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Rapid plant uptake of isothiazolinone biocides and formation of metabolites by hydroponic *Arabidopsis*†

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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 that significantly increased in the BIT-exposure treatment compared to the control included: endogenous plant compounds 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 it 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.

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Environmental significance

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 it 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 1600 ng L^{-1} benzisothiazolinone (BIT),[5] 150 ng L^{-1} of methylisothiazolinone (MIT),[7] 41 ng L^{-1} chloromethylisothiazolinone (CMI),[7] and 67 ng L^{-1} octylisothiazolinone (OIT).[5] These four compounds (Table S1†) are classified by the European Chemicals Agency as 'very toxic to aquatic life

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with long-lasting effect'; thus, the presence of these biocidal compounds in environmental waters through stormwater runoff could negatively impact wildlife.[8–11] Additionally, 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 lower 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.*, transformations such as hydroxylation, hydrolysis, and dealkylation.[21,27–29] These reactions, mainly catalyzed by cytochrome P-450 enzymes, generate 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 first undergoing 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 identify *Arabidopsis* plant metabolites following isothiazolinone exposure. 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.*, were 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 (M_W = 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 1*H*-benzotriazole (CAS 95-14-7, Fluka Analytical, ≥98%). Other chemicals are described in the ESI.† 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† (ref. 6, 7 and 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 ESI†). 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 ESI†).

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 ESI.† 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^{-1}, 112 μg L^{-1}, 678 μg L^{-1}, and 2127 μg L^{-1}. 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^{-1}), 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 ESI.†

A repeated exposure kinetics experiment was also conducted with BIT. Medium was spiked to 1050 μg BIT per 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 per 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 per 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 ESI,† 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 ESI.†

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 per L (the measured concentration targeted to be similar to the nominal 150 μg BIT per 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 per 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 (see below). 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 per L, and (b) a nominal mixture of 50 μg BIT per L and 50 μg benzotriazole per L. For tryptophan, a nominal mixture of 50 μg BIT per L and 67.4 μg L-tryptophan per L (a molar-equivalent concentration to 50 μg BIT per 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 ESI.† 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 per L and 25 mL of plant medium per box. Samples were taken at $t = 0$ h, 2 h, 24 h, and 48 h, 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 (rather than C/Co basis). 10 μL of 1.3 mg L^{-1} 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 ESI.† 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^{-1}, 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 ESI.†

2.3.2 Metabolomics investigation *via* high-resolution mass spectrometry. The extracted plant tissues (extraction details in the ESI†) 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 ESI† 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 ESI†). 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" (Fig. 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 (Fig. 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 (ESI†).

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 (Fig. 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 (Fig. 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 h, 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 (Fig. 1, $p \geq 0.05$).

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 (Fig. 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 directly measure potential abiotic interactions with exudates for BIT, previous work in the same experimental setup[25] found that *Arabidopsis* root exudates did not create a significant loss of another organic xenobiotic, benzotriazole.

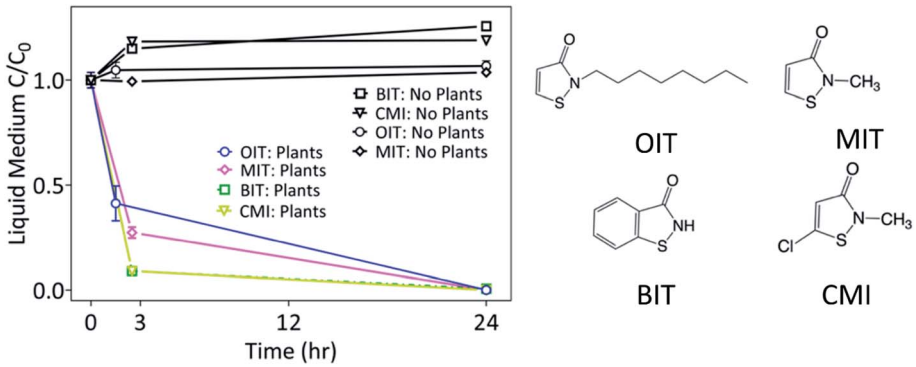


Fig. 1 Plant-facilitated depletion kinetics of isothiazolinone biocides from hydroponic medium. OIT (nominal C_0 of 150 μg L^{-1}), BIT (nominal C_0 of 100 μg L^{-1}), and MIT and CMI (as a mixture, nominal MIT C_0 of 33 μg L^{-1}, nominal CMI C_0 of 100 μg L^{-1}) were all rapidly taken up by *Arabidopsis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

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^{-1} and 28-fold for a C_0 of 49 μg BIT per L (calculations shown in the ESI†). 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 2.4–2127 μg L^{-1}, BIT uptake by *Arabidopsis* was rapid, resulting in complete or near-complete removal within 24 hours (Fig. S4†). This rapid removal limited the collection of early data points, but with a total of 252 data points from 11 starting concentrations (Fig. S4,† summarized in Fig. 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: 2.4–2127 μg L^{-1}, Fig. 2), 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

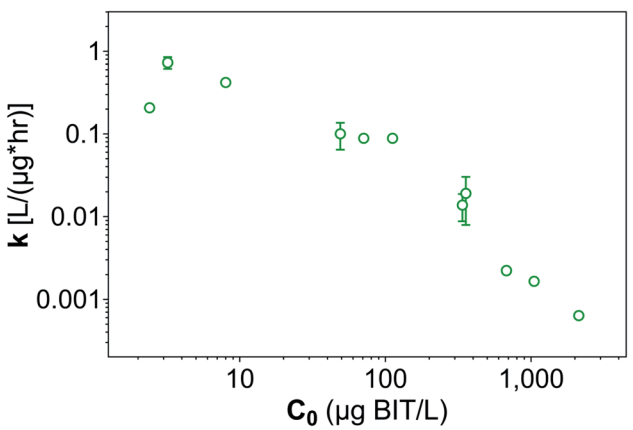


Fig. 2 Second-order rate constants for 11 different initial concentrations of BIT in plant medium with *Arabidopsis* (see Methods for experimental details). The concentrations range from 2.4 μg L^{-1} to 2127 μg L^{-1}. Each point represents the rate constant for 4 replicates and 4–8 timepoints, for 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 Fig. S4.† Error bars are ± one standard error, with some error bars obscured by the data point symbol.

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^{-1}, and BIT stormwater concentrations have been reported up to 1.6 μg L^{-5}. Lower initial concentrations, *e.g.* 0.9 μg BIT per 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 Fig. 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 (Fig. S4†) including 0.9 μg BIT per L when the BIT medium concentration reached zero so rapidly that a curve could not be constructed. These results suggest the potential for high BIT plant uptake rates at environmentally-relevant concentrations.

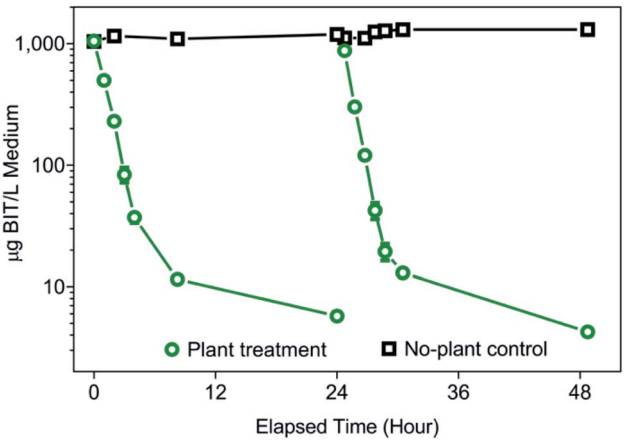


Fig. 3 Concentration of BIT in the hydroponic medium over time during a repeated spiking experiment. The first spike at $t = 0$ h was 1050 μg BIT per L. The second spike at $t \cong 25$ h was 875 μg BIT per L. A second-order reaction rate fit both curves the best (*vs.* zero- and first-order reaction rates). The reaction rate (k) for the first spike was 0.008 L μg^{-1} h^{-1} and spike 2 is 0.01 L μg^{-1} h^{-1}. 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.

3.3 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 (Fig. 3) demonstrated that the rate of BIT removal increased from 0.008 L μg^{-1} h^{-1} in spike 1 to 0.01 L μg^{-1} h^{-1} 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 (1050 μg BIT per L) and second (875 μg BIT per 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 2100% of 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 this process being inducible rather than the slight increase between BIT spikes observed in this study (*i.e.*, 5–21X for mercaptobenzotiazole *vs.* 1.25X for BIT) that suggests a constituently active process.

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 (Fig. S5–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 (Fig. S8†), further demonstrating the robustness of BIT plant uptake. Following plant health experiments to determine an appropriate inhibitor concentration, numerous inhibitors (listed below 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. Inhibitors of the following processes 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 $\alpha = 0.05$), there were 546 HRMS features that increased in BIT-exposed plant tissue, and 453 decreased (shown visually in Fig. 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 identity 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, 472-fold in hydroponic 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.

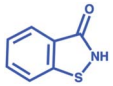
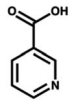
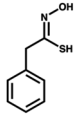
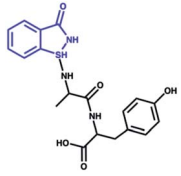
We propose a BIT–amino acid conjugate (BIT–alanine–tyrosine) as a possible structure for another of the compounds upregulated in BIT-exposed plant tissue *vs.* unexposed plant tissue (at a seven-fold increase). The mass deviation of this compound is 8 ppm, with a level 3b confidence[67] based on MS fragments (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 ($pK_a = 4.0$)[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 ESI† 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 ESI†) 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 (Fig. S9†). Future work is needed to more conclusively identify both TP 410 and TP470.

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 (Fig. S4†). 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

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 ESI (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 h *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)
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)

by near-complete metabolism, consistent with prior literature,[40] as well as a lack of any substantial product-to-parent reversion.[33]

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 occured 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 the 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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