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Rapid Plant Uptake of Isothiazolinone Biocides and Formation of Metabolites by Hydroponic *Arabidopsis*

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Abstract

Isothiazolinones biocides are water-soluble, low molecular weight, nitrogenous compounds widely used to prevent microbial growth in a variety of applications including personal care products and building façade materials. Because isothiazolinones from buildings wash off and enter stormwater, interactions with terrestrial plants may represent an important part of the environmental fate of these compounds (e.g., in green stormwater infrastructure). Using the model plant *Arabidopsis thaliana* grown hydroponically, we observed rapid (≥99% within 24 hours), plant-driven removal of four commonly used isothiazolinones: benzisothiazolinone (BIT), chloromethylisothiazolinone, methylisothiazolinone, and octylisothiazolinone. No significant differences in uptake rate occurred between the four compounds; therefore, BIT was used for further detailed investigation. BIT uptake by Arabidopsis was concentration-dependent in a manner that implicates transporter-mediated substrate inhibition. BIT uptake was also minimally impacted by multiple BIT spikes, suggesting constituently active uptake. BIT plant uptake rate was robust, unaffected by multiple inhibitors. We investigated plant metabolism as a relevant removal process. Proposed major metabolites significantly increased in the BIT-exposure treatment compared to control included: endogenous nicotinic acid (confirmed with a Reference Standard) and phenylthioacetohydroximic acid, a possible amino acid-BIT conjugate, and two accurate masses of interest. Two of the compounds (phenylthioacetohydroximic acid and TP 470) were also present in increased amounts in the hydroponic medium after BIT exposure, possibly via plant excretion. Upregulation of endogenous plant compounds is environmentally significant because this demonstrates that BIT impacts plant biology. The rapid plant-driven isothiazolinone removal observed here indicates that plant-isothiazolinone processes could be relevant to the environmental fate of these stormwater compounds.

Environmental Significance Statement

Isothiazolinone biocides are used in outdoor building products, such as paint and building facades, to prevent microbial growth. These chemicals can wash off and enter stormwater, and thus interactions with terrestrial plants may represent an important part of isothiazolinone's environmental fate. This work uses a hydroponic model plant to demonstrate rapid (<24 h), concentration-dependent plant uptake of isothiazolinones, including previously untested compounds. This removal is not disrupted by several known plant uptake inhibitors, increasing the likelihood of its environmental relevance. The rapid removal kinetics implicate active uptake, an understudied mechanism that can inform the environmental behavior of other compounds. Upregulation of endogenous plant compounds following isothiazolone exposure is environmentally significant because this is an indication that plant biology is impacted.

1. INTRODUCTION

Isothiazolinones are water-soluble, low molecular weight, nitrogenous biocides widely used to prevent microbial growth in a variety of applications including in industrial compounds, personal care products (e.g., shampoo,[1] cosmetics[2,3]), and building façade materials (e.g., paint[4]). Isothiazolinones from building products wash off of buildings and enter stormwater during precipitation events[5,6] with concentrations (measured in separate stormwater sewer pipes or an underground stormwater storage pond) of up to 1,600 ng/L benzisothiazolinone (BIT),[5] 150 ng/L of methylisothiazolinone (MIT),[7] 41 ng/L chloromethylisothiazolinone (CMI),[7] and 67 ng/L OIT.[5] These four compounds (Table S1) are classified by the European Chemicals Agency as 'very toxic to aquatic life with long-lasting effect'; thus, the presence of these biocidal compounds in environmental waters through stormwater runoff could negatively impact wildlife.[8–11] Octylisothiazolinone (OIT) has been reported in three of 17 soil samples collected under home façades in Denmark and was attributed to stormwater.[12] Isothiazolinones can degrade in soil, with relatively short half-lives (e.g., $t_{1/2}$: MIT=0.28, BIT=0.52, OIT=9.3 day).[12,13] Nevertheless, those half-lives allow sufficient time for plant interaction with these compounds, particularly with repeated dosing through multiple storm events. Due to their high water solubility, isothiazolinones in stormwater can also infiltrate: OIT has been reported in groundwater, attributed to stormwater infiltration.[14] Conventional wastewater activated sludge[15,16] treatment can decrease isothiazolinone concentrations;[5,17] however, measurable isothiazolinones have been found in wastewater effluent.[5] Thus, in locations where recycled wastewater is used for irrigation, isothiazolinones may interact with food crops and potentially be taken up into the plants.[18]

The extent and rate of plant uptake of anthropogenic chemicals such as isothiazolinones by vegetation must therefore be understood, both for beneficial applications (*i.e.*, phytoremediation) and to characterize potential exposure risk (*i.e.*, groundwater used as a drinking water source, possible crop uptake during water recycling). Plants are known to take up a variety of anthropogenic chemicals from water[19,20] via multiple pathways. Some contaminants are taken up by plants passively with water in the transpiration stream.[21] The transpiration stream concentration factor (TSCF, the ratio of the concentration of the chemical in the xylem sap over the concentration in the solution surrounding the plant)[22] is a common measurement of xenobiotic plant uptake; however, the TSCF does not distinguish between plant uptake routes. Other chemicals, including some organic nitrogen compounds,[23,24] are transported into the plant via transporter proteins that can cause the compound's accumulation rate in the plant to exceed the transpiration rate. For example, uptake of the anticorrosive benzotriazole and tire rubber vulcanizer mercaptobenzothiazole into hydroponic *Arabidopsis thaliana* (Arabidopsis) exceeds the transpiration rate.[25,26] Thus, understanding the kinetics of plant uptake of a compound is critical to probe plant uptake mechanisms and predict if plant uptake is likely to occur in a given contact time between the chemical and plant (e.g., during stormwater infiltration through the root zone).

In addition, identifying in-plant transformation products generated following plant uptake is important to understanding the metabolism of xenobiotic compounds. The classical model of xenobiotic plant metabolism begins with "Phase I" functionalization of a compound, e.g., oxidations such as hydroxylation, hydrolysis, and dealkylation.[21,27–29] These reactions, mainly catalyzed by cytochrome P-450 enzymes, create increased-polarity products.[28,29] The product is conjugated with plant molecules, such as glutathione, in "Phase II" metabolism,[29–31] which may

proceed with multiple different pathways for a single xenobiotic[27] and generally forms a more water-soluble product with lowered toxicity.[30,31] Some xenobiotics, e.g. 2,4,6-tribromophenol with its hydroxyl group in the parent compound,[32] can proceed directly to Phase II metabolism without Phase I metabolism. Thus, the functional group chemistry of the parent compound is important to plant metabolism. After Phase II, conjugated metabolites can be more easily sequestered into vacuoles or bound residues in the plant ("Phase III" metabolism) than the parent compound, which removes them from harming plant processes.[29,31]

Nevertheless, recent evidence demonstrates that some xenobiotic compounds do not follow all the phases of classical plant metabolism. For example, recent evidence indicates that conjugated metabolites can subsequently deconjugate[33,34] and/or be excreted from the plant,[25–27,32,35–37] presenting previously unknown exposure routes. Additionally, amino-acid conjugated xenobiotics may closely resemble natural plant compounds and be incorporated into those biosynthesis pathways rather than simply being sequestered. For example, benzotriazole can conjugate with the amino acid alanine to form structural analogues of tryptophan and storage forms of the plant hormone auxin, likely by following the tryptophan and auxin biosynthesis pathways.[25] Benzotriazole was not incorporated into proteins,[38] but its presence in biosynthetic pathways may still produce important and as-of-yet undocumented plant physiological responses. Thus, xenobiotic plant metabolites may present important consequences for plants / plant consumers, or potential phytoremediation routes.

Despite the potential for plant-isothiazolinone interactions, including both plant uptake and metabolism, knowledge of these interactions is limited. Two papers describe the same set of experiments and are limited to aquatic plants.[39,40] Both CMI and MIT were rapidly (first measurement at 20 hours) taken up by both a duckweed (*Lemma minor*) and an aquatic fern

(*Salvinia braziliensis*).[39] The parent CMI was not found in the plant tissue after one day of exposure, suggesting rapid metabolism of the compound.[40] Nevertheless, much more information is needed to understand the fate of isothiazolinones interacting with plants. Plant uptake of BIT or OIT to our knowledge has not been studied. Given the presence of these compounds in stormwater,[5–7] this represents a critical knowledge gap. Additionally, stormwater-relevant kinetic data are needed. Understanding initial (<20 hours of exposure) plant uptake and metabolism kinetics is critical to determining the environmental fate of these compounds in stormwater situations, when water may rapidly infiltrate into the soil or engineered soil medium. More detailed kinetics will also inform plant uptake mechanisms, including if uptake rate is inducible by repeated biocide exposure, as would occur with repeated rain events. To the best of our knowledge, there are no published data on terrestrial plant uptake and processing of isothiazolinones, which is critical for stormwater flowing across a landscape or in green infrastructure (e.g., bioretention cells[41]).

Therefore, the objective of this work was to quantify the plant uptake kinetics of four isothiazolinones [benzisothiazolinone (BIT), chloromethylisothiazolinone (CMI), methylisothiazolinone (MIT), and octylisothiazolinone (OIT)] by Arabidopsis, a model plant, as well as Arabidopsis plant metabolites. Metabolites are defined herein as inclusive of BIT-conjugates and endogenous plant compounds whose presence was significantly increased or decreased following BIT exposure. Because in this work we were primarily interested in BIT plant transformation products, we quantified those metabolites that increased after BIT exposure (i.e., upregulated), such as BIT conjugates with endogenous plant compounds that are typically formed as part of plant xenobiotic metabolism. We hypothesized that all of the tested isothiazolinones would be rapidly taken up by the Arabidopsis plants via a transporter protein

(i.e., not passively transported with the transpiration stream). All four molecules are relatively small (MW=149.6–213.3 Da) and water-soluble (log K_{ow} values of -0.49–2.61),[4,6,42] but feature different functional groups. To determine the detailed kinetics and possible mechanisms of plant uptake of BIT, as a representative isothiazolinone, removal kinetics at a range of starting BIT concentrations were measured. We tested if Arabidopsis BIT uptake was constituently active and probed the route of plant uptake by testing BIT plant uptake in the presence of molecules with similar structures or known plant uptake inhibitors. Additionally, we investigated metabolites in both plant tissue and the hydroponic medium that increased following BIT exposure, and tentatively propose possible structures for these compounds.

2. METHODS

2.1 Chemicals

Chemicals used in these experiments include: benzisothiazolinone ("BIT", CAS 2634-33-5, Alfa Aesar, 97%), a chloromethylisothiazolinone (CMI) and methylisothiazolinone (MIT) mix (mix of CAS 26172-55-4 and CAS 2682-20-4, Combi-Blocks, Inc., 68%), octylisothiazolinone ("OIT," CAS 26530-20-1, Tokyo Chemical Industry, >98%), L-tryptophan (CAS 73-22-3, Research Products International, >99%), and 1H-benzotriazole (CAS 95-14-7, Fluka Analytical, ≥98%). Other chemicals are described in the SI. All LC-MS/MS solvents (acetonitrile, water, and formic acid) were Fisher Optima LC/MS grade.

Using previously established protocols for growing Arabidopsis hydroponically,[25,26] liquid plant medium was generated by combining (for 1 L medium): 4.43 g Murashige and Skoog Basal Medium powder (PhytoTech Labs M519), 0.5 g 2-morpholin-4-ylethanesulfonic

acid (MES) free acid monohydrate (Fisher), and deionized water to ~900 mL. Hydroponic medium pH was corrected to 5.6 with potassium hydroxide, then 5 g sucrose (Research Products International) and DI water to 1 L was added. Medium pH was rechecked and adjusted to 5.7 as needed with potassium hydroxide or hydrochloric acid. Before experimental use, the medium was filter sterilized using a bottle top filter (Corning #431118, 0.22 μm pore size) into an autoclaved bottle.

2.2 Experimental Design

2.2.1 Experimental compound selection

Isothiazolinone compounds were selected to represent a variety of chemical features. BIT is the only compound of the four to contain an aromatic ring. The other three tested molecules (CMI, MIT, and OIT) have other functional groups: chloro (CMI), methyl (CMI and MIT), or a carbon chain (OIT). OIT's eight-carbon chain results in a higher log K_{ow} value than the other three molecules (i.e., 2.6 vs log K_{ow}<1 for the other three compounds, Table S1[6,7,43]); log K_{OW} is known to influence plant uptake rates.[44,45] All four compounds tested are used as preservatives in building materials and are thus relevant to stormwater.[7]

2.2.2 Arabidopsis growth preceding isothiazolinone exposure

Arabidopsis seeds were surface-sterilized using a previously-published bleach procedure,[25,26,38,46] with minor modifications (detailed in the SI). Seeds were then grown aseptically in the sterile hydroponic Murashige and Skoog-based medium above, in washed and autoclaved Magenta GA-7-3 boxes (Bioworld), also using a previously published procedure with minor modifications (detailed in the SI).

The Arabidopsis plants were grown hydroponically in this work to enable the rapid quantification of relevant plant uptake kinetics and route without competition from soil abiotic mechanisms and abundant microbes. Because Arabidopsis is a model plant, the translation of results to other plant species in soil conditions would require further testing. Previous work has demonstrated that such translation is possible and useful: multiple xenobiotic (benzotriazole) metabolites first discovered in hydroponic Arabidopsis were also found in soil-grown strawberries.[38] Thus, the Arabidopsis results allowed for the prediction of plant uptake and targeted metabolite discovery in other plant species.

2.2.3 Plant isothiazolinone exposure experiments

The exposure experiments were modeled on previous work[25,26] and described in detail in the SI. Briefly, after a 10–11 day period of growth in unspiked sterile hydroponic medium, the medium was exchanged for sterile, isothiazolinone-spiked medium. A medium-only abiotic control was also created to quantify non-plant related losses (e.g., photolysis, hydrolysis). Each treatment and control consisted of n=3 or n=4. Sampling of the hydroponic medium occurred throughout the duration of the experiment. Except during active sampling, boxes were maintained in a Percival growth chamber alternating between 16 hours light at 23° C and 8 hours dark at 21° C.

2.2.4 BIT uptake kinetics under varied concentrations / multiple exposures

To determine if the plant uptake rate of BIT changed with increasing BIT concentration, a plant isothiazolinone exposure experiment was conducted with four starting BIT concentrations run in parallel with an abiotic control. Measured initial concentrations were 8 µg/L, 112 µg/L, 678 µg/L, and 2,127 µg/L. Medium samples were collected at six timepoints over 48 hours and

BIT was quantified with LC-MS/MS. The data were fit using nonlinear regression (curve fit, GraphPad Prism 9.0) with zero, first, and second-order equations. Additionally, data from seven other BIT starting concentrations (lowest concentration = 2.4 µg/L), using the same experimental design but with each concentration conducted independently rather than in parallel, were curve fit in the same manner and plotted along with the four rate values from the parallel experiment to make a total of 11 data points. Calculations of observed vs. expected passive (transpiration-driven) BIT removal rate from the medium are described in the SI.

A repeated exposure kinetics experiment was also conducted with BIT. Medium was spiked to 1,050 µg BIT/L at t=0, and medium samples were taken at 0, 1, 2, 3, 4, 8, and 24 hours. After the 24-hour sample, the plant medium was drained from the boxes (in the laminar flow hood biosafety cabinet) while retaining the plant biomass (for the plant treatments) by keeping the lid on the box and tilting until the medium poured out of the box. Then, 25 mL of freshly spiked 875 µg BIT/L medium (although the aim was to have both spikes be the same concentration; this is difficult to achieve as evidenced by other similar studies[25,26]) was added to each box for the second spike. Medium samples were collected using the same timepoints as the first spike, with t=0 as the time of the second spike.

2.2.5 Plant metabolomics experiments

To determine plant metabolites increased after BIT exposure, a nominal 200 µg BIT/L treatment group of Arabidopsis plants was grown in parallel with a "clean" (unspiked with BIT) positive control group of Arabidopsis plants. Each group had n=9 sample boxes. Additional details are in the SI, briefly: plant tissue was harvested at 24 h by straining out the liquid medium as above, inverting the box onto a paper towel, gently patting the plant tissue dry with the paper towel to remove any residual medium, and freezing the plant tissue at -20 °C until plant tissue

extraction. Freeze drying and plant tissue extraction into a liquid sample followed a previously published procedure,[25] detailed in the SI.

2.2.6 Quantification of BIT sorption to plant tissue

Each cleaned and autoclaved magenta box received 25 mL of plant medium spiked to 213 µg BIT/L (the measured concentration targeted to be similar to the nominal 150 µg BIT/L of the inhibitor experiments) and one vial of freeze-dried plant tissue. Given the rapid uptake and phytotransformation of BIT, freeze-dried (lyophilized) Arabidopsis plant tissue (n=4 plant boxes) was used rather than live plants. Freeze-dried plant tissue has been used to quantify sorption of chemicals to plants in other studies.[46,47] This decouples plant uptake from sorption, while providing a normalized dry-weight basis for sorption and preserving the tissue more than other methods of removing water.[48] Each vial contained plant tissue grown from 30±2 seeds for 14 days in hydroponic plant growth medium with no biocide present, which had been harvested per the procedure above, placed into a vial, and freeze dried overnight. Hydroponic medium (1.0 mL) was sampled from each box setup described above (213 µg BIT/L spike with freeze-dried plant tissue) at t = 0, 1 h, and 24 h. BIT sample concentration was determined via LC-MS/MS (see below for LC-MS/MS details).

2.2.7 Competitive inhibition experiments

Two competitive inhibition experiments were performed to test if BIT uptake would be inhibited by chemically similar molecules in the liquid plant medium. Using the procedure for plant isothiazolinone exposure experiments above, benzotriazole and tryptophan were tested separately in mixtures with BIT. Such mixtures are environmentally relevant, as both compounds are known to be taken up by plants,[25,49] and both tryptophan (an amino acid)-containing

compounds and benzotriazole (a corrosion inhibitor) are found in stormwater.[25,50,51] Treatments were: (a) 50 µg BIT/L, and (b) a nominal mixture of 50 µg BIT/L and 50 µg benzotriazole/L. For tryptophan, a nominal mixture of 50 µg BIT/L and 67.4 µg L-tryptophan/L (a molar-equivalent concentration to 50 µg BIT/L) was used for the (b) treatment.

2.2.8 Pathway inhibitor experiments

Known inhibitors to plant uptake and xenobiotic metabolic pathways were used to test likely BIT plant uptake pathways. Equivalent Arabidopsis-inhibitor experiments to our experimental design were not found in the literature for all of the tested inhibitors. Thus, prior to conducting a full Arabidopsis-inhibitor experiment, we conducted plant health experiments to determine an appropriate inhibitor concentration for our experimental setup to ensure plant health was not significantly impaired (based on visual inspection) by the inhibitor concentration; full details are in the SI. Then, BIT-inhibitor experiments were run in the same manner as the plant health experiments, except an abiotic control was added and medium samples were taken non-sacrificially over time. Each inhibitor was tested separately, with a nominal BIT C_0 of 150 µg BIT/L and 25 mL of plant medium per box. Samples were taken at t = 0 hr, 2 hr, 24 hr, and 48 hr, with n=3–4 for each timepoint and no replacement of plant medium.

Known inhibitors of several different pathways were used. Diethylpyrocarbonate is an amino acid,[52] peptide,[53] and sucrose[54] plant uptake inhibitor. Glycerol is an aquaporin inhibitor.[55,56] Quinidine inhibits organic cation transporters (as used in uptake experiments in *Typha latifolia*).[57] Anthracene-9-carboxylic acid (9-AC) inhibits anion channels[58] (demonstrated in algal plasma membranes,[59,60] and through the inhibition of auxin movement in Arabidopsis[61] and oat coleoptile tissue[62]). 2,4-dinitrophenol (2,4-DNP) is a cellular metabolism inhibitor, which is known to inhibit amino acid/peptide uptake of dileucine (for an Arabidopsis membrane

transport protein expressed in yeast),[63] glutamine (in excised castor bean cotyledons),[64] and Gly-Sar (in barley embryos).[65] 1-aminobenzotriazole (1-ABT) inhibits cytochrome P450,[66] which is involved in xenobiotic metabolism in plants.[27]

2.3 Analytical Methods

2.3.1 LC-MS/MS methods

All samples except for the metabolomics samples were analyzed via high-performance liquid chromatography (Agilent 1260) coupled to a triple-quadrupole mass spectrometer (LC−MS/MS; Agilent 6460 Triple Quadrupole MS with Mass-Hunter, version B.07.00) operating in multiple-reaction monitoring (MRM) positive mode and electrospray ionization (ESI) (Table S2). An isotopically labeled (d4) imidacloprid-normalized external calibration curve was used to account for matrix effects during ionization for those samples that were quantified on a mass per L basis. 10 µL of 1.3 mg/L d4 imidacloprid in LCMS-grade acetonitrile were added to each 1 mL of sample or standard. The chromatography column was an Agilent Eclipse Plus C18 (5 µm, 4.6×150 mm) for all but the OIT samples (which used an Agilent XDB-C18 ZORBAX, 3.5 µm, 2.1×50 mm). The mobile phases were Fisher Optima LC/MS Water with 0.1% Optima LC/MS grade formic acid (A) and Fisher Optima LC/MS acetonitrile with 0.1% Optima LC/MS grade formic acid (B). The mobile phase gradients are given in the SI. The injection volume was 10 µL. The MS/MS was set in multiple reaction monitoring mode (MRM, Table S2). Two MRM transitions were used for each compound (Table S2) for quality control. The instrument response was linear throughout the calibration range. Between non-detect values and the lowest non-zero standard with a reliable peak (≤500 ng/L, representing ≤0.02–21% of the

starting concentrations in the experiments) for the given MS/MS run, the standard curve was extended and used to estimate the concentration. The signal-to-noise ratio was ≥2 for samples between non-detect and the lowest non-zero standard. Full analytical and quality assurance details are provided in the SI.

2.3.2 Metabolomics investigation via high-resolution mass spectrometry

The extracted plant tissues (extraction details in the SI) were analyzed on a Thermo Q-Exactive Orbitrap High-Resolution Mass Spectrometer using Full MS scans with data-dependent MS/MS acquisition, in the manner of previous literature.[67] Both ESI positive and negative modes were used. Polarity switching was used for the MS scan (i.e., both positive and negative modes were run in the same sample run). Data dependent MS2 (ddMS2) scans were performed in positive and negative modes separately for composited plant tissue extracts (see SI for details). The chromatography and method parameters from the Agilent Triple Quadrupole MS BIT method (Table S2) were used on the Q-Exactive for both the full scan and data-dependent scans of these samples. Specific parameters for the Q-Exactive are given in Tables S3 and S4.

Q-Exactive data of the metabolomics samples were analyzed via Compound Discoverer 3.1 (details given in the SI). Because of the explicit experimental design wherein the entire MS spectra of BIT-exposed plant extracts were compared to unexposed plant extracts, the data can include both endogenous plant compounds that increased with BIT exposure and conjugates formed from BIT. The established workflow within Compound Discoverer, "Untargeted Metabolomics with Statistics Detect Unknowns with ID using Online Databases and mzLogic" (Figure S1) was used. The results were filtered to remove background with the "background is false" filter. Analysis of plant hydroponic medium samples were composite samples from multiple biological replicates, thus no p-value calculation was possible. In the plant tissue

extracts, compounds were screened for further analysis based on a p-value ≤0.05 in the fold-change ratio of the peak area of the treatment (BIT-exposed) plant tissue extracts to the peak area in the positive control (BIT-unexposed) plant tissue extracts. Several hundred compounds met these criteria for both the plant extracts and the medium samples, e.g., 546 compounds increased in BIT-exposed plants (Figure S2), which were then sorted from greatest-to-least peak area ratio for each sample type (plant extract or medium). Results with a peak area ratio of five or greater (i.e., increased five-fold or more in BIT-exposed plants vs. unexposed plants) were selected for further analysis. Features decreased in peak area in BIT-exposed vs. unexposed plants were not examined further in this work. Those compounds were then sorted by retention time, and similar retention time compounds (within 0.04 min) were grouped for further examination as possible in-source fragments of the metabolite. Candidate proposed metabolites were drawn in ChemDraw 20.0 (PerkinElmer) and the exact mass of a proposed formula was compared with the accurate mass of the mass spectrometry results. Fragments were also drawn and compared with the spectra for each proposed compound (SI).

2.3.3 Statistics

GraphPad Prism 9 (GraphPad, La Jolla, CA) was used for all statistics. Matched-pairs t-tests were used to compare two treatments and/or controls, with $\alpha=0.05$. Departure from the linear null slope at the 95% confidence interval determined if a significant change in compound concentration occurred over time. A repeated measures mixed-effects model in Prism (REML, under one-way ANOVA, $\alpha=0.05$) with Geisser-Greenhouse correction was used to assess differences in plant uptake rate between the four different biocides, given that some timepoints (0 and 24 h) were the same for all biocides and one timepoint varied (1.5 h vs 2.5 h) for OIT vs the three other biocides.

3. RESULTS AND DISCUSSION

3.1 Rapid hydroponic removal of isothiazolinone biocides is likely due to transporter-mediated plant uptake

The presence of different functional groups and isothiazolinone structure did not significantly (p=0.48) alter the Arabidopsis-facilitated removal rate from the hydroponic medium for any of the four tested molecules, given the available data from three timepoints (Figure 1). Although additional data points may reveal subtle differences among uptake rates, all four molecules behaved similarly in our experiments such that ≥99% of the initial concentration of a given spiked compound was removed from the medium at 24 hours in the presence of Arabidopsis plants (Figure 1). Neither the number of rotatable bonds (previously reported to influence plant uptake[45] and greater in OIT than in the other tested molecules due to the OIT carbon tail), the presence of carbon or methyl groups (i.e., in MIT and CMI,), nor the presence of an aromatic ring (i.e., BIT) impacted isothiazolinone removal extent at 24 hr, and with the given limited data significant uptake rate differences were not observed. Plant-driven process(es) accounted for the majority of isothiazolinone removal, as no significant removal of the four isothiazolinones was found in the abiotic controls (Figure 1, $p \geq 0.05$).

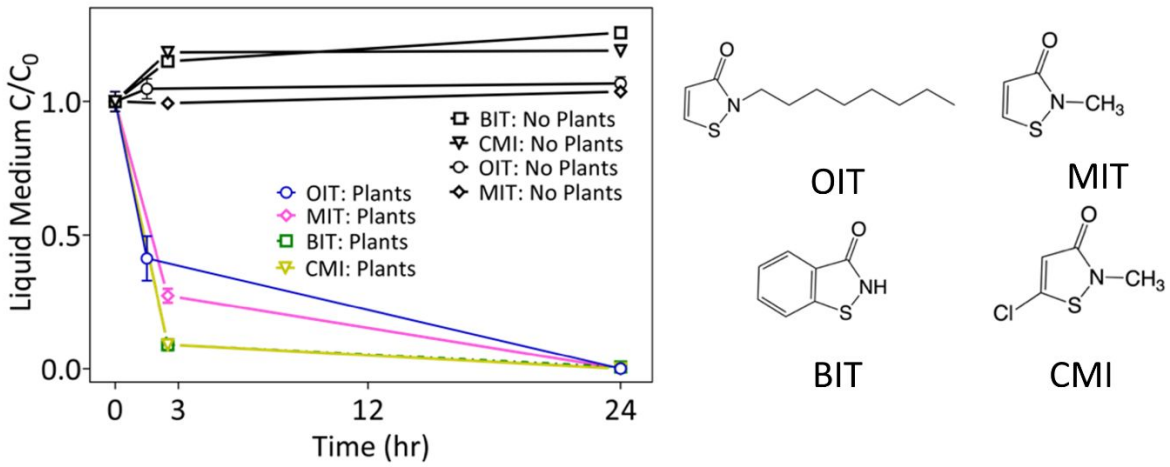


Figure 1: Plant-facilitated depletion kinetics of isothiazolinone biocides from hydroponic medium. OIT (nominal C_0 of 150 µg/L), BIT (nominal C_0 of 100 µg/L), and MIT and CMI (as a mixture, nominal MIT C_0 of 33 µg/L, nominal CMI C_0 of 100 µg/L) were all rapidly taken up by *Arabidopsis thaliana*, at a statistically indistinguishable rate (p=0.48 for the plants treatments).

Because the hydroponic plant depletion kinetics of all four molecules were similar, we chose to use BIT as a representative isothiazolinone compound for the remaining more-detailed plant uptake investigation. Removal of BIT from the medium was rapid. Sorption of BIT to plant tissue occurred, representing roughly a quarter of total removal of BIT from the medium (Figure S3); however, the majority of total BIT removal was due to other process(es). It is possible that some BIT is transformed (and thus would appear to be removed) from the medium due to root exudates. Although we did not measure potential abiotic interactions with exudates for BIT directly, previous work in the same experimental setup[25] found that Arabidopsis root exudates did not create a significant loss of another organic xenobiotic, benzotriazole.

Such rapid depletion of BIT from the hydroponic medium implicates BIT active plant uptake, which likely proceeds through a transporter-mediated process rather than merely passive movement of BIT with the transpiration stream into the plant. Indeed, measured BIT removal from the medium exceeded the expected transpiration rate by 21-fold for a C_0 of 357 µg/L and

28-fold for a C_0 of 49 µg BIT/L (calculations shown in the SI). This matches literature precedent for uptake of benzotriazole and mercaptobenzothiazole into Arabidopsis plants, which also substantially exceeded the transpiration rates.[25,26] Combined with the evidence for BIT phytotransformation based on a likely BIT-conjugate present in plant tissue extracts (described below), it is likely that the primary mechanism for BIT removal from the medium is plant uptake via a transporter protein. Uptake of a variety of other xenobiotics, such as herbicides 2,4-D and paraquat, is known to occur via transporters.[25,68–70] The ability of transporters to move xenobiotics into plants is attributed to the relatively nonselective nature of the transporters and the similarity of xenobiotics to the intended substrates of the transporters, *e.g.*, amino acids.[25,68]

3.2 Concentration-dependent BIT uptake by Arabidopsis implicates substrate inhibition

For all tested BIT concentrations in the range of 2.4–2,127 µg/L, BIT uptake by Arabidopsis was rapid, resulting in complete or near-complete removal within 24 hours (Figure S4). This rapid removal limited the collection of early data points, but with a total of 252 data points from 11 starting concentrations (Figure S4, summarized in Figure 2), a second-order rate model fit the data best (r^2 of 0.92 for first-order and 0.95 for second-order, Table S5). A second-order rate constant is also consistent with plant uptake of other xenobiotics such as mercaptobenzothiazole into Arabidopsis[26] and triclosan into carrot cells.[71] In this work, the BIT removal rate increased between the lowest and second-lowest tested concentration then decreased with increasing initial BIT concentration throughout the remainder of the tested concentration range (initial concentration range: 2.4–2,127 µg/L, Figure 2; representing 252 total samples), a behavior consistent with enzymatic substrate inhibition. Substrate inhibition, wherein the reaction rate increases and then decreases with increasing concentration of substrate instead

of rising to a steady reaction rate following traditional Michaelis-Menten kinetics,[72] is not uncommon within the normal operating substrate concentration range of enzymes.[72] Indeed, this inhibition is crucial to the normal functioning of many metabolic processes; for example, substrate inhibition can regulate reaction velocity to a more stable range than would occur without inhibition in order to maintain appropriate concentrations of substrates and/or products.[72] Other plant uptake studies have reported evidence of substrate inhibition of plant uptake over at least part of the tested range of substrate concentration, e.g., uptake of mercaptobenzothiazole;[26] however, substrate inhibition occurred at a higher concentration for mercaptobenzothiazole than for BIT. This result contrasts with the reported plant uptake rate of metformin, which is proposed to enter the plant through transporter proteins but whose plant uptake rate did not vary with initial metformin concentration (possibly due to the concentrations affected by substrate inhibition not being tested).[57] BIT concentrations tested in this study were slightly higher than typical environmental concentrations: the lowest tested initial concentration in this study was 2.4 µg/L, and BIT stormwater concentrations have been reported up to 1.6 µg/L[5]. Lower initial concentrations, e.g. 0.9 µg BIT/L, did not yield full curves from which a reaction rate could be quantified due to the rapid plant uptake/sorption of BIT to below detection limits. Although Figure 2 indicates that the highest BIT plant uptake rates occur at concentrations slightly above the environmentally relevant concentrations, BIT plant uptake was rapid (≥99% removal from the medium within 24 hours) at all tested concentrations (Figure S4) including 0.9 µg BIT/L when the BIT medium concentration reached zero so rapidly that a curve could not be constructed, suggesting the potential for high BIT plant uptake rates at environmentally-relevant concentrations.

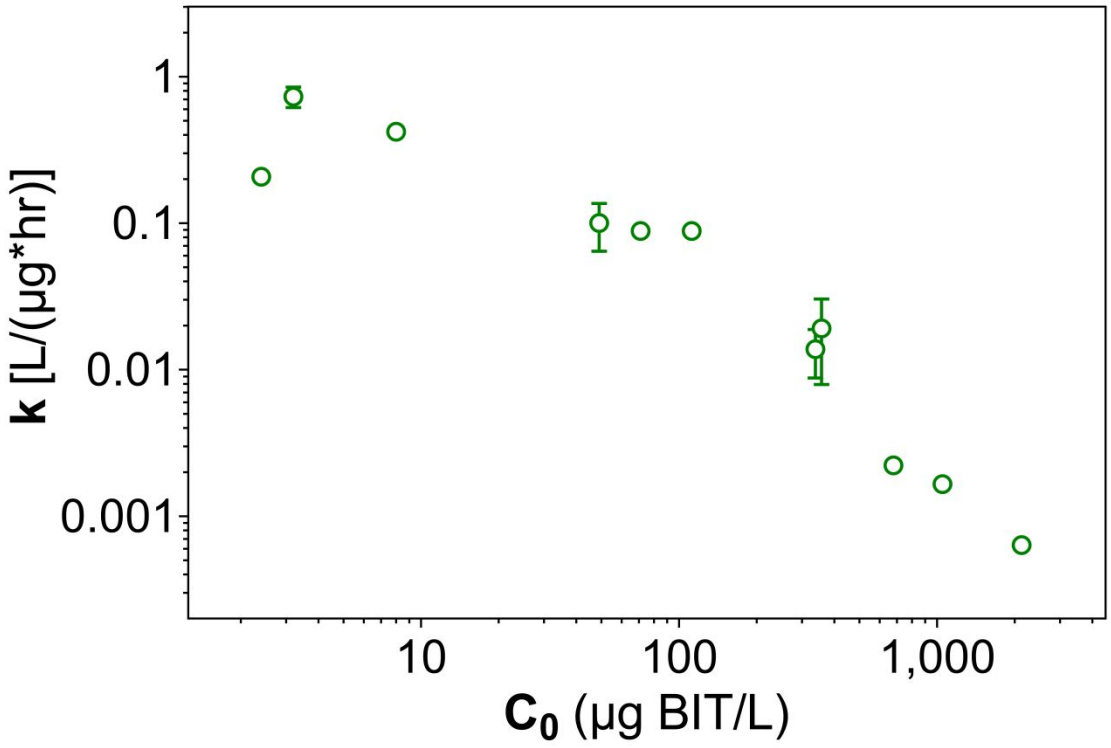


Figure 2: Second-order rate constants for 11 different initial concentrations of BIT in plant medium with Arabidopsis (see Methods for experimental details). The concentrations range from 2.4 µg/L to 2,127 µg/L. Each point represents the rate constant for 4 replicates and 4–8 timepoints, for a total number of 252 data points represented in this graph. A second-order rate best fit the full data set (Table S5). Full kinetic concentration depletion data from all of the experiments represented in each point in this figure are provided in Figure S4. Error bars are +/- one standard error, with some error bars obscured by the data point symbol.

3.3 Evidence that Arabidopsis BIT uptake is a constituently active process

Uptake of BIT by Arabidopsis appears to proceed via a pathway that is largely constituently active rather than inducible. Some plant xenobiotic processes involve "housekeeping" enzymes that are continually produced regardless of conditions, termed constituently active.[73] Other enzymes, termed induced enzymes, increase greatly in number in response to the presence or absence of organic compounds, including xenobiotics.[73,74] The repeated BIT spiking experiment (Figure 3) demonstrated that the rate of BIT removal increased

from 0.008 L/(µg-h) in spike 1 to 0.01 L/(µg-h) in spike 2. The relatively small difference between the removal rates (25% increase) between the two spikes was statistically significant (p=0.045), but this may in fact be merely due to a slight increase in plant biomass between the first and second spikes (which could not be measured *in situ* during the experiment) and/or the difference in measured C_0 between the first (1,050 µg BIT/L) and second (875 µg BIT/L) spike rather than evidence of induction. Generally, the uptake rate was repeatable and did not increase greatly between spikes, indicating an uptake pathway that is likely constituently active. In contrast, a second spike of mercaptobenzothiazole in similar Arabidopsis systems resulted in a reported rate constant between 500% and 2,100% the spike 1 kinetics at any of the various tested concentration levels[26]—much greater than what we observed for BIT. The large rate increase reported for mercaptobenzothiazole with repeated spiking may be due to being an inducible process rather than the slight increase between BIT spikes in this study (*i.e.*, 5-21X for mercaptobenzotiazole vs. 1.25X for BIT) that suggests a constituently active process.

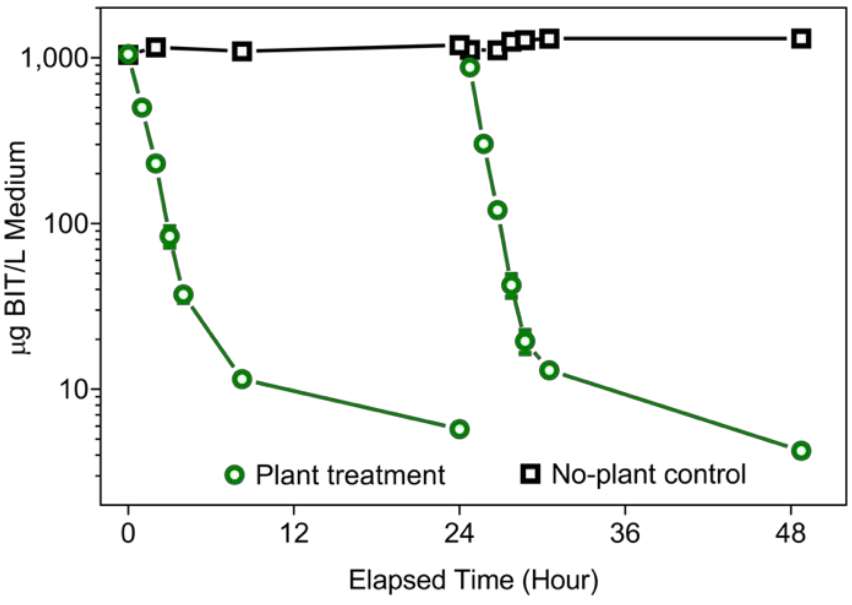


Figure 3: Concentration of BIT in the hydroponic medium over time during a repeated spiking experiment. The first spike at t=0 hr was 1,050 µg BIT/L. The second spike at t≅25 hrs was 875

μg BIT/L. A second-order reaction rate fit both curves the best (vs. zero- and first-order reaction rates). The reaction rate (k) for the first spike was 0.008 L/(μg-h) and spike 2 is 0.01 L/(μg-h). The rates were significantly different (p=0.045) but within 25% of each other. n=4 for all plant treatment data points, and n=1–4 for all no-plant control points. Error bars are ± one standard error, with some error bars obscured by the data point symbol.

3.4 BIT uptake by Arabidopsis is robust, as shown by it being unaffected by several structurally similar compounds and known inhibitors of plant uptake and metabolism pathways

BIT plant uptake proceeds through a pathway that was not significantly affected by multiple tested structurally similar compounds (possible competitive inhibitors) added individually to the plant medium. Neither OIT, benzotriazole, nor tryptophan—all with similar chemical structures to BIT—significantly changed the BIT uptake rate (Figures S5, S6, S7). This lack of competitive inhibition suggests either a robust shared uptake pathway[70] or different uptake pathways[75] for BIT and the tested molecules. The exact mechanism(s) of uptake, however, for the four molecules was not determined. Such mixtures are environmentally relevant because all compounds are known to be taken up by plants,[25,49] and both benzotriazole (a corrosion inhibitor) and tryptophan (an amino acid)-containing compounds are found in stormwater.[25,50,51] Uptake of a single compound being unaffected by similar organic solutes in plant medium solution has previous precedent, e.g., the influx of amino acids into *Zea mays* roots was independent of other organic solutes in solution.[24]

Additionally, the tested inhibitors of known plant uptake and metabolism pathways did not have a significant impact on BIT plant uptake (Figure S8), further demonstrating the robustness of BIT plant uptake. Following plant health experiments to determine an appropriate inhibitor concentration, numerous inhibitors (listed here in parentheses) were used at a single

concentration to test pathways known to be important to the plant uptake and metabolism of a variety of compounds. These inhibitors yielded no significant impact on BIT plant uptake (p>0.05): peptide and amino acid uptake (diethyl pyrocarbonate and 2,4-dinitrophenol), sucrose uptake (diethyl pyrocarbonate), aquaporins (glycerol), organic cation transporters (quinidine), anion channels (anthracene-9-carboxylic acid), and cytochrome p450 (1-aminobenzotriazole). Therefore, these pathways do not appear relevant to BIT uptake—at the inhibitor concentrations used in this work. Further testing at higher inhibitor concentrations may reveal that these pathways are involved in BIT uptake; however, these results most clearly suggest that a different pathway or pathways not tested with these inhibitors is/are the mechanism of Arabidopsis BIT uptake.

3.5 Possible plant metabolites observed to increase in response to BIT exposure

Following 24 hours of BIT exposure (with α=0.05), there were 546 HRMS features that increased in BIT-exposed plant tissue, and 453 decreased (shown visually in Figure S2). Of the increased compounds, we observed five metabolites (for some metabolites, one metabolite was represented by more than one HRMS feature) upregulated five-fold or greater. Two of the increased metabolites were also present in the plant medium at 24 hours (vs. the unexposed plant medium at t=0), implicating possible excretion of plant metabolites.

Two of the proposed upregulated metabolites are endogenous plant compounds rather than transformation products or conjugates of BIT. Nicotinic acid was upregulated by 6-fold in the plant tissue and was initially discovered with a MS2 spectral library match (reported as an mzCloud Best Match score of 86.9 by Compound Discoverer). We subsequently used a commercially-available reference standard and performed a standard addition to confirm the

compound identify to the highest confidence (Level 1 Confidence[76]). Nicotinic acid is active in numerous normal plant metabolic processes,[77] including the formation of NADPH.[78] Thus, the upregulated nicotinic acid in BIT-exposed plants may be due to the increased NADPH needed for glutathione conjugation,[79–81] cytochrome P450,[82] or other processes potentially related to detoxification metabolism. The other endogenous plant compound significantly upregulated (11-fold in plant tissue, 473-fold in hydrponic medium) we propose as phenylthioacetohydroximic acid (mass deviation of 3 ppm; 3a confidence[67]). Phenylthioacetohydroximic acid is part of the glucotropaeolin synthesis pathway. Glucotropaeolin is a glucosinolate, which are activated as part of the plant defense system,[83] and thus may increase in response to xenobiotics. Additionally, the glucotropaeolin pathway requires UDP-glucose, which is known to be used for the glycosylation of xenobiotics.[84] It is possible that a BIT-glucose conjugate was formed but not detected, thus occupying much of the UDP-glucose pool in the plants and causing the accumulation of phenylthioacetohydroximic acid. It is environmentally significant that one or more endogenous plant compounds is proven to be upregulated following exposure to BIT because this demonstrates that xenobiotic compounds found in stormwater can impact fundamental plant biology.

We propose a BIT-amino acid conjugate (BIT-alanine-tyrosine) as a possible structure for another of the compounds upregulated in BIT-exposed plant tissue vs unexposed plant tissue (at a seven-fold increase). The mass deviation of this compound is 8 ppm, with a level 3b confidence[67] based on MS fragments (no MS2 data) and experimental data. Structures in Table 1 are shown in their unionized state for consistency; however, we would expect (based on the accurate mass measured) that the compound is likely deprotonated in the at ambient conditions (pKa= 4.0)[85] and tyrosine is known to ionize in either ESI+ or ESI- modes.[85] Amino acid

conjugation is becoming an increasingly well-documented mechanism of xenobiotic metabolism by plants.[25,68,86] Specifically, conjugation with alanine and tyrosine was also previously reported for Arabidopsis metabolism of di-*n*-butyl phthalate, where the amino acids replaced a hydroxyl group on the parent molecule.[87] Thus, a similar pathway may occur for BIT. The full metabolic implications of amino acid conjugation of xenobiotics are poorly understood.[88] Despite the xenobiotic benzotriazole forming tryptophan analogues via amino acid conjugation, benzotriazole was not integrated into plant proteins in Arabidopsis;[38] however, other metabolic influences may occur.

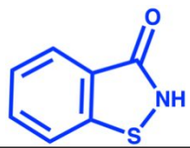
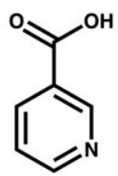
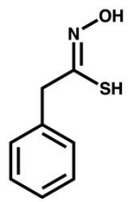
Lastly, two compounds, TP410 and TP470, both with a plant tissue fold change of 8, could not be structurally resolved at acceptable mass deviations (i.e., 10 ppm or less) and are thus reported here merely as a Level 5 accurate masses of interest. TP470 contains fragments that suggest possible glutathione conjugation (see SI spectra), a well-known plant detoxification process for many xenobiotics.[30,79,89] Nevertheless, the high mass deviation (199 ppm between TP470 and a BIT glutathione conjugate; see SI) prevents tentative identification. The retention time for TP470 (1.9 min) was consistent and distinct enough to be detected repeatedly in the nine replicates, eluting just after many other compounds early in the sample run (Figure S9). Future work is needed to more conclusively identify these unknown metabolites.

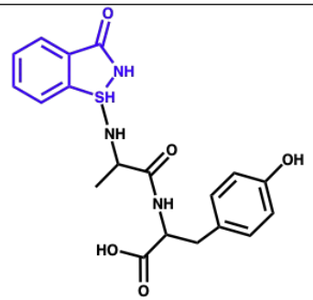
Two of the upregulated plant metabolites in the plant tissue, phenylthioacetohydroximic acid and TP470, were also present in the hydroponic medium after 24 hours of BIT exposure (fold changes >1, Table 1), possibly due to plant excretion. There was no significant decrease in the concentration of BIT in the abiotic control medium, demonstrating that minimal if any abiotic BIT transformation occurred. The proposed alanine-tyrosine BIT conjugate was not present as a major metabolite in the medium. This corresponds with previous work in which a

benzotriazole-amino acid conjugate was not excreted into the medium.[25] The formation of metabolites through interaction of BIT with exudates in the medium cannot be fully ruled out. Previous work with benzotriazole did not find that interactions with plant exudates created new compounds,[25] but further work would be needed to definitively prove a similar lack of interaction between plant exudates and BIT.

The BIT parent compound was not a major compound in the plant tissue or medium at 24 hours, although a small amount was present in BIT-exposed plant tissue (fold change of 1.8 vs unexposed tissue), and in the medium (1% of the BIT at t=0). This observation indicates BIT plant uptake occurred followed by near-complete metabolism, consistent with prior literature,[40] as well as a lack of any substantial product-to-parent reversion.[33]

Table 1: Summary of metabolites whose production increased ≥5 times more in BIT-exposed Arabidopsis plant tissue vs unexposed plant tissue. BIT itself is also provided for reference. The BIT structure is shown in blue, and other structures in black. We employed the Schymanski framework of communication of confidence in novel product discovery.[76] Accurate and exact metabolite masses, ionization mode, mass spectra, and fragment information are provided in the Supplementary Information (Table S6 and Mass Spectra section).

Compound Name	**Proposed Structure** (shown unionized)	**Proposed Formula**	**Confidence Level**[76]	**Plant Tissue Fold Change Peak Area in BIT Treatment vs Unexposed Plant Tissue**	**Medium Fold Change: Peak Area in BIT Treatment at 24 hr vs Unexposed Medium**	**Retention Time (min)**	**Ionization mode, Measured m/z** [Accurate Mass Deviation (ppm) from proposed ionized formula]
BIT (parent compound, for reference)		C_7H_5NOS	Level 1: Confirmed with standard	1.8	0.01	14.59	$[M+H]^+$ 151.00928 (3)
Nicotinic Acid		$C_6H_5NO_2$	Level 1: Confirmed with reference standard (additionally, Library Spectrum Match, MS^2)	6	0.4	12.91	$[M-H]^-$ 122.02349 (10)
Phenylthioacetohydroximic acid		C_8H_9NOS	Level 3a: [67] Tentative Candidate (based on MS, fragments, exp. data, MS^2)	11	472	14.77	$[M+H]^+$ 168.04785 (3)

Compound Name	**Proposed Structure** (shown unionized)	**Proposed Formula**	**Confidence Level**[76]	**Plant Tissue Fold Change Peak Area in BIT Treatment vs Unexposed Plant Tissue**	**Medium Fold Change: Peak Area in BIT Treatment at 24 hr vs Unexposed Medium**	**Retention Time (min)**	**Ionization mode, Measured m/z** [Accurate Mass Deviation (ppm) from proposed ionized formula]
BIT-Alanine-Tyrosine Conjugate		$C_{19}H_{21}N_3O_5S$	Level 3b:[67] Tentative Candidate (based on MS fragments, exp. data)	7	1	13.89	$[M+H]^+$ 403.11679 (8)
TP 470 [Unknown Accurate Mass of Interest Significant Upregulated]	N/A	N/A	Level 5: Accurate Mass of Interest	8	50	1.92	$[M-H]^-$ 470.15134 (N/A)
TP 410 [Unknown Accurate Mass of Interest Significant Upregulated]	N/A	N/A	Level 5: Accurate Mass of Interest	8	0.8	6.73	$[M-H]^-$ 410.86249 (N/A)

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4. Conclusions

This work demonstrates that four commonly used isothiazolinones are rapidly removed from plant medium by aseptic Arabidopsis grown hydroponically. This removal is likely primarily due to plant uptake, with no significant difference in removal rate among the compounds. Rapid, likely transporter-mediated removal of isothiazolinones from water is a novel and promising finding for removing isothiazolinones from stormwater even in situations where there may be a fairly short amount of interaction time, e.g., in bioretention cells designed for infiltration of stormwater. In the initial BIT concentration ranges tested in this paper, rapid (≥99% removal from the medium within 24 hours) rates of uptake were found at all concentrations with a pattern suggesting substrate inhibition. Repeated BIT exposures implicated constituently active uptake. These data together suggest the potential for high uptake rates at environmentally relevant concentrations. Further work would be needed to demonstrate the plant uptake kinetics under field conditions.

Additionally, isothiazolinone plant uptake was robust even in mixtures with other similar compounds as potential competitive inhibitors and with the addition of known inhibitors of plant uptake pathways and metabolism. This discovery lends further support to our findings being environmentally relevant in the compound mixtures that occur in stormwater green infrastructure systems. The rapid plant uptake of isothiazolinones coupled with the plant metabolism proposed in this work present the possibility for phytoremediation of BIT and possibly other isothiazolinones. The potential BIT conjugation and upregulation of endogenous plant compounds indicate plant metabolism of BIT and potential to impact plants. Further work is needed to verify the identity of the metabolites identified as accurate masses of interest. Overall, the ability of Arabidopsis to rapidly take up and metabolize isothiazolinone compounds without

visual impacts to plant health indicates the potential for phytoremediation of these compounds from stormwater.

Conflicts of Interest

The authors declare no competing financial interest.

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