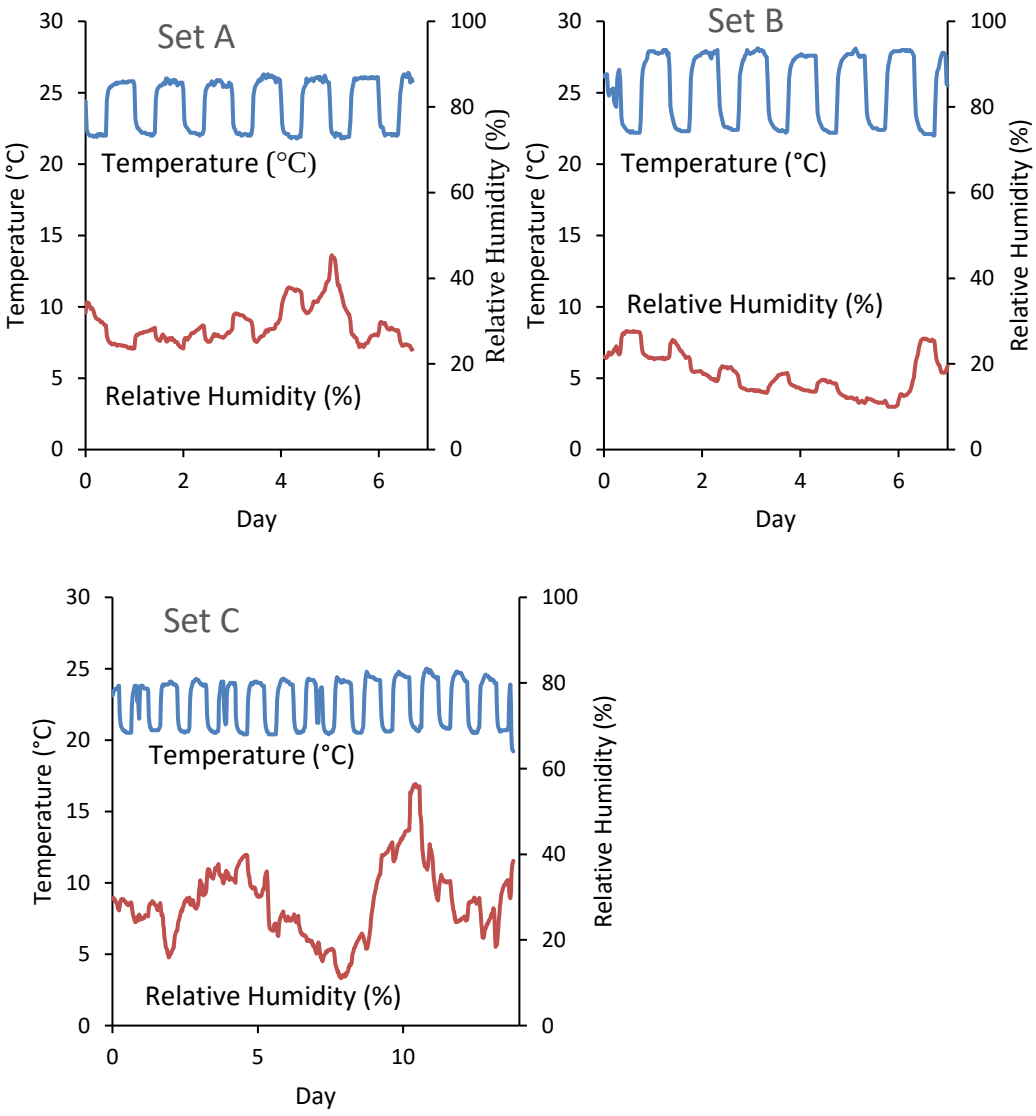

Figure C1. Temperature and humidity measurements during the three sets of spinach exposure experiments. Measurements were recorded every 30 minutes using an automated system for the duration of each experiment. Differences in temperature and humidity likely influenced the differences in plant size and transpiration we saw between batches (Table C4).

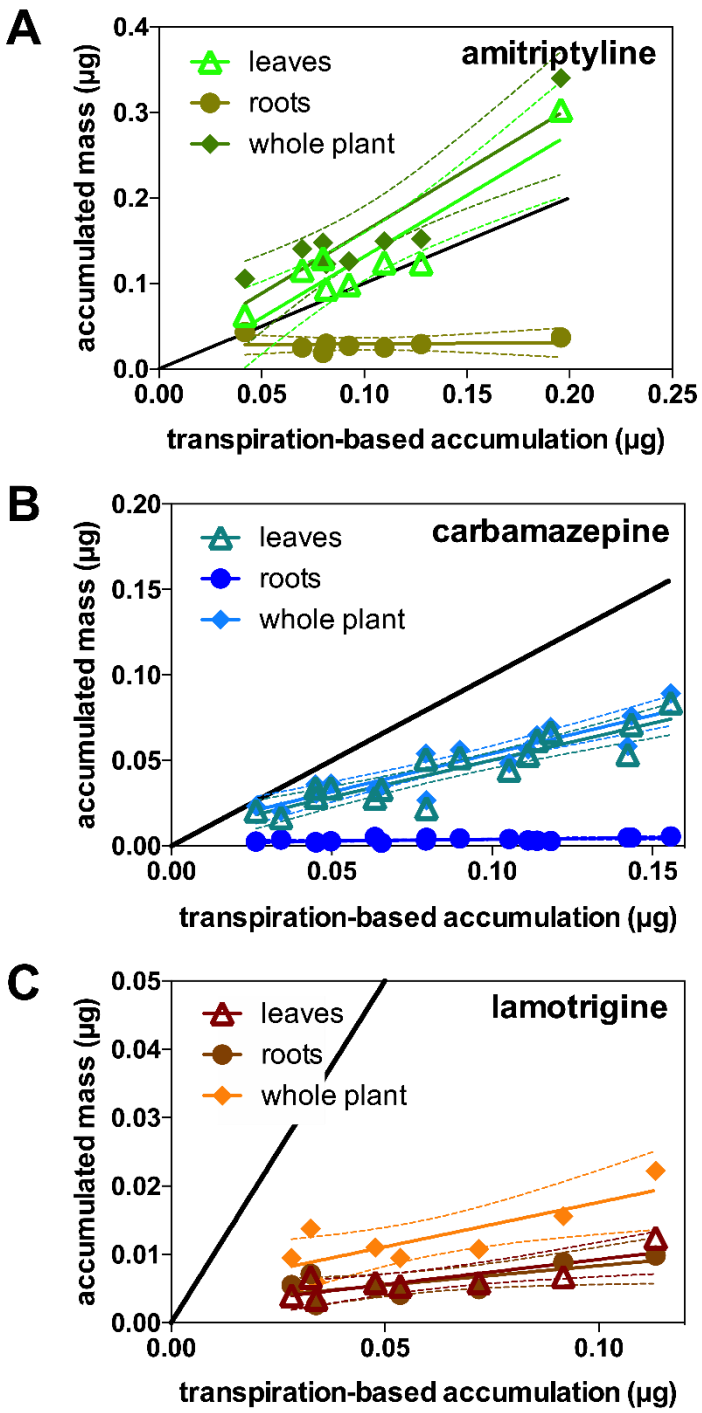

Figure C2. Transpiration-based and actual accumulation for plants exposed to 1 µg·L^{-1} for (A) amitriptyline, (B) carbamazepine, and (C) lamotrigine. Transpiration-based accumulation correlates with actual accumulation, but the slope varies significantly between compounds and none of them are close to one (black line). Dashed lines correspond to 95% confidence intervals. Transpiration-based accumulation was calculated according to Eq 4.1 in the main text.

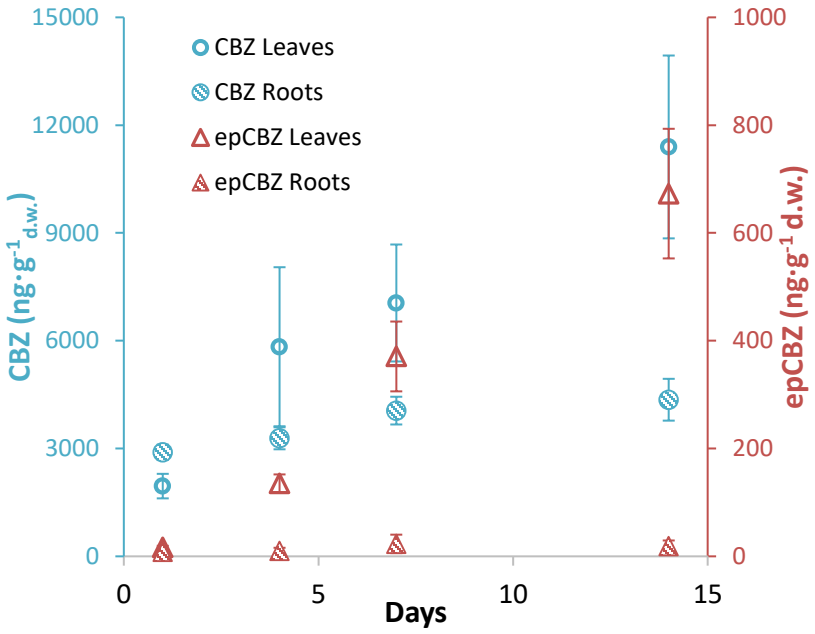

Figure C3. Concentration of carbamazepine (CBZ) and 10,11-epoxycarbamazepine (epCBZ) in spinach roots and leaves during a 14-day exposure period**.** Nutrient solution contained 100 $\mu g \cdot L^{-1}$ of CBZ. Error bars show one standard deviation ($n \geq 3$). Abbreviation: d.w., dry weight.

Appendix D. Rhizosphere impacts on phytoavailability of ionizable contaminants – Supplemental Information

Note: The portion of this work describing results obtained using quartz sand is reproduced with permission from Nason, S.L.; Miller, E.L.; Karthikeyan. K.G.; Pedersen. J.A. Plant-induced changes to rhizosphere pH impact leaf accumulation of lamotrigine but not carbamazepine. *Environ. Sci. Technol. Lett.*, **2018**, 5 (6), pp 377–381. DOI: 10.1021/acs.estlett.8b00246.

D.1. SUPPLEMENTAL METHODS

D.1.1. Chemical and Soil 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 (Quebec, Canada). 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. Elliott silt loam soil was obtained from the International Humic Substances Society (IHSS), St. Paul, MN. Richford loamy sand soil was collected in Portage County, WI. Bluestem sandy clay loam was collected from the Bluestem landfill in Marion, IA. Plano soil was collected from the Arlington Agricultural Research Station, Arlington, WI.

D.1.2. Plant Growth Methods.

D.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 C1. Growth cell design and construction is modeled after the setup used for the RHIZOtest.[1–3]

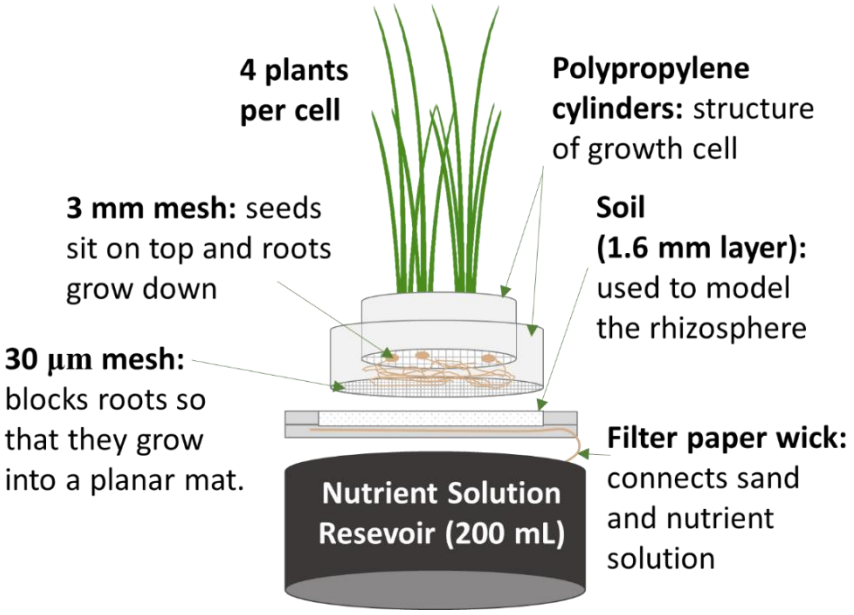

Figure D1. Exploded view of a full rhizosphere setup**.** Growth cells were based on the RHIZOtest[1–3] and were composed of two polypropylene cylinders (i.d. 25 mm and 35 mm) with 3 mm polypropylene mesh stretched across the bottom of the smaller and 30 µm nylon mesh attached to the bottom of the larger. 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 $\mu g \cdot L^{-1}$.

D.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).

D.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 or nutrient solution (days 8-21) up to the level of the coarse mesh (replenished twice daily).

On day 22, 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 soil 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. Pharmaceuticals were added to the nutrient solution reservoir and wicked up the filter paper with the nutrient solution to reach the plants. The nutrient solution (and the CBZ and LTG, where appropriate) was renewed every 2 days.

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 placed 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.

D.1.3. 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 D1. 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 C1) and sat at room temperature overnight prior to extraction. Internal standards used were carbamazepine-d_{10}, 10,11 epoxycarbamazepine-d_{10}, and lamotrigine-$^{13}C_3$ (CDN Isotopes). 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 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.

D.1.4. LC-MS/MS Analysis.

D.1.4.1. MRM Method Details. 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) with a four-minute linear gradient of 5% to 95% organic phase (0.25 mL·min^{-1} flow rate) and seven-minute re-equilibration time (0.3 mL·min^{-1} flow rate). A buffered aqueous mobile phase (10 mM ammonium acetate, 1% acetic acid, 2.5% acetonitrile, 2.5% methanol) and a four-minute linear gradient of 10% to 95% organic phase (0.25 mL·min^{-1} flow rate) and seven-minute re-equilibration time (0.3 mL·min^{-1} flow rate) was used for rhizosphere and sand sorption samples to prevent variation in pH during analysis. Column temperature was held at 30 °C. Internal standard-based calibration and measurement was used for plant extracts only. Ion masses used for detection via LC-MS/MS are listed in Table D1.

Table D1. Mass-to-charge ratios (*m/z*) for precursor, quantitative and confirmatory ions

compound	*ion* m/z 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

Table D2. Recovery of analytes from plant tissues [a]

		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

[a] Abbreviations: IS, internal standard; NA, not applicable.

D.1.4.2. Extraction Recoveries and Limits of Detection/Quantification. Extraction recoveries are shown in Table D2. 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 D2. For both CBZ metabolites we used 10,11 epoxycarbamazepine-d_{10} as the IS.

Limits of detection (LOD) and quantification (LOQ) (Table D3) were determined by running a low concentration sample seven consecutive times and taking the standard deviation of the measurements (σ). The LOD and LOQ were calculated using the equations:[6]

$$LOD = 3\sigma \qquad \text{(Eq. D.1)}$$

$$LOQ = 10\sigma \qquad \text{(Eq. D.2)}$$

D.1.4.3. Calibration and quality control (QC) information. We calibrated the instrument before each use, and ran a QC standard approximately every 10 samples to verify that signal drift over time was minimal. Representative QC information from our LC-MS/MS run to determine compound concentrations in plant leaves is shown in Table S4. In general, calibration curves and QC samples run throughout our analysis showed that our measurements were precise and accurate.

Table D3. Limits of detection and quantification

Compound	plant tissue (ng/g dw)				porewater (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

Table D4. Example Calibration and QC information [a]

Compound	Calibration curve R^2	Accuracy range of calibration points	Precision of QC standards [b]
carbamazepine	0.99993	93%-108%	4%
epCBZ	0.9998	95%-106%	8%
diOH-CBZ [c]	0.999	97%-128%	30%
Lamotrigine [d]	0.99999	97%-101%	7%

[a] Abbreviations: epCBZ, 10,11-epoxycarbamazepine; diOH-CBZ, *trans*-10,11-dihydroxy-carbamazepine.

[b] QC standards stayed within X% of the original measurement used for calibration

[c] The accuracy and precision of diOH-CBZ measurements were lower than for the other compounds because mass-labeled diOH-CBZ was not used for internal standardization. Instead, internal standardization relied on mass-labeled epCBZ .

[d] Lamotrigine calibration curve was quadratic rather than linear, as the concentrations were near the top of the instrument range.

D.1.5. 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 all other analysis including assessment of normality and equality of variance, ANOVAs with Tukey's *post hoc* analysis for comparison of data with equal variance, ANOVAs with Welch's correction for data with unequal variance, and Wilcoxon/Kruskal-Wallis tests with Steel-Dwass *post hoc* analysis for data with unequal variance and non-normal distribution. We used Shapiro-Wilk tests to assess normality and Levene's tests to assess equality of variance. All comparisons were made at the $\alpha = 0.05$ level of significance. Retrospective calculations of regression statistical power were made using the adjusted power function ($\alpha = 0.05$) in JMP Pro 12.2.0 (SAS). Retrospective statistical power represents the probability that a new set of similar samples with an identical variability profile and effect size would show a statistically significant trend.

D.2. SUPPLEMENTAL RESULTS AND DISCUSSION

D.2.2. Analyte Concentrations and pH of Nutrient Solution Reservoirs and Porewater. Bulk nutrient solution was analyzed at the beginning and end of the replenishment cycle that commenced on day 4. Concentrations of CBZ and LTG in the nutrient solution reservoirs varied <15% between the beginning and end of replenishment cycles. For Experiment A, 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 and did not differ between nutrient solutions. 10,11-*Trans*-dihydroxycarbamazepine was below the limit of detection in all nutrient solution samples.

Addition of CBZ or LTG did not affect rhizosphere sand porewater pH (Figure D3). For experiment A, the CBZ metabolite 10,11-epoxycarbamazepine was present at up to 0.9 $ng \cdot mL^{-1}$ in some rhizosphere porewaters, but 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 porewater samples. We did not detect any difference in plant accumulation of CBZ metabolites between nutrient solution treatments.

We accounted for pharmaceuticals that remained adsorbed to the soil (<11% for sand after rehydration) using the K_d values determined in the adsorption experiments presented in Part A to determine the rhizosphere porewater concentrations shown in Figure D4. This was only necessary for LTG, as CBZ did not adsorb to sand to a measureable extent. Using the total amount (dissolved and adsorbed) of LTG in the rhizosphere, we estimated the dissolved concentration in the rhizosphere porewater using the K_d values and the rhizosphere solid-to-solution ratio (Table D5). Between 0% and 40% of LTG present in the rhizosphere was adsorbed to the sand, depending on pH. Carbamazepine and LTG accumulated in porewater as water evaporated and/or transpired more quickly than the compound was taken up. Therefore, porewater concentrations were 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 note that our estimation of LTG adsorption to sand in the rhizosphere does not account for the possible effects of root exudates on sorption.[8,9] Surface active compounds in root exudates can promote desorption of hydrophobic organic contaminants such as polychlorinated biphenyls, polyaromatic hydrocarbons, and polybrominated diphenyl ether flame retardants, from rhizosphere soils.[8,10–12] Root exudate effects on desorption of polar and ionizable organic compounds from soils has not been investigated to our knowledge. This topic warrants investigation in future studies. We further note that the ionic composition and strength of the solution used in our sorption experiments may have differed from those of the rhizosphere porewater. While we matched the composition of the major cations in the initial nutrient solution provided to the plants, the plants may have taken up water and nutrients at different rates, causing accumulation or depletion of some cations in the rhizosphere porewater. Despite these uncertainties, we are confident in the reported trends in bioaccumulation; they hold whether or not adsorption to the rhizosphere sand is accounted for.

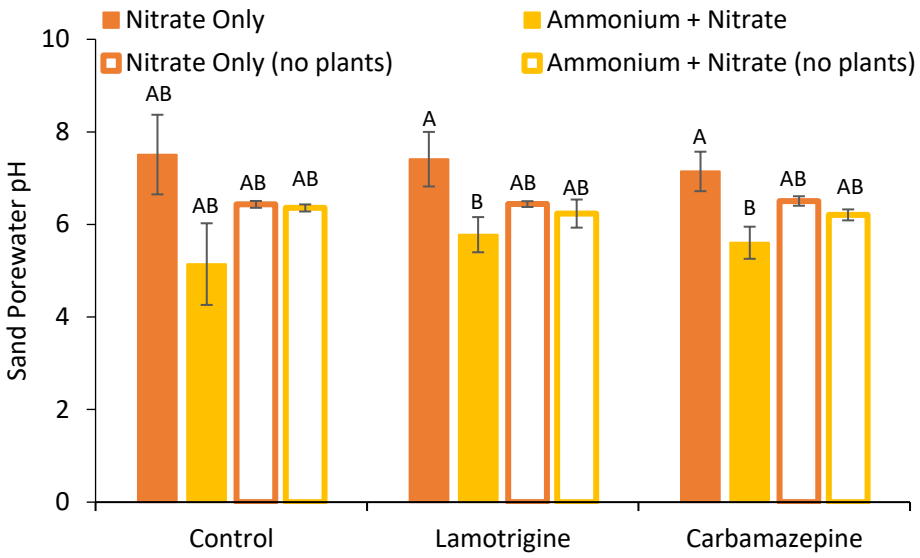

Figure D2. Porewater pH in rhizosphere sand with and without plants. Addition of CBZ or LTG did not affect pH for any treatment. Letters indicate statistically significant differences (Wilcoxon/Kruskal-Wallis test with Steel-Dwass post hoc analysis, $p < 0.05$). Error bars denote one standard deviation. Control treatments do not show differences due to the lower number of replicates.

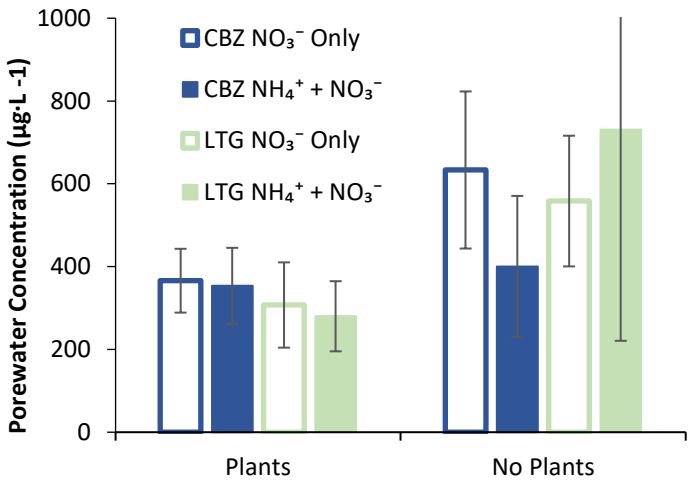

Figure D3. Total CBZ and LTG in porewater. Error bars indicate one standard deviation. Porewater concentrations did not differ within the CBZ or LTG treatments (ANOVA with Welch's correction, $p > 0.05$).

D.2.3. Masses of Plants, Sand, and Transpired Water. Experiment A root fresh masses, leaf fresh masses, leaf dry masses, the mass of transpired water, mass of harvested sand, and porewater volume (Table D5) 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 to 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.

D.2.4. Correlation between Porewater Concentration and Plant Accumulation. Lamotrigine accumulation in leaves correlated with the concentration of neutral LTG (LTG^0) in rhizosphere porewater. Correlations between total CBZ and LTG concentration in porewater and concentration in the plants are not statistically significant ($p > 0.05$).

D.2.5. Temperature and Humidity Measurements. Temperature and humidity readings are shown in Figure D5. 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 was not controlled and varied between 9 % and 38 %. These low relative humidity values are characteristic for Madison, WI, where the experiments took place.

Table D5. Masses of plant tissues and transpired water for experiment A

Exposure	Nutrient solution	Leaf mass (g)		Root mass (g)		Transpired water mass (g)	Porewater volume (mL)	Rhizosphere sand mass (g)	Solid-to-solution ratio
		f.w.	d.w.	f.w.	d.w.				
Control	NO_3^-	1.0 ± 0.3	0.08 ± 0.02	1.3 ± 0.2	0.19 ± 0.02	101 ± 9	1.77 ± 0.05	4.59 ± 0.04	2.6 ± 0.1
Control	$NH_4^+ + NO_3^-$	1.0 ± 0.1	0.06 ± 0.02	1.5 ± 0.3	0.20 ± 0.04	90 ± 10	1.78 ± 0.03	4.60 ± 0.08	2.6 ± 0.4
CBZ	NO_3^-	1.2 ± 0.4	0.05 ± 0.01	1.1 ± 0.2	0.16 ± 0.03	80 ± 20	1.76 ± 0.03	4.47 ± 0.06	2.5 ± 0.4
CBZ	$NH_4^+ + NO_3^-$	1.1 ± 0.2	0.04 ± 0.01	1.2 ± 0.2	0.17 ± 0.03	80 ± 20	1.82 ± 0.04	4.6 ± 0.1	2.2 ± 0.5
LTG	NO_3^-	1.3 ± 0.5	0.05 ± 0.02	1.2 ± 0.3	0.18 ± 0.04	80 ± 20	1.78 ± 0.06	4.53 ± 0.08	2.5 ± 0.9
LTG	$NH_4^+ + NO_3^-$	1.0 ± 0.4	0.04 ± 0.01	1.2 ± 0.3	0.16 ± 0.03	80 ± 20	1.81 ± 0.02	4.53 ± 0.05	2.5 ± 0.3

Table D6. Coefficients for correlations between plant and porewater concentrations for experiment A [a]

Tissue Concentrations ($\mu g \cdot g^{-1}$ d.w.)	Porewater Concentrations ($\mu g \cdot L^{-1}$)	R^2	*p* value	Regression Power
$[CBZ]_{leaves}$	$[CBZ]_{porewater}$	0.02	0.56	0.05
$[CBZ]_{roots}$	$[CBZ]_{porewater}$	0.11	0.18	0.12
$[LTG]_{leaves}$	$[LTG_{Total}]_{porewater}$	0.19	0.07	0.28
$[LTG]_{roots}$	$[LTG_{Total}]_{porewater}$	0.01	0.65	0.05
$[LTG]_{leaves}$	$[LTG^0]_{porewater}$	0.73	1.4×10^{-5}	1.00
$[LTG]_{roots}$	$[LTG^0]_{porewater}$	0.27	0.03	0.45

[a] Notation: LTG_{Total}, all lamotrigine species; LTG^0, neutral lamotrigine.

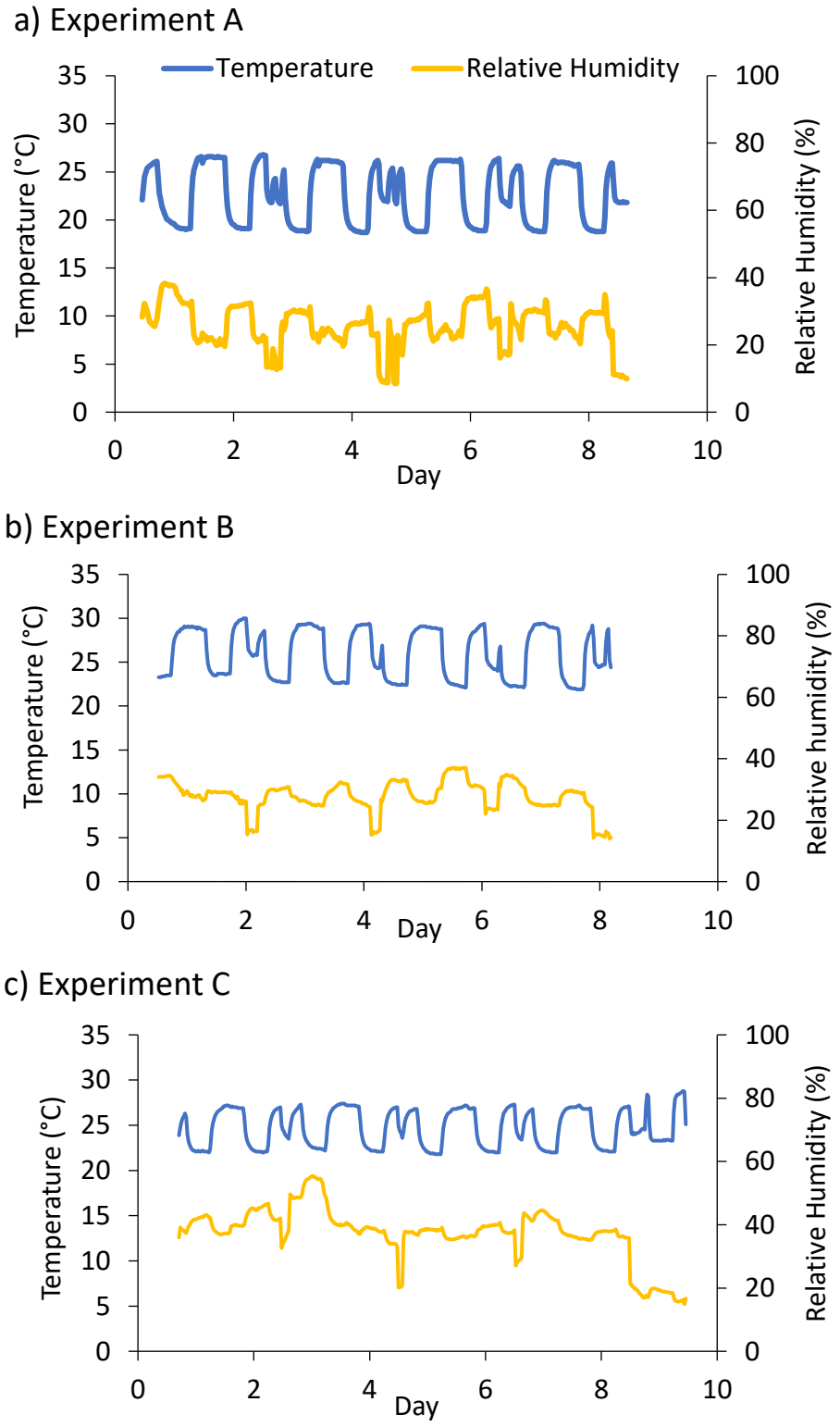

Figure D4. Temperature and humidity variation over the course of the wheat uptake experiments**.** Temperature (blue) and humidity (yellow) measurements were taken every 30 min using an automated system for the duration of the exposure period.

D.3. SUPPLEMENTAL REFERENCES

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