



# Harnessing plant-microbiome interactions for bioremediation across a freshwater urbanization gradient

Anna M. O'Brien<sup>\*,1,a,b</sup>, Zhu Hao Yu<sup>1,c</sup>, Clara Pencer<sup>a</sup>, Megan E. Frederickson<sup>a</sup>, Gregory H. LeFevre<sup>d</sup>, Elodie Passeport<sup>c,e</sup>

<sup>a</sup> Department of Ecology and Evolutionary Biology, University of Toronto, 25 Willcocks St, Toronto, ON, M5S 3B2, Canada

<sup>b</sup> Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, 46 College Rd, Durham, NH, 03824, USA

<sup>c</sup> Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, ON, M5S 3E5, Canada

<sup>d</sup> Department of Civil & Environmental Engineering and IIHR-Hydroscience & Engineering, University of Iowa, 4105 Seamans Center, Iowa City, IA, 52242, USA

<sup>e</sup> Department of Civil and Mineral Engineering, University of Toronto, 35 St George St, Toronto, ON, M5S 1A4, Canada

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## ABSTRACT

Urbanization impacts land, air, and water, creating environmental gradients between cities and rural areas. Urban stormwater delivers myriad co-occurring, understudied, and mostly unregulated contaminants to aquatic ecosystems, causing a pollution gradient. Recipient ecosystems host interacting species that can affect each others' growth and responses to these contaminants. For example, plants and their microbiomes often reciprocally increase growth and contaminant tolerance. Here, we identified ecological variables affecting contaminant fate across an urban-rural gradient using 50 sources of the aquatic plant *Lemna minor* (duckweed) and associated microbes, and two co-occurring winter contaminants of temperate cities, benzotriazole and salt. We conducted experiments totalling >2,500 independent host-microbe-contaminant microcosms. Benzotriazole and salt negatively affected duckweed growth, but not microbial growth, and duckweeds maintained faster growth with their local, rather than disrupted, microbiota. Benzotriazole transformation products of plant, microbial, and phototransformation pathways were linked to duckweed and microbial growth, and were affected by salt co-contamination, microbiome disruption, and source sites of duckweeds and microbes. Duckweeds from urban sites grew faster and enhanced phytotransformation, but supported less total transformation of benzotriazole. Increasing microbial community diversity correlated with greater removal of benzotriazole, but taxonomic groups may explain shifts across transformation pathways: the genus *Aeromonas* was linked to increasing phototransformation. Because benzotriazole toxicity could depend on amount and type of *in situ* transformation, this variation across duckweeds and microbes could be harnessed for better management of urban stormwater. Broadly, our results demonstrate that plant-microbiome interactions harbour manipulable variation for bioremediation applications.

## 1. Introduction

Urban landscapes are expected to triple in area from the year 2000 to 2030 alone (Seto et al., 2012), and thus represent a new major type of ecosystem into which existing species assemble. Urban environments confront organisms with unique challenges and stressors, and in response, many organisms evolve along urban-to-rural gradients (Johnson and Munshi-South, 2017). The influx of highly complex mixtures of anthropogenic contaminants into aquatic systems via

stormwater is one such unique pressure of urban environments that elicits ecological and evolutionary responses (Brans and De Meester, 2018; Hale et al., 2016; Kern and Langerhans, 2018; Masoner et al., 2019; Nacci et al., 2010; Peter et al., 2018; Tian et al., 2021; Walsh et al., 2005).

Interactions between organisms in systems receiving stormwater, such as ponds, wetlands, rivers, lakes, and marine environments, can simultaneously alter both biological consequences of contaminants and contaminant fate (e.g., Tondera et al., 2021). Contaminant

\* Corresponding author.

E-mail address: [anna.obrien@unh.edu](mailto:anna.obrien@unh.edu) (A.M. O'Brien).

<sup>1</sup> Authors contributed equally

transformation in receiving systems is particularly affected by resident “microbiomes” (assemblages of microbial species living together in a community). Host-associated microbiomes can comprise hundreds or thousands of species, each with unique metabolic capabilities and potentially distinct effects on contaminants. Because plant carbon increases microbial growth and metabolic rates, plant-microbe combinations often synergistically enhance contaminant transformation (Ashraf et al., 2018; Fester et al., 2014), and plant-associated microbiomes can often transform contaminants faster than environmental microbiomes (Chaudhry et al., 2005). Engineering microbiomes to improve human or plant health shows great promise (Busby et al., 2017; Lawson et al., 2019). Likewise, engineered plant-microbe systems have been proposed as a way to manage stormwater contaminant influxes as part of constructed, wetland-mimicking infrastructure (Ashraf et al., 2018; Ishizawa et al., 2020). Constructed wetlands demonstrate variable contaminant removal efficacy, and therefore opportunity for improvement (Brisson and Chazarenc, 2009; Varma et al., 2021).

One representative and widespread source of stormwater contaminants is the co-use of anti-corrosives, such as benzotriazole, and salt in temperate latitudes during winter. Benzotriazole is a common anti-corrosive agent in many applications, including de-icers for aircraft and cars, which may explain its higher abundance as a winter contaminant in more urban stormwater (Alvey et al., 2016; Kiss and Fries, 2012; Parajulee et al., 2017). Environmental concentrations are generally well below effect concentrations (environmental in ng/L to low  $\mu\text{g/L}$ , rarely  $>100\text{ mg/L}$ ; effect  $\approx 10\text{ }\mu\text{g/L}$  for sensitive species, Cancilla et al., 1998; Parajulee et al., 2017; Tangtian et al., 2012). Yet, benzotriazole is relatively resistant to biodegradation, has a long half-life ( $>>100$  days, Liu et al., 2011a), and its environmental fate is poorly understood. At the same time, winter salt application in many temperate-region urban areas has shifted salinities near the U.S. EPA aquatic life threshold criterion, and contributes to expanding freshwater salinization across North America (Dugan et al., 2017; Kaushal et al., 2018). Impacts of contaminant mixtures are often poorly predicted from components alone due to interactive effects (Cedergreen, 2014), and both occurrence in mixture and interactive effects are especially likely for salt and benzotriazole (Asheim et al., 2019; Parajulee et al., 2017; Rhodes-Dicker and Passetport, 2019).

Given the potential for contaminant responses or transformation to vary across plant genotypes, microbial community composition, and background environmental conditions (Hijosa-Valsero et al., 2012; Inui et al., 2008; Rhodes-Dicker and Passetport, 2019; Tondera et al., 2021; Wu et al., 2021), harnessing microbiomes for stormwater treatment requires understanding how these factors interact. Large experiments can simultaneously reveal more effective plant-microbe combinations for specific purposes (i.e. constructed wetlands) and develop tools for leveraging plant microbiomes across applications. Duckweeds (*Lemna minor*) are tiny plants common in stormwater ponds in northern temperate regions, foster biotransformation of benzotriazole even in the presence of salt, and have plant growth-promoting microbiomes that vary in composition (Ishizawa et al., 2017; O'Brien et al., 2020; 2019). We used duckweed as a model wetland plant, leveraging these characteristics in high-throughput constructed microcosm experiments. Our objective was to identify how variation in co-applied salt, duckweed genotype, and interacting microbes across an urban-to-rural gradient may alter benzotriazole fate.

## 2. Materials and Methods

We conducted two microcosm experiments to reveal interactive effects between benzotriazole biotransformation and duckweed microbiomes. Combined, these experiments tested  $>100$  differences in plant and microbiome manipulation (“biotypes”) in  $>2,500$  independent microcosms.

### 2.1. Biological materials

We simultaneously collected duckweeds and associated microbes from 50 field sites in the Greater Toronto Area and adjacent rural areas in 2016–2018 (Supplementary Material, Table S1). We generated near isogenic lines (NILs) from each duckweed collection by clonally propagating a single frond. We cultured the microbial community living in, on, or in water adhering to, a field-collected frond on yeast mannitol agar plates. We call these “local microbes” or “local microbiomes” for short, though they contain only microbial taxa that can survive in co-culture under our lab conditions (e.g., O'Brien et al., 2020; 2019). We grew NILs in a growth chamber and stored microbial communities at  $4\text{ }^{\circ}\text{C}$ . We characterized cultured communities via 16S rDNA sequencing performed at Genome Québec (Table S2). We profiled the resulting reads using QIIME 2 (Bolyen et al., 2019) to clean, trim, process, identify amplicon sequence variants, and calculate diversity metrics. We inserted remaining reads into a phylogenetic tree (McDonald et al., 2012). We used QIIME 2's gneiss tool to compute the contrast in relative abundance of taxa comprising each daughter branch from each node of the tree in each cultured community (“balances,” Morton et al., 2017).

We prepared duckweeds from each NIL for experiments by thoroughly rinsing, and bleaching with 1% bleach for 30 seconds to disrupt microbes prevalent in lab culture, followed by 7 days recovery. To prepare microbes for experiments, we stirred a swab from the stored culture of each duckweed line's accessioned microbial community into liquid yeast-mannitol media (YM), and shook for 1 day ( $30\text{ }^{\circ}\text{C}$  and 200 rpm). We diluted each high density inoculum to an expected cell density of 3,000 cells/ $\mu\text{L}$  by adding water, relying on the relationship between optical density and colony forming units we verified previously (O'Brien et al., 2020). See Text S1 for further details on biological materials.

### 2.2. Testing responses of duckweeds and microbes from more urban sites to benzotriazole, and effects on benzotriazole fate

Our first experiment measured 11-day plant and microbial growth, as well as benzotriazole removal and transformation, across treatments of salt, initial benzotriazole, and microbial manipulations, using 50 duckweed genotypes and microbial communities collected across an urbanization gradient.

#### 2.2.1. Experiment

We crossed two environmentally relevant levels of salt (0, 0.8 g/L NaCl, Transportation Services Division, 2016; Lawson and Jackson, 2021) and benzotriazole (0, 1 mg/L, Loos et al., 2010; Parajulee et al., 2017), and two microbial treatments (microbiome disruption, or microbiome disruption followed by application of local microbes) with our 50 duckweed NILs. We prepared salt and benzotriazole treatments in Krazčič's media (Krazčič et al., 1995). Solutions were autoclaved after adding all but benzotriazole, which was added after autoclaving as a high purity powder (as it may be unstable at high temperature and pressure).

We added the four treatment solutions to 24-well plates in 2.5 mL per well, to which we then added approximately one bleached duckweed unit (attached mother and daughter fronds). In inoculated treatments, duckweeds received the cultured microbial community from the same site as the duckweeds. Uninoculated duckweeds received sterile liquid YM diluted similarly with water. The uninoculated duckweeds did not support fewer microbes (Fig. S1), thus we call this treatment “disrupted microbiomes” or “disrupted microbes” herein. We sealed plates with BreatheEasier (Millipore-Sigma, Diversified Biotech, Dedham, MA, USA) membranes, and placed in the growth chamber for 11 days (conditions above). The membranes allowed high diffusion, so we added sterile water to, and resealed, each plate midway through the experiment. We measured microbial growth at the end (optical density, BioTek Synergy HT plate reader). We scored duckweed growth from images throughout

the experiment by hand counting live fronds (days 0, 3, 5, 7, 9, and 11) and using ImageJ for frond area (Schneider et al., 2012, days 0, 7, and 11, see Table S3).

### 2.2.2. Transformation analysis

We quantified both benzotriazole remaining and various transformation products on a subset of benzotriazole-treated wells (four salt  $\times$  microbial treatments, 17 genotypes, three replicates for most, 200 total). We used high-performance liquid chromatography with direct injection, gradient elution, and electrospray ionization tandem mass spectrometry for quantification of benzotriazole and benzotriazole transformation products (Table S4). Three groups of transformation products were investigated in samples, all using authentic standards: phototransformation products (3-aminophenol, phenazine, Liu et al., 2011b; Weidauer et al., 2016; Wu et al., 2021), phytotransformation products (glycosylated benzotriazole, benzotriazole alanine, and benzotriazole acetylalanine, LeFevre et al., 2015), and microbial transformation products (1-methylbenzotriazole, 4-methoxybenzotriazole, and 5-methoxybenzotriazole, Liu et al., 2011a, see Text S1).

### 2.2.3. Statistical tests

We analyzed biological growth variables and benzotriazole transformation using linear models in R (package MCMCglmm, Hadfield, 2010; R Core Team, 2019). For each response variable, we began with a model including all fixed effects (salt, benzotriazole, and microbial treatments, as well as road density at sources of duckweeds and local microbes) and all interactive effects. Starting with this most complex model, we identified best models in a reverse, stepwise approach. We removed terms singly beginning with the least significant highest-order interaction term, retaining components of significant interaction terms (using Bayesian equivalents of p-values, pMCMC). We refit models after each removal, and checked fit improvement (Deviance Information Criterion, Spiegelhalter et al., 2002). We used this stepwise procedure for all linear models, here and below. Biological responses included change in frond area of duckweeds from start to end, final microbial optical density, and duckweed growth through time in frond number. Frond number models included all fixed effects as functions of time and included random effects for each experimental well.

We fit additional models with benzotriazole removed and each transformation product as response variables, and duckweed growth in frond surface area, optical density, and their interaction as explanatory variables. To improve model fitting, we logged response variables when this improved normality (evaluated with Shapiro-Wilk tests), or we scaled by subtracting the mean and dividing by standard error. As we could not fully distinguish 4-methoxybenzotriazole and 5-methoxybenzotriazole chromatographically, we summed the two isomers ("methoxybenzotriazole"). We summed benzotriazole alanine and benzotriazole acetylalanine because we expect their production is linked (LeFevre et al., 2015). We also evaluated whether changes to the scale of variance among duckweed and microbe sources or changes in their rank order contributed more to benzotriazole transformation differences across salt and microbial treatments using variance partitioning (Cockermham, 1963; Muir et al., 1992).

We related variation in taxonomic makeup of local, cultured microbial communities across source sites to response variables for the subset of microcosms treated with both benzotriazole and local microbes. We first fit separate linear models between taxonomic diversity and the proportion of benzotriazole removed, duckweed growth, and microbial growth. We then averaged response variables (biological growth and transformation products) for each microcosm treatment, calculated the balances at each phylogenetic node (contrasts of relative abundance of descendant taxa, Morton et al., 2017) across the microbial inocula, and computed correlations between averages and balances. We performed this separately for each salt treatment. For each response variable and salt treatment, we counted the nodes subtending each ASV that fell outside the 95th percentile of correlations as a metric of

ASV-response association. We permuted treatment averages across the microbial communities 1,000 times and re-calculated the metric, yielding a 95% interval for random ASV-response associations.

### 2.3. Testing how co-occurring algae, duckweeds, and microbes shift kinetics

Our second experiment followed benzotriazole removal kinetics in microcosms of a single duckweed genotype across both levels of salt for 21 days, with additional microcosm types. We spiked concentrated benzotriazole (filter-sterilized at 0.2  $\mu$ m) at 5  $\mu$ g/L in 2.5 mL of autoclaved Krazčič's media in 24-well plates with or without: 0.8 g/L of salt (NaCl), one bleached unit of duckweed fronds from the Lion's Head site (Table S1), inoculation with local microbes, and added algae (approximately 5.91 mg wet weight of algae *Chlorella vulgaris*). We crossed treatments in a semi-factorial manner with 18 replicates each (treatments listed in Table S5). Experimental plates were sealed with BreatheEasy membranes (Millipore-Sigma, Diversified Biotech, Dedham, MA, USA). We sampled three replicates per treatment sacrificially after 0, 3, 7, 11, 17, and 21 days (see Table S3). Chemical analysis of remaining benzotriazole was conducted almost identically to the previous experiment. We estimated first-order kinetic constants by non-linear regression, using the profile likelihood method to calculate confidence intervals in R (95%, Perrin, 2017; R Core Team, 2019). For the final timepoint, 21 days, we tested differences between each biotic treatment and the abiotic treatment using linear models with treatment as a fixed effect in R. See Text S1 for more details.

## 3. Results

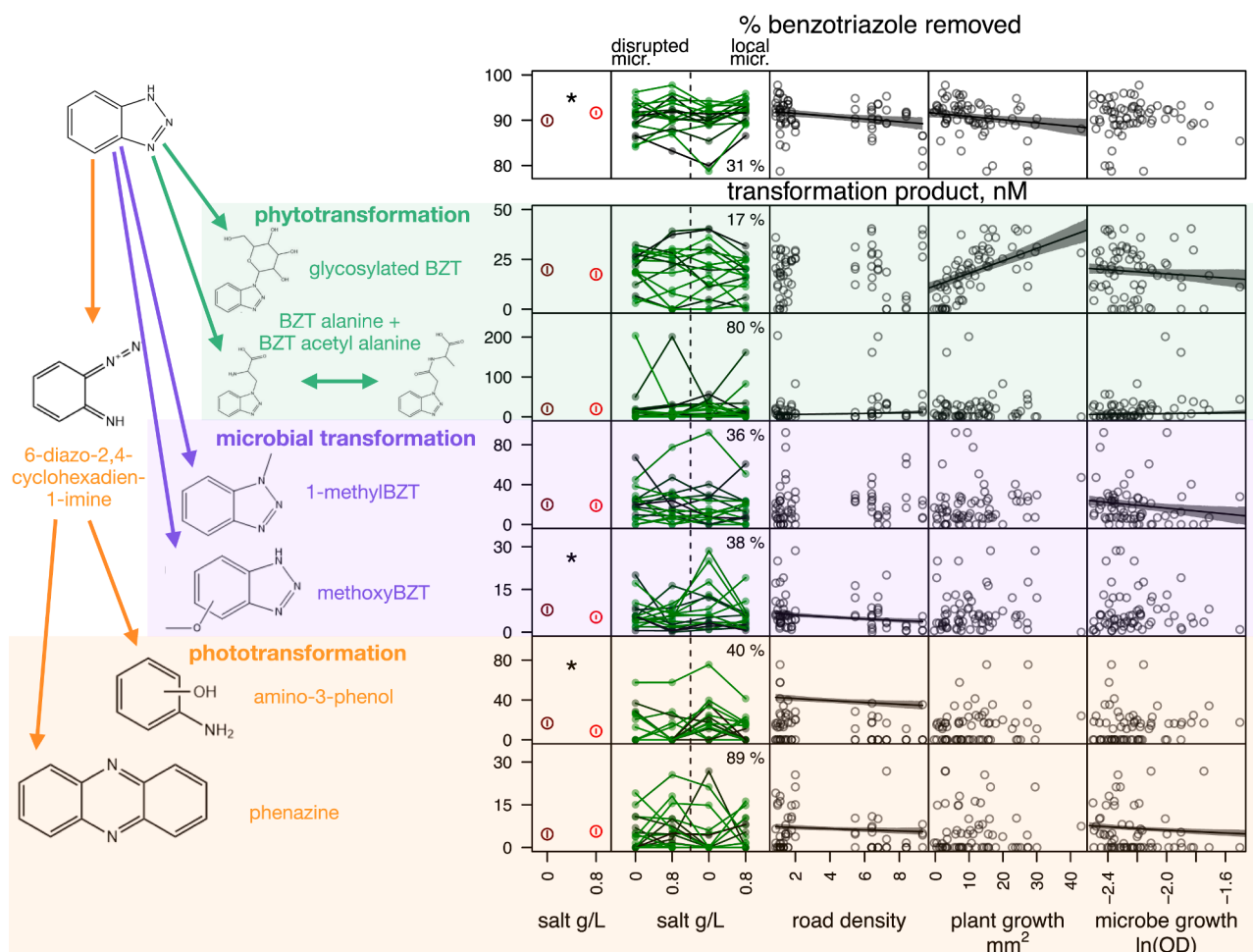
The vast majority of benzotriazole was transformed or otherwise removed in our microcosms during our 11-day experiment (73-99% across treatments, Fig. 1). Estimated half-lives of benzotriazole in our 21-day experiment reached as low as 3.9 days (95% confidence interval 3.2-5 days, Table S5, Fig. S2). However, transformation varied across microcosms depending on salt and biological context.

### 3.1. Growth, salt, and biotype variation linked to benzotriazole transformation

Duckweed and microbial growth were correlated to contrasting benzotriazole fate. Greater duckweed growth was associated with less total transformation but more of a phytotransformation product (glycosylated benzotriazole), and benzotriazole decreased duckweed growth (all  $p < 0.05$ , Figs. 1, 2, Tables S6, S7, S8). Benzotriazole had no effect on microbial growth (Fig. 2, Table S9). Microbial growth was positively correlated with phytotransformation products (summed benzotriazole alanine and benzotriazole acetyl alanine), and negatively correlated to microbial and phototransformation products (1-methylbenzotriazole and phenazine, all  $p < 0.05$ , Fig. 1, Table S6).

Co-contamination with urban runoff levels of salt resulted in more benzotriazole removal ( $p < 0.05$ , Fig. 1, Table S6), but decreased the amount of the phototransformation product 3-aminophenol ( $p < 0.05$ ), and possibly the microbial transformation product methoxybenzotriazole ( $p < 0.1$ , Fig. 1, Table S6). Salt co-contamination altered the rank order of biotypes in total benzotriazole removal, with effects depending on microbial treatments (crossing lines, Fig. 1, 2<sup>nd</sup> column). Indeed, rank shifts in the amount of transformation products measured among duckweed lines were frequent across treatments. Rank shifts contributed >30% to the variance of biotype means across salt and microbial treatments, for all except glycosylated benzotriazole (Fig. 1).

Despite the interactive effects of salt and biotype, differences in benzotriazole fate existed among biotypes along the urban-rural ecotone. Duckweeds from urban sites both grew faster and decreased benzotriazole removal compared to those from rural sites. This may underlie decreased benzotriazole removal in microcosms with more



**Fig. 1.** Transformation pathways of benzotriazole and abundance of each detected product. Benzotriazole is in percent of initial 1 mg/L removed, the rest are in nM concentration. The 1<sup>st</sup> column compares across NaCl salt treatments (significant differences marked with ‘\*’). The 2<sup>nd</sup> column compares duckweed genotype-treatment means, with genotype means across salt and microbiome treatments connected by lines (from more rural areas in lighter green; from more urban areas in darker green to black). The % of variance in genotype means across salt and microbiome treatments that is due to changes in rank among genotypes is shown. The 3<sup>rd</sup> column compares genotype origin. The 4<sup>th</sup> compares across growth of duckweeds in frond area change, while the 5<sup>th</sup> compares across growth of microbes in optical density at 600 nm (OD, logged). In the 3<sup>rd</sup>-5<sup>th</sup> columns, fit lines indicate significant relationships (except for glycosylated benzotriazole and microbe growth, which is marginal). Fit lines are predicted means, surrounded by shaded 95% highest posterior density intervals (HPDI, Bayesian equivalent of confidence intervals). Points in the 3<sup>rd</sup>-5<sup>th</sup> columns are means for each unique source-site and treatment combination. Models, estimates and p-values (Bayesian, pMCMC) in Table S6. Benzotriazole is abbreviated ‘BZT.’

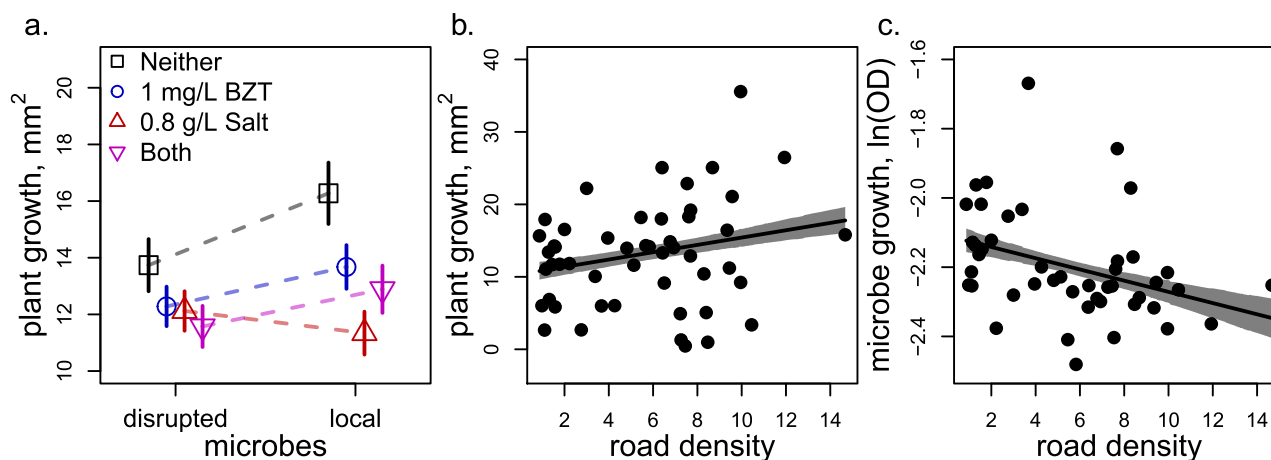
plant growth (all  $p < 0.05$ , Figs. 1, 2, Tables S6, S7, S8). Duckweeds from urban sites were also associated with more phytotransformation products (summed benzotriazole-alanine and benzotriazole-acetyl-alanine), but less photo- and microbial transformation products (3-aminophenol and methoxybenzotriazole), and less microbial growth (all  $p < 0.05$ , Figs. 1, 2, Tables S6, S9).

Benzotriazole transformation is expected to decrease toxicity. Transformation products of benzotriazole have limited empirical toxicity data, so we leveraged ECOSAR (Ecological Structure Activity Relationships Predictive Model, Mayo-Bean et al., 2019). We used ECOSAR to estimate toxic units for each measured mixture (summed concentration of each compound divided by its predicted fish 96-hour 50% lethal dose). Phytotransformation products were predicted to have the least toxicity (Table S10). Benzotriazole was the most abundant compound, and was predicted to be substantially toxic by ECOSAR (Fig. 1, Table S10). Because they removed the most benzotriazole, rural duckweeds decreased predicted toxicity the most (45% lower than microcosms with urban duckweeds,  $p < 0.05$ , Table S11).

### 3.2. Drivers of variation across biotypes in benzotriazole transformation

We evaluated whether local adaptation to contaminants in urban duckweeds or shifts in microbial community composition may underlie variation among biotypes in growth and benzotriazole transformation. We did not detect local adaptation to benzotriazole or salt in urban biotypes: benzotriazole and salt equally decreased growth of duckweeds from urban and rural sites, and did not affect growth of local duckweed microbes (Tables S7, S9, but see complex effects for frond accumulation, Figs. S3, S4, Table S8). However, duckweeds exhibited faster growth in local relative to disrupted microbiome treatments across source sites ( $p < 0.05$ , Fig. 2, Tables S7, S8).

In contrast, microbial community composition was linked to growth and benzotriazole fate. Higher microbial richness positively correlated with benzotriazole removal, and negatively correlated with duckweed growth (all  $p < 0.05$ , Figs. 3a, S5, Table S12). Cultured local microbial communities varied substantially across collection sites (Fig. S6). The relative abundance of different phylogenetic clades correlated with differences in benzotriazole transformation in treatments with local microbial communities (Fig. 3b). Within the highly abundant *Aeromonadaceae*, greater relative abundance of *Aeromonas* compared to



**Fig. 2.** Significant effects of treatment and source site on duckweed and microbial growth. Duckweed growth is shown in (a) and (b), microbial growth in (c), and includes only treatments with local microbes. Points are treatment (a) or source site (b, c) means, vertical bars are  $\pm 1$  standard error (a). Benzotriazole is abbreviated 'BZT.' Optical density at 600 nm is abbreviated OD, and is transformed by the natural log. Road density is km road per km<sup>2</sup> land area. Fit lines in panels (b) and (c) show predicted mean and 95% HPDI. See [Tables S7, S9](#) for fitted parameters, p-values, and model with additional microbial data.

*Tolumonas* correlated with more phototransformation (3-aminophenol across salt treatments, and phenazine at 0.8 g/L NaCl). Similarly, some inocula communities had higher abundance of *Pseudomonadaceae*, which positively correlated with the amount of microbial transformation (1-methyl benzotriazole, only in salt treatments). Others had more *Rhizobiaceae* relative to *Caulobacteriaceae*, which positively correlated with phytotransformation products (sum of benzotriazole alanine and benzotriazole acetyl-alanine, all relationships  $p < 0.05$ , [Figs. 3b, S6, S7, Table S13](#)). No similarly strong links were observed between microbial community composition and duckweed or microbial growth ([Fig. S8](#)).

### 3.3. Plant-microbiome interactions altered benzotriazole removal rates

By the end of the 21-day kinetics experiment, microcosms removed 58–90% of the 5  $\mu\text{g/L}$  benzotriazole initially added. Microbial communities added alone removed less benzotriazole by day 21 than abiotic treatments without organisms added, in which phototransformation is the presumed mode of removal (average 58% versus 76%,  $\pm 10\%$  and 0.6% standard error, respectively,  $p < 0.05$ ). Duckweeds and algae added together resulted in the most benzotriazole removal, and significantly more than abiotic treatments (average 90%  $\pm 1\%$  standard error,  $p < 0.05$ , [Fig. S2, Table S5](#)). The kinetics of removal also varied, with some microcosms reaching a lower concentration of benzotriazole earlier. Microcosms removed benzotriazole faster when duckweeds were inoculated with *Chlorella vulgaris* than when inoculated with their local microbiome (half-lives and 95% CI 3.9, 3.1–5.0 d and 10, 6.2–12 d, respectively). Microbial communities without duckweeds removed benzotriazole no faster than abiotic controls (half-lives and 95% CI 20, 8.2–30 and 15, 7.9–21 d, respectively, difference n.s., [Fig. S2, Table S5](#)).

## 4. Discussion

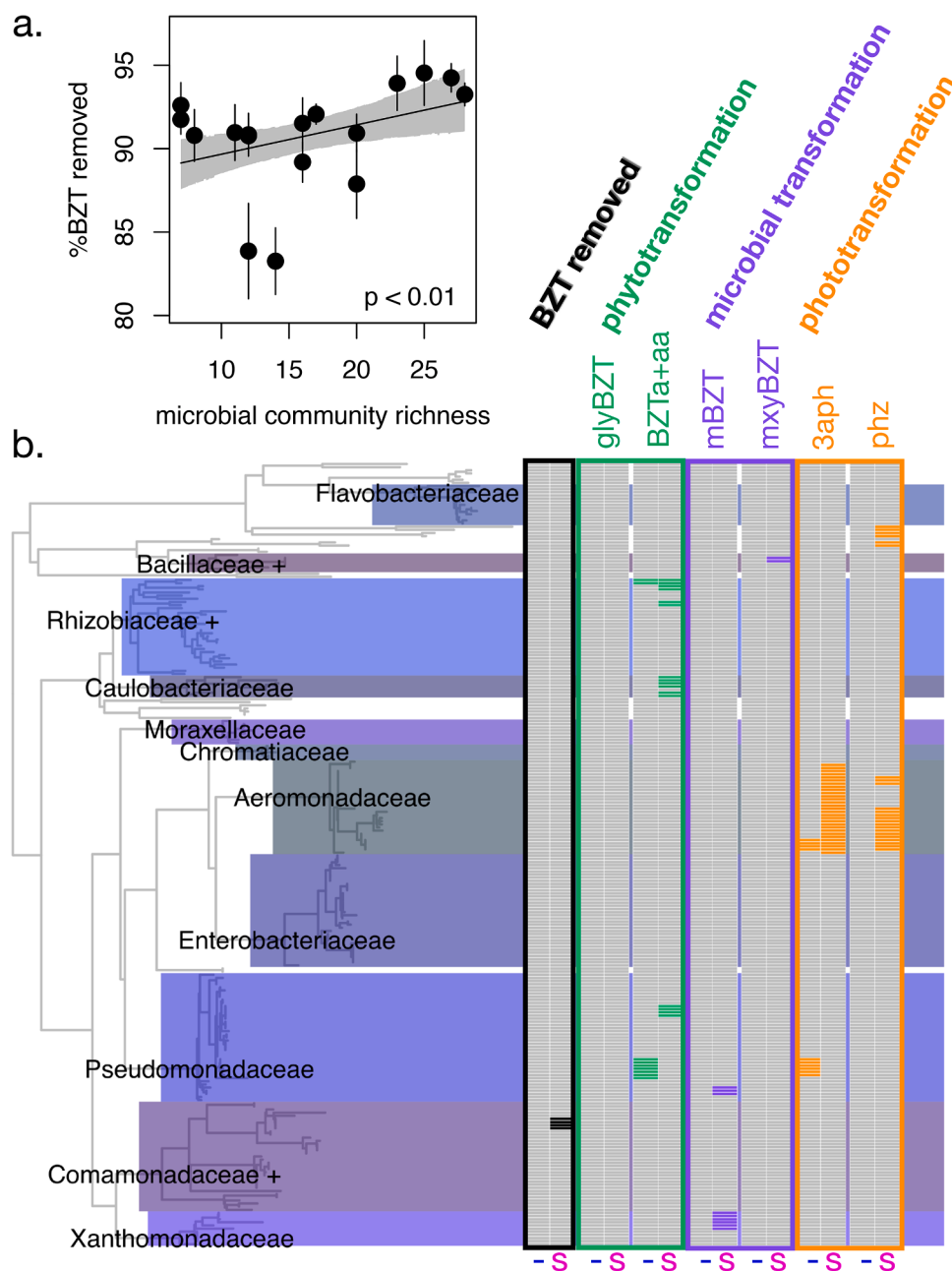
Rapid, continued urbanization brings potentially harmful consequences, such as release of anthropogenic contaminants into stormwater ([Masoner et al., 2019](#)), and makes leveraging services that can be provided by urban ecosystems, such as bioremediation in wetlands, more critical than ever ([Elmqvist et al., 2015; Johnson and Munshi-South, 2017](#)). Here, we observed 73–99% of the common contaminant benzotriazole was transformed in microcosms with a model wetland plant, duckweed, and its associated microbes. Transformation occurred in 1.5 weeks via phyto-, microbial, or photo- transformation, substantially faster than previously reported with microbes ([Table S5, Herzog et al.,](#)

[2014; Liu et al., 2011a](#), except in activated sludge, [Huntscha et al., 2014](#)). This supports the use of plants (like duckweed) in nature-based water treatment systems such as constructed wetlands and stormwater ponds, to enhance benzotriazole transformation. All measured and manipulated aspects of microcosms affected benzotriazole fate, indicating that optimizing plants, microbes and environmental conditions could enhance benzotriazole removal and minimize toxic transformation products.

Duckweed growth positively correlated with the amount of phytotransformation products in microcosms (glycosylated benzotriazole, [Fig. 1, Table S6](#)). [LeFevre et al. \(2015\)](#) first observed benzotriazole phytotransformation products in *Arabidopsis thaliana*, though other plants can generate the same products ([Gu et al., 2021; LeFevre et al., 2017; Pritchard et al., 2018](#)). [LeFevre et al. \(2015\)](#) suggested that plants may form benzotriazole acetyl-alanine and benzotriazole alanine by converting benzotriazole into an analog of the plant growth hormone auxin, and processing it through auxin pathways. Glycosylated benzotriazole could conceivably also form from the plant auxin pathway, which includes glycosylation ([Lim and Bowles, 2004](#)), but a separate detoxification pathway may be more likely ([LeFevre et al., 2015](#)).

Microbial growth in our microcosms was linked to more than just microbial transformation products ([Fig. 1, Table S6](#), photo- and phytotransformation). Microbes can degrade a wide array of compounds, and higher microbial growth can allow product biodegradation rates to exceed formation rates from benzotriazole (as found for 5-methylbenzotriazole, [Liu et al., 2011a](#)). Similar differences in formation and biodegradation rates could explain negative correlations between microbial growth and both microbial and phototransformation products in microcosms ([Fig. 1, Table S6](#)). Microbes can also indirectly affect auxin regulation in plants via their effects on plant nutrition ([Lu et al., 2018](#)), and many microbes can synthesize auxin via pathways overlapping with or dissimilar to those of plants ([Spaepen et al., 2007](#)), including some duckweed-associated microbes ([Gilbert et al., 2018](#)). Both mechanisms could affect benzotriazole phytotransformation pathways by augmenting plant contributions, or diverting benzotriazole from phytotransformation. Indeed, in the absence of their duckweed hosts, duckweed-associated microbes may not transform benzotriazole at appreciable rates (95% confidence intervals for kinetic constant largely overlaps abiotic control, [Fig. S2, Table S5](#)).

Salt proved to be a critical background context for benzotriazole fate. The increased removal of benzotriazole when salt was added is promising given that benzotriazole and salt are common winter co-contaminants ([Parajulee et al., 2017](#)). However, it is unusual:



**Fig. 3.** Association between aspects of microbial communities and benzotriazole transformation. (a) Relationship between amount of benzotriazole removed and increasing microbial inoculum community richness (corrected for sampling depth), with model fitted prediction (line), 95% HPDI (shaded region), means (points, across both salt treatments) and  $\pm 1$  standard error of the mean (vertical bars). (b) Taxa (amplicon sequence variants, ASVs) associated with removal of benzotriazole or a transformation product. Color other than gray indicates an ASV where there is a stronger association with a transformation product than expected by chance (see Methods, Text A.1). Each response variable has one column for each salt treatment: 0 (left, '-') and 0.8 g/L (right, 'S') NaCl. All include data from treatment with benzotriazole and inoculation with local microbes. Colors separate transformation pathways. Abbreviations: BZT=benzotriazole; glyBZT = glycosylated benzotriazole; BZTa+aa = the sum of benzotriazole alanine and benzotriazole acetyl-alanine; mBZT = 1-methylbenzotriazole; mxyBZT = methoxybenzotriazole; 3aph = 3-aminophenol; phz = phenazine.

increased salinity more often decreases biotransformation of organic contaminants (e.g. Jing et al., 2018; Okpokwasili and Odokuma, 1990). Further, salt shifted which biotype (source site of duckweeds and microbes) we would suggest deploying in constructed wetlands to maximize benzotriazole removal or transformation through a particular pathway (crossing lines, Fig. 1, 2<sup>nd</sup> column).

One explanation for variation in benzotriazole effects, removal, and transformation in microcosms with different biotypes is that those from urban sites may have adapted to contaminants. Consistently polluted sites often elicit evolutionary responses in organisms (e.g., Whitehead et al., 2012), and salt and benzotriazole occur at the levels we used in our experiments only in urban areas (Division, 2016; Lawson et al., 2019; Parajulee et al., 2017). However, benzotriazole and salt equally decreased growth in urban and rural duckweeds, and salt did not affect local microbes—suggesting that the duckweeds and microbes we studied are not locally adapted to benzotriazole and salt runoff (Fig. 2, Tables S7, S9). Yet adaptation may still play a role: urban duckweeds

grew faster than rural duckweeds (Fig. 2, Table S7), and faster or earlier growth has been linked to plant adaptation to urban environments (Gorton et al., 2018; Santangelo et al., 2020). In contrast, urban duckweeds supported less microbial growth (Fig. 2, S9). This pattern could be explained either by contaminant stressors in urban sites favoring stress-tolerant microbes (usually slower-growing, Moreno-Gómez et al., 2020; Zakrzewska et al., 2011), or by adaptation in urban duckweeds to inhibit the growth of microbes. Indeed, increased duckweed growth in local relative to disrupted microbiome treatments across source sites suggests a role of local adaptation between duckweeds and microbes (Fig. 2). Regardless of the mechanism, consistent differences from urban to rural source sites suggest deterministic biological processes underlie the decreased transformation of benzotriazole by urban duckweeds.

Relationships between community diversity and community function permeate ecological systems, including microbiomes (Saleem et al., 2019; Schulze and Mooney, 2012). Benzotriazole removal was greatest in duckweeds inoculated with microbial communities of high taxonomic

richness (Fig. 3a), as has been observed for other contaminants (Lv et al., 2017; Zhang et al., 2018). However, ecosystem and microbiome function can sometimes be driven more by individual taxonomic groups than by diversity (Finkel et al., 2020; Ruppelt et al., 2020; Schulze and Mooney, 2012). Indeed, the relative abundance of certain groups was linked to different benzotriazole transformation pathways (Fig. 3b). Specifically, relative abundance of genera within the *Aeromonadaceae* correlated with phototransformation products (*Aeromonas* versus *Tolomonas*, Figs. 3b, S7, Table S13), and microbial communities with greater abundance of *Pseudomonadaceae* had more microbial transformation products (Figs. 3b and S7, Table S13). Although this analysis is exploratory, we suggest that duckweed microbiome engineering efforts to increase transformation of benzotriazole into less toxic products should focus on manipulating members of these clades, e.g. in decreasing the relative abundance of *Aeromonas* relative to *Tolomonas*. Such efforts could also explore benzotriazole degrading functions in uncultured fractions of duckweed microbiomes, and leverage approaches to identify or enrich organisms with contaminant degrading functions (see Duarte et al., 2001).

Transformation of contaminants in the environment does not always decrease toxicity (Lundstedt et al., 2007; Tian et al., 2021). Because benzotriazole was still the most abundant compound, its relative removal drove estimated total ECOSAR toxicity. Biotypes from rural sites removed the most benzotriazole, and ECOSAR suggested they would most decrease toxicity (Fig. 1, Table S11). Yet, benzotriazole was not the most toxic compound. If the rapid transformation of benzotriazole in duckweed-microbe systems briefly pushes more toxic compounds to high levels, urban sources for duckweeds and microbes that divert benzotriazole to phytotransformation may be preferred (Figs. 1, S2, Tables S5, S6). One caution to concluding that phytotransformation decreases toxicity is that transformation products of organic contaminants can sometimes undergo product-to-parent reversion in both biological and phototransformation pathways (e.g., Fu et al., 2017; Qu et al., 2013). The transformation products for which we had authentic standards accounted for 0.30–0.37% of the decrease in benzotriazole on average (Table S14, including products potentially arising from multiple transformation pathways), but accounted for a greater proportion when benzotriazole or salt was applied at higher levels (up to 10%; see Text S2). This implies that further transformation of these products (Chen et al., 2018; Liu et al., 2011a) or other transformation products (Huntscha et al., 2014; Llorca et al., 2017) could account for the balance.

Engineering plant-microbe biotypes for constructed wetlands will be effective only if manipulations have measurable effects. We experimentally verified that manipulating microbes can change benzotriazole fate with an example microbe: when *Chlorella vulgaris* was added to the kinetics experiment, more benzotriazole was transformed, and it was transformed at a faster rate (Fig. S2, Table S5). Indeed, duckweeds float on the water surface, limiting the proportion of contaminating benzotriazole that they may interact with, while algae such as *Chlorella vulgaris* may enhance functioning of constructed wetlands by colonizing more of the water column (Park et al., 2018; Renuka et al., 2015). While microcosms are ideal for high-throughput comparisons, considering the potential for similar complimentary spatial zones may be especially important when implementing treatments at the *in situ* scale.

## 5. Conclusions

Here, we quantified effects of physical and biotic context on biotransformation outcomes for a model stormwater contaminant, and demonstrated that these factors offer substantial variation to consider when constructing urban wetlands for bioremediation. In high-throughput microcosm scale experiments, our model plant (duckweed, *Lemna minor*) rapidly transformed our model contaminant (benzotriazole). Certain manipulations of variation in duckweed and its associated microbial communities increased benzotriazole transformation: duckweeds from rural sites, hosting more diverse communities with

fewer *Aeromonas*, or with added algae, maximized bioremediation potential. Our workflow exhibits how harnessing natural or augmented variation in plants and their microbiomes could improve bioremediation of contaminated urban stormwater. Our results indicated a role for evolution in outcomes, thus evaluating responses to selection and long-term trajectories of engineered urban ecosystems will be a critical avenue for future research.

## Data Accessibility

Data including raw sequence files and code are on figshare at <https://doi.org/10.6084/m9.figshare.20311758>. Code and some data are available at <https://github.com/amob/benzotriazole-microcosms>.

## Author contributions

AMO, ZHY, MEF, and EP conceived of the idea and designed experiments. AMO, ZHY, and CP conducted experiments and collected data. GHL synthesized chemical standards. AMO and ZHY analyzed the data, with MEF, EP, and GHL advising. AMO and ZHY provided the first draft of the manuscript, and AMO, ZHY, MEF, EP, and GHL edited the manuscript. All authors approved the submitted manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.watres.2022.118926](https://doi.org/10.1016/j.watres.2022.118926).

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