Environmental Processes

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Effects of Binary Mixtures and Transpiration on Accumulation of Pharmaceuticals by Spinach

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ABSTRACT. Many pharmaceuticals are present in reclaimed wastewater and effluent-dominated water bodies used to irrigate edible crops. Previous research has shown that 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 effects of exposure to 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.

KEY WORDS. Plants, pharmaceuticals, transpiration, mixture effects, carbamazepine, amitriptyline

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, are frequently found in treated and untreated wastewater,[4,5] and use of reclaimed wastewater for irrigation can result in human exposure to contaminants via consumption of irrigated crops.[6]

A large body of literature demonstrates that crop plants can accumulate pharmaceuticals under field conditions,[7,8] but monitoring agricultural produce for all potential wastewater-derived contaminants is impractical. Thus, predicting plant accumulation of pharmaceuticals is an important goal. Most attempts to predict pharmaceutical accumulation 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]

Plant uptake of contaminants is partially driven by water flow through the plant via transpiration.[7,11–13] Multiple studies have reported a positive relationship between transpiration and removal of non-polar organic compounds from growth media,[14–16] but the relationship between transpired water and accumulation in plant tissues was not evaluated and may would in principle be affected by transport pathways within the plant, *in planta* metabolism, and for some compounds, volatization from plant leaves.[64] To our knowledge, three prior studies explicitly addressed

correlation between transpiration and plant accumulation of nonvolatile polar and ionizable, organic compounds.[11,12,17] However, none of them present a compound-level comparison of structurally diverse contaminants, and only one considers the effects of *in planta* transformation.[17]

Most pharmaceuticals are nonvolatile, polar or ionizable, and 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.[17]

In this study, we used four structurally diverse pharmaceuticals to investigate the effects of binary mixtures on accumulation and metabolism in spinach (*Spinacia oleracea*) and 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] We selected the psychoactive compounds carbamazepine (CBZ), lamotrigine (LTG), amitriptyline (AMI), and fluoxetine (FLX) for study based on their

presence in treated wastewater[25,26] and demonstrated accumulation in plants.[27–30] 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]

MATERIALS AND METHODS

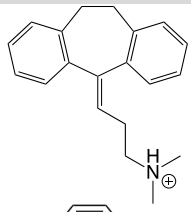
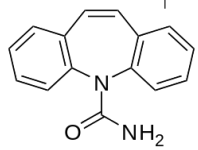
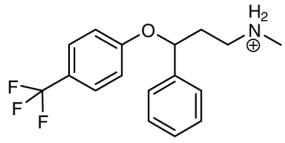
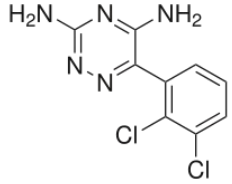
Materials. Chemicals used, suppliers, and purities are described in the Supporting Information (Text S1.1). Structures and selected physico-chemical properties of the four pharmaceuticals are displayed in Table 1.

Plant 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 S1.2). 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 S1.3). 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] 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

93 on day 7). Our experiments included control plants not exposed to pharmaceuticals and plant-free
94 controls which contained pharmaceuticals. At least three replicates were used for each treatment.

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

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	–	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.[32] [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.[33] using compound descriptors from the LSER Dataset 2017 for CompTox users.[34] [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[35] using compound descriptors from the LSER Dataset 2017 for CompTox users.[34] [e] From Cheney et al., 2010.[36]

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 S1).

Extraction and Analysis. The extraction and analysis methods were similar to those we previously reported.[37] 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 S1.4). 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 S1.4).

RESULTS AND DISCUSSION

Pharmaceutical Accumulation and Metabolism. The studied pharmaceutical compounds differed in their extent of accumulation and tissue distribution in spinach (Figure 1). 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.[17,28,29,38–42] Our results for AMI contrast with a previous study that reported accumulation primarily in roots for strawberry plants.[30] Possible explanations include more rapid degradation in above-ground tissues of strawberry plants or phloem mobility in spinach but not in strawberry plants.

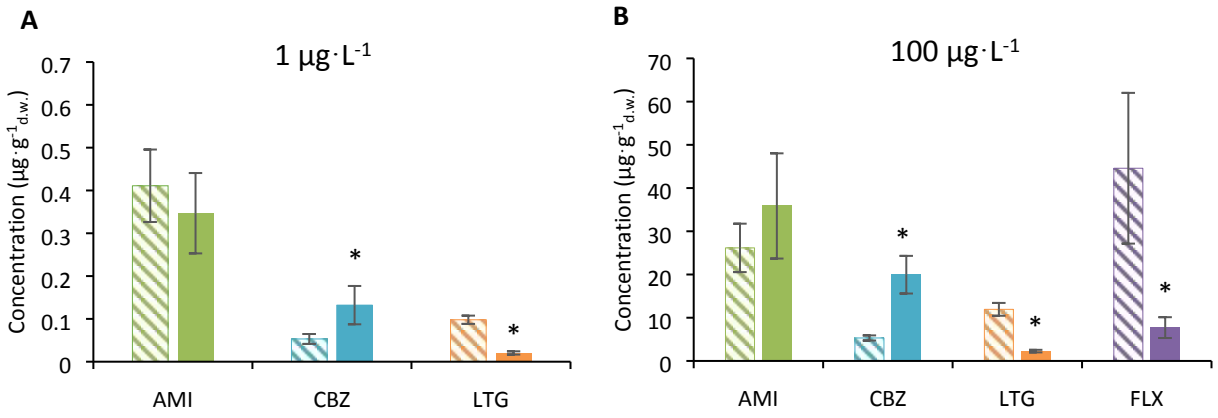


Figure 1. Dry weight (d.w.) concentrations of parent compounds in roots (striped bars) and leaves (solid bars) of spinach plants exposed to (A) 1 µg·L^{-1} or (B) 100 µg·L^{-1} of the indicated compounds. Data from mixture and single compound exposures were combined because accumulated amounts did not 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.

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[18,43] (Figure 2). Leaf concentrations of epCBZ were higher than those in roots by a factor of 12 ± 8 in the 100 µg·L^{-1} exposure, indicating that CBZ metabolism primarily occurs in the leaves, consistent with prior reports.[17,18,28,29] Concentrations of epCBZ were below the limit of quantification in the roots of plants exposed to 1 µg·L^{-1} CBZ. Concentrations of diOH-CBZ 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.

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.[17] Leaf accumulation of epCBZ was lower in the plants co-exposed to AMI or FLX than in those exposed to CBZ alone (Figure 2). 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,[31] while CBZ induces the CYP450s responsible for metabolizing AMI, FLX, and itself.[31] 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.[17] 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.

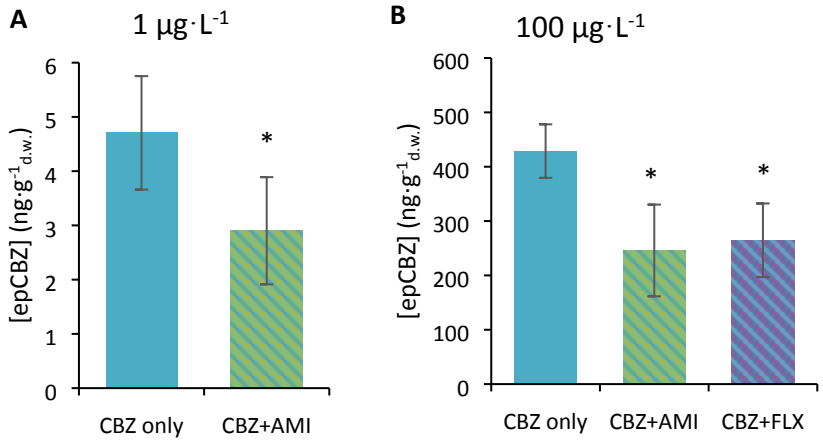


Figure 2. Accumulation of 10,11-epoxycarbamazepine in spinach leaves was lower in plants exposed to carbamazepine in combination with amitriptyline or fluoxetine relative to those exposed to CBZ alone at both the (A) 1 µg·L^{-1} and (B) 100 µg·L^{-1} exposure levels ($p < 0.05$, Dunnett's tests, indicated 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.

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 3). For FLX and AMI, mole balances were incomplete for the 100 µg·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 3 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 S4).

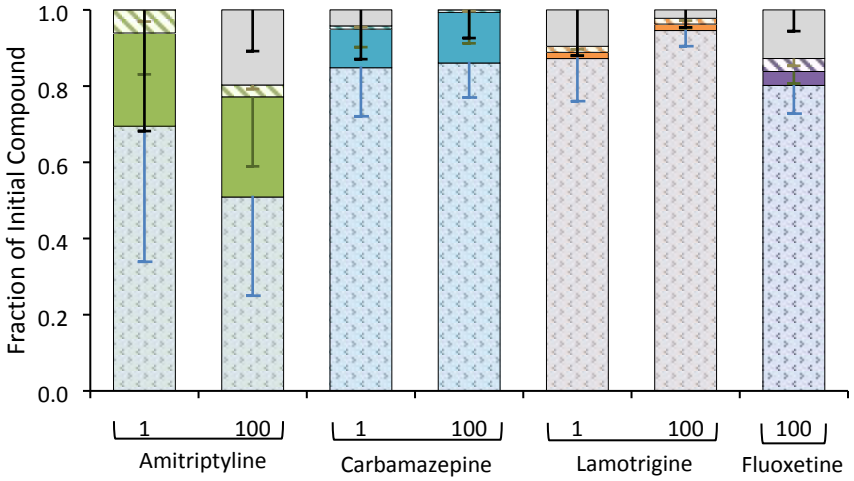


Figure 3. Mole balances for pharmaceuticals in experimental systems after 7-day exposure to spinach plants. Fractions shown for each treatment bottom to top of bars: solution (dotted), leaves (solid), roots (stripes), and missing (grey). The missing fraction refers to the difference between the initial 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 ($n \geq 7$).

Transpiration-Based Accumulation. 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)[46] 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):

$$\mathrm{TBA} = \overline{C}_{\mathrm{solution}} \times V_{\mathrm{transpired}} \quad (1)$$

where $\overline{C}_{\mathrm{solution}}$ 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 $V_{\mathrm{transpired}}$ 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.

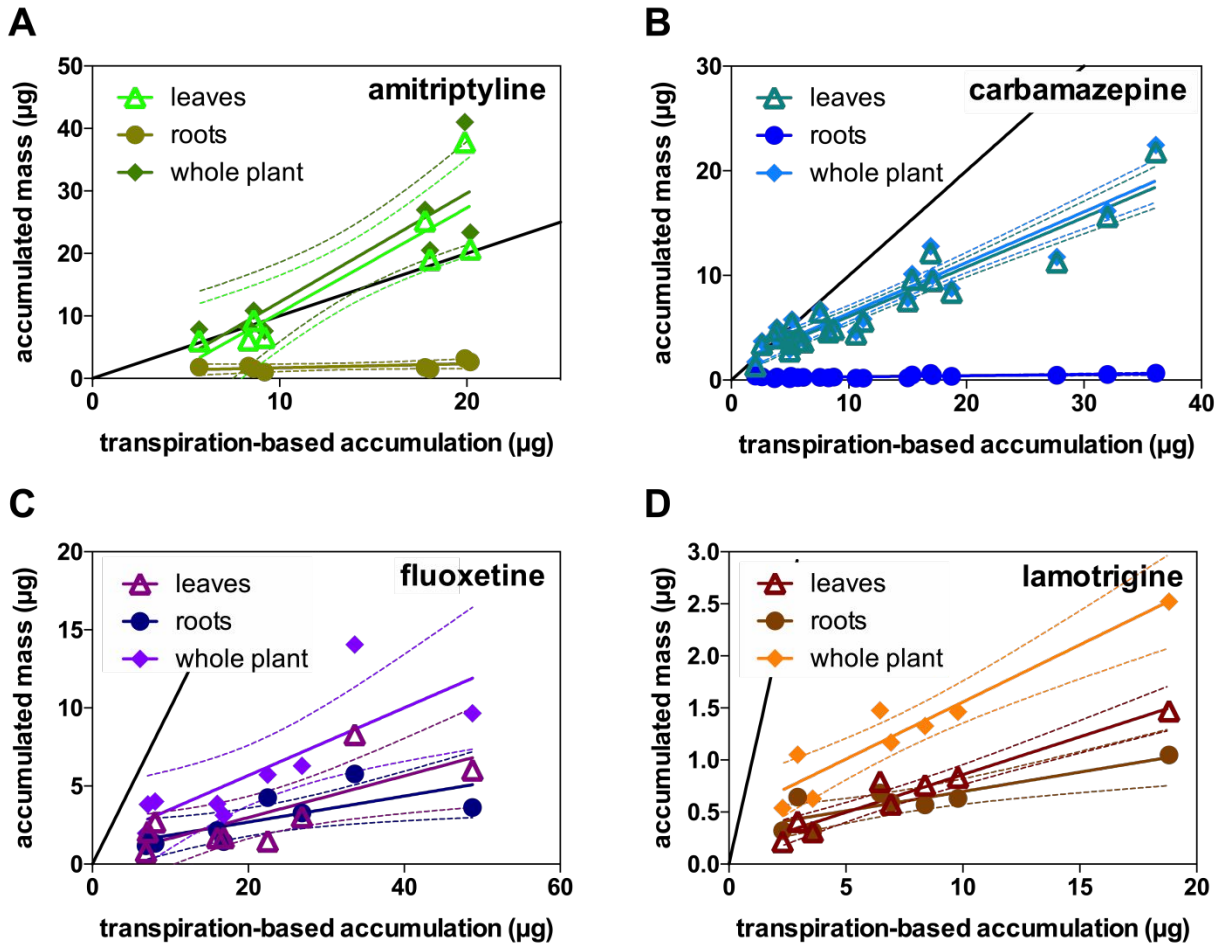


Figure 4. Correlation of actual accumulation with transpiration-based accumulation in leaves, roots, and the whole plant for (A) amitriptyline, (B) carbamazepine, (C) fluoxetine, and (D) lamotrigine. Dashed lines represent 95% confidence intervals. All correlations shown are statistically significant ($p < 0.05$) 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.

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 and S2 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 S5).

Leaf accumulation correlated strongly with TBA and explained a large proportion of inter-replicate variability (Table S5). 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 S5). The weak correlations for the roots likely reflect sorption to root membranes and negatively charged cell walls (for cationic species)[17] 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 and S2). 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 5). Phytovolatilization is unlikely for any of the studied compounds due to their high octanol–air partition coefficients (Table 1).[64]

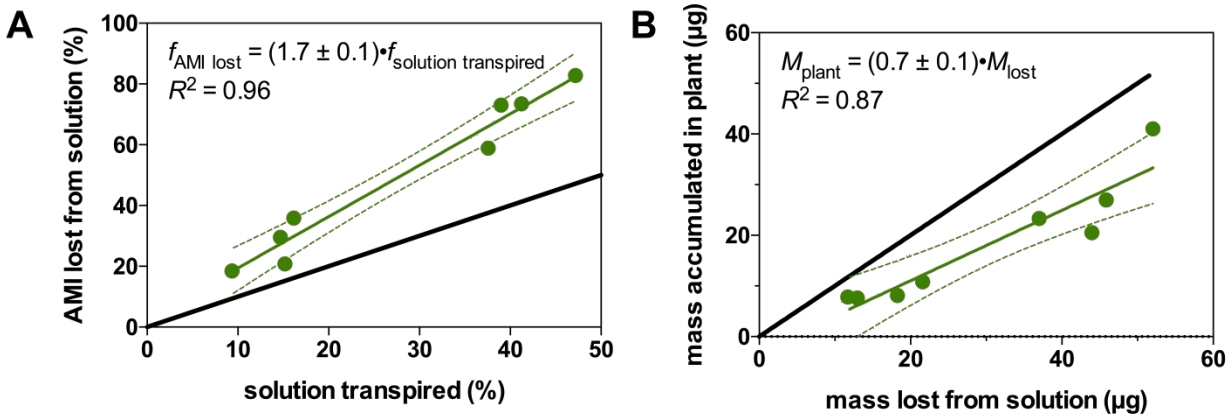


Figure 5. Amitriptyline (AMI) loss from solution correlated with (A) transpiration and (B) accumulation in the plant ($p < 0.001$) (green points and lines). Black lines show a slope of one. Strong correlation between transpiration and loss from solution indicates that uptake into the plant is the main 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 ($p > 0.05$).

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.[7,46] 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.[47] 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).[46,48] 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.[49] 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),[7,47] 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.[17,28,29,38–41] Lamotrigine (pK_a = 5.7) may also be subject to ion trapping in root cell vacuoles (pH ~5.5).[28,37,46,50]

As a neutral molecule with a relatively high membrane lipid-water partition coefficient (K_{lipw}; Table 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.[17,18,28,29,38,41] 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.[46]

Interestingly, accumulation of AMI was higher than would be predicted based solely on transpiration, despite clear evidence of *in planta* metabolism (Figures 3 and 5). 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.[47] The K_{lipw} for neutral AMI is higher than those for the other cationic compounds studied (Table 1) 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).[46] Trapping of AMI cations in root cell vacuoles (pH ~5.5)[46] 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.[30] 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.[47] Passive transporters are important for plant

accumulation of positively charged molecules that are essential for plant nutrition such as potassium, calcium, and urea,[47] and are necessary for elongation and growth, maintaining membrane potential, and responses to stress and pathogens.[51] 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,[52] phenanthrene uptake into cells (mediated by a proton symporter),[53,54] and antibiotic resistance in plants (connected to membrane transporters such as members of the ATP-binding cassette (ABC) and major facilitator superfamilies of proteins).[55,56] These latter proteins are found in all organisms.[59]

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.[57] 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.[57] For drugs that have been specifically studied, substantial evidence exists for protein-mediated transport.[57] 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.[58] 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.[60,61] In plants, similar ABC transporters function in transport of auxins, secondary metabolites, and xenobiotic compounds.[59] 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]

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 $ng \cdot mL^{-1}$ 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 S3); 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 6). 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 $ng \cdot mL^{-1}$ and 102 ± 2 $ng \cdot mL^{-1}$ 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.[17]

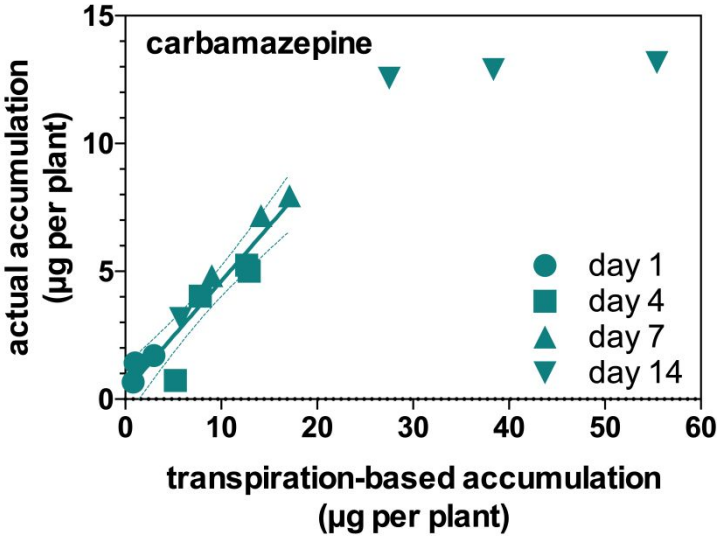


Figure 6. Transpiration-based and actual accumulation for whole spinach plants exposed to 100 µg·L^{-1} carbamazepine and harvested at various time points. The linear correlation shown is for days 1-7: actual accumulation = transpiration-based accumulation × (0.43 ± 0.05), R^2 = 0.90; dashed lines indicate 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.

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.

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%). 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.[18,20,21,62] 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.;[11] however, those authors reported correlations for all compounds taken together rather than for individual ones. Lamshoeft et al.[12] 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.[28–30,41] 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.[53,54] If this is the case, models of plant accumulation of organic contaminants[63] 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. SI contains information on experimental design, extraction recoveries, limits of detection and quantification, additional results details as noted, and a record of temperature and humidity during plant exposure.

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Notes

The authors declare no competing financial interest.

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