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The Impacts of Anthropogenic Chemicals on the Soil Microbiome Associated With Crop Plants and how Plant Growth Promoting Rhizobacteria can Help Plants Cope With Stress

A Dissertation submitted in partial satisfaction
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in

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by

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ABSTRACT OF THE DISSERTATION

The Impacts of Anthropogenic Chemicals on the Soil Microbiome Associated With Crop Plants and how Plant Growth Promoting Rhizobacteria can Help Plants Cope With Stress

by

Nathan Kenneth McLain

Doctor of Philosophy, Graduate Program in Microbiology
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Maintaining efficient plant yields is critical to protect agricultural output levels that can support the global population. Unfortunately, crop yields can be devastated by drought conditions, pathogen attacks, and abnormal root growth due to stress. Recycled wastewater (RWW) can be employed to alleviate drought conditions, however, this source of water can deliver a set of chemicals referred to as chemicals of emerging concern (CECs). These compounds can have deleterious effects on the soil microbiome associated with the crop plants, thus reducing crop yields. Pathogens can be addressed with the application of fungicidal agents, but these often have off target effects that impact the soil microbiome. Plant growth promoting rhizobacteria (PGPR) can interact with plants through root development pathways and ensure that plant root development continues or is altered to cope with the stressful conditions. In our studies we examined how CECs and off target effects from fungicidal agents impact the soil microbiome associated with plants. We also examined how the PGPR *Bradyrhizobium japonicum*

interacts with plants to facilitate root development. Overall the results suggest that CECs and off-target effects of fungicidal agents can impact the soil microbiome and alter their community functions. It was also observed that PGPRs were able to facilitate root development through auxin transport pathways.

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INTRODUCTION

Water scarcity and supply issues are some of the most critical problems of the 21st century. Water shortages are a multifaceted issue that are connected to and drive many others (Corwin 2021; Howitt et al. 2015). As less water becomes available natural and artificial aquifers are reduced. Excess draining of these reservoirs have adverse impacts on the aquatic environment, their associated upland environments, and all the ecotones in between that depend on the water source. Drought conditions are major stressors for natural and agriculture plants (Lal et al. 2013; Corwin 2021), as well as their associated soil microbial communities (Geng et al. 2015). Droughts cause the loss of or reduce the yield of many crops every year (Seleiman et al. 2021; Shakeel et al. 2011). Additionally, biotic stress from plant pathogens can cause great harm to plants, while plants subjected to abiotic stress can be more susceptible to pathogen attack. (Schoeneweiss 1975). Crops can be treated with fungicidal agents to deter pathogens, however off-target effects to the microbial community associated with the plants has been observed. Some of these impacts include altering the growth of growth promoting rhizobacteria (PGPR) (Mubeen et al. 2006; Gallori et al. 1991) and altering nitrogen cycling carried out by soil microorganisms (Mikael Pell et al. 1998; Gallori et al. 1991).

Conservation efforts have reduced the amounts of water being used from natural and artificial aquifers. These efforts can only stretch available water supplies so far and alone may not be sufficient to offset the growing demand for clean water (Moglia et al. 2018; Grant et al. 2012). To further protect clean water supplies, the use of recycled waste

water (RWW, aka reclaimed water) has been adopted for many uses (Warsinger et al. 2018; Parsons et al. 2010; Schulte 2011). Normally, wastewater is treated at a basal level and allowed to return to the natural water cycle, however, wastewater can also be treated to a greater extent and used to supplement potable water use as reclaimed water (Warsinger et al. 2018; see figure 1 in chapter 1). Potable water supplies can also be replenished using RWW by allowing it to mix with potable water reservoirs (Warsinger et al. 2018).

In many arid regions, such as Southern California, the majority of RWW is used for the irrigation of crops and landscapes (Warsinger et al. 2018; Schulte 2011). Overall the act of using RWW greatly increases the supply of usable, clean water and preserves potable water supplies for direct human consumption (Parsons et al. 2010; Schulte 2011; Cooley and Phurisamban 2016). Many regions have planned to begin implementing or expanding upon RWW systems due to its ability to have a significant impact on water conservation outcomes (Parsons et al., 2010; Schulte, 2011; Cooley and Phurisamban, 2016; Warsinger et al., 2018). Therefore, RWW use will most likely continue and be expanded upon in the future.

The technology for cleaning water for recycled use is primarily focused on the removal of biological, disease-causing elements (Kolpin et al. 2002; Kinney et al. 2006; Batt et al. 2007; Gros et al. 2010) and are less efficient at the removal of a wide range of chemicals collectively referred to as contaminants of emerging concern (CECs, aka chemicals of

emerging concern in some sources). CECs are compounds ranging from pharmaceutical products, personal care products, detergents, nanoparticles, and etc. that are delivered directly to environments in which RWW is used, and indirectly to neighboring environments (Kinney et al. 2006; Gros et al. 2010; Epa 2019). These compounds have also been observed to accumulate and increase in concentration in soils and plants that are irrigated with RWW (Kinney et al. 2006; Boxall et al. 2006; Wu et al. 2013; Bartha et al. 2010). The presence of these compounds act as anthropogenic disturbances and have been observed to impact insects, plants, the soil microbiome, and even plant microbe interactions in which plant health is intimately related to (Wang and Gunsch 2011; Barra Caracciolo et al. 2015; Pennington et al. 2017; Pennington et al. 2018; Christou et al. 2018). The increase to plant stress from the presence of these chemicals could also exacerbate soil plant diseases caused by soil borne plant pathogens, therefore disease severity of *Verticillium* wilt disease was evaluated in eggplants irrigated with a select set of CECs (acetaminophen (APAP), trimethoprim, (TMP), sulfamethoxazole (SMX), and gemfibrozil (GEM)).

Since APAP and TMP are found commonly in many different recycled waste water sources, these compounds were evaluated further to determine if they impact the soil microbiome associated with *Solanum melongena* (eggplants) (Kolpin et al. 2002; Gros et al. 2010; Sim et al. 2011; Fram and Belitz 2011; Li et al. 2014). APAP has been observed to be concentrated in the soil by 604% up to 4,860% of the amount found in the RWW source used for irrigation in a given field (Kinney et al. 2006). APAP can be broken

down into a glycoside by soil fungi or plants (Bartha et al. 2010; Huang et al. 2006; Huber et al. 2009). APAP can also be broken down into a carboxylic acid by soil microbes (Li et al. 2014). These breakdown products, or even APAP itself, may be able to act as a carbon source and select for microbes that can utilize them. Since it has been previously observed that APAP can impact soil microbial communities directly, we evaluated their impacts to the soil microbial community associated with the agriculture plant, *S melongena*. Shifts in the microbial community may lead to suboptimal community structure and functions leading to deleterious effects on the associated plant community, such as decreasing crop yields (Bais et al. 2006; Van der Heijden et al. 2008; Berendsen et al. 2012).

Evaluation of fungicidal agents on the soil microbial community

We examined the impact of the fungicidal agents Ridomil Gold (a chemical fungicide) and SoilGard (a biofungicide) on the soil microbial community associated with carrots. These are two industry standard fungicides used to treat carrots to protect them from *Pythium*, but they may cause off target effects to microbes present in the soil, thus altering the microbial community (Zhang et al. 2021; Gasoni et al. 2008; Ma et al. 2021; Al-Assiuty et al. 2014; Abbey et al. 2019). The impacted soil community may arrive at a new community structure with different functions that are not optimal for carrot growth and production (Barra Caracciolo et al. 2015). Therefore we evaluated how the use of a biofungicide or a chemical fungicide could alter the microbial community associated with plants.

Bradyrhizobium japonicum IRAT FA3 alters Arabidopsis thalana root architecture – a collaboration on Schoreder et al., 2022

In addition, normal plant root development is impacted by factors such as drought or salinity stress (Galvan-Ampudia and Testerink 2011; Gupta et al. 2020)**.** Abnormal root development can have detrimental impacts to plant health and plant yield (Gupta et al. 2020). Auxin is the major plant hormone involved with healthy, normal root development (Overvoorde et al. 2010; Hodge et al. 2009; Du and 2018). In order for this hormone to function correctly however, it needs to be correctly and accurately transported to specific plant tissues at specific times (Overvoorde et al. 2010; Du and Scheres 2018). Thus interference to auxin transport will disrupt normal root development in plants. However, a group of plant beneficial bacteria, referred to as plant growth promoting rhizobacteria (PGPR), have been observed to stimulate the expression of auxin transport genes when they are associated with a given host (Shi et al. 2010; Spaepen et al. 2007). Therefore, we evaluated auxin expression in *Arabidopsis* plants inoculated with the PGPR *Bradyrhizbioum japonicum* in order to elucidate the microbes specific influence on auxin expression and transportation in root cells.

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CHAPTER 1

Chemicals of Emerging Concern in treated wastewater impact microbial growth

The works used in this chapter were previously published in the journal Frontiers in Environmental Science (McLain and Gachomo 2019)

Abstract

Agriculture production in California is negatively impacted by soilborne fungi, such as *Verticillium dahliae*, and limited water availability for irrigation. Some regions have adapted the use of recycling wastewater, i.e., reclaimed water, to supplement the potable water supply. Wastewater purification is not fully efficient at removing all contaminants and small amounts of pharmaceutical products, known as chemicals of emerging concern (CECs), remain. Acetaminophen, trimethoprim, sulfamethoxazole and gemfibrozil are some of the most common CECs found in treated wastewater and were therefore used in this study. These CECs were evaluated for their potential to interact with microorganisms directly, or for their ability to alter the development of Verticillium wilt disease in eggplants. The microorganisms *Verticillium dahliae*, *Fusarium oxysporum* f. sp. *lycopersici*, *Piriformospora indica*, *Phytopthora capsici*, and *Bradyrhizobium japonicum* were used for in vitro growth assays in the presence of CECs. CECs induced varying responses in strains of the same fungi by promoting growth of one strain while inhibiting growth of the other. CECs influenced spore germination of *V. dahliae* and *F. oxysporum*. Greenhouse experiments in which *Solanum melongena* (eggplants) were inoculated with *V. dahliae* and irrigated with CECs were used to evaluate the impacts of these chemicals on disease development. Overall, our results found that most of the organisms we tested were sensitive to the CECs. *P. capsici* was found to be the most

sensitive microorganism, while *B. japonicum* growth was unaffected by the CECs at the concentrations used. The greenhouse assays results indicated that plant disease severity may be influenced by given CECs at certain stages of plant growth. Overall, the results of this study indicate that the concentrations of CECs found in reclaimed water are occurring at biologically relevant concentrations.

Introduction

Extended periods of drought and the presence of soilborne plant pathogens jeopardize California's agricultural production, which contributes a significant portion to the United States food supply. Recent drought conditions and continued population growth in Southern California have place great demands on the region's limited potable water supply (AghaKouchak et al., 2015; Howitt et al., 2015). This valuable resource is consumed by the population directly or indirectly through irrigation of crops and landscape vegetation. Since potable water is in such high demand, California has taken steps to conserve this limited resource. Recycling wastewater has been successfully employed to protect the supply of potable water to satiate the growing demands for clean water (Parsons et al., 2010; Schulte, 2011; Cooley and Phurisamban, 2016; Warsinger et al., 2018). Normally wastewater is treated to a limited capacity and allowed to exit the potable water supply and re-enter the natural water cycle. Alternatively, wastewater can be treated to a far greater extent, and used to supplement potable water supplies as reclaimed water (Figure S1; Warsinger et al., 2018). Reclaimed water can be used to recharge potable water supplies directly by injecting it into ground water reservoirs, or

allowing it to mix with potable water aquifers (Warsinger et al., 2018). Reclaimed water can also be used to irrigate crops or landscape vegetation, thereby preserving potable water supplies for direct human consumption (Warsinger et al., 2018).

Chemicals of emerging concern (CECs) such as pharmaceuticals, personal care products, detergents, nanoparticles, etc. continue to be introduced in the environment, and have potential to impact human and aquatic life that would otherwise not be exposed to them (Kinney et al., 2006; Gros et al., 2010; EPA, 2019) Unfortunately, even after extensive treatment, CECs remain in the treated water (Kinney et al., 2006; Gros et al., 2010). The past consensus with these chemicals is that they do not pose much danger for human and environmental health since they occur in such low concentrations, and in the case of pharmaceuticals, well below therapeutic doses used for humans (Boxall et al., 2006; Wu et al., 2013; Zimmermann and Curtis, 2017). However, this notion has been eroded by the observations that sub-therapeutic concentrations of certain pharmaceuticals can impact microbial, plant, and insect life (Wang and Gunsch, 2011; Pennington et al., 2017, 2018). Additionally, some CECs have been observed to accumulate in soils upon repeated irrigation with reclaimed water containing the compounds (Wu et al., 2015). Therefore, the concentrations of CECs found in reclaimed water can be at biologically relevant concentrations or can be raised to that level with repeated irrigation or in the presence of chemicals that can increase their potency.

Given that CECs accumulate in plants (Wu et al., 2015), they may impact plant physiology, potentially altering a plants ability to interact with microorganisms (pathogenic or beneficial). Exacerbation of already prevalent soilborne fungi (or plant pathogens), and consequently plant diseases, found in agricultural soils is one potential outcome of chemical interference from the CECs that can impact plants, and/or the microbial community that is associated with plants. CECs may interact with soil microbes directly by stimulating or inhibiting their growth, thus impacting their ability to colonize or infect a host. CECs may alter microbial population equilibrium by promoting some, while inhibiting others and can thereby increase pathogen populations by reducing their competitors or antagonist in the soil (Mulligan et al., 1982; De Vries-Hospers et al., 1991; Azevedo et al., 2015).

Verticillium dahliae, the causative agent of Verticillium wilt, is a soilborne pathogen that may benefit from the anthropogenic inputs of CECs from reclaimed water. *V. dahliae* has a wide host range for many crops that are important to California's agriculture industry including bell peppers, eggplants, strawberries, tomatoes, and watermelon (Pegg, 1984; Aguiar et al., 1998; Bhat and Subbarao, 1999; Klosterman et al., 2009). California leads the nation in the production of the above-mentioned crops (California Department of Food Agriculture, 2018) and major reductions in their yields could have devastating consequences to food supply and income. Plant diseases already reduce California's agriculture output and increase cost of production, and they may be exacerbated by anthropogenic input of CECs into agriculture soils.

Acetaminophen (APAP), trimethoprim (TMP), sulfamethoxazole (SMX), and gemfibrozil (GEM) are synthetic pharmaceutical products that are commonly found in recycled wastewater. APAP is an antipyretic pain killer that has been observed to have antimicrobial properties at concentrations above therapeutic use (Zimmermann and Curtis, 2017), and observed to have growth promoting effects on microorganism at therapeutic concentrations (Carvalho et al., 2010). TMP and SMX are antibiotics that are commonly taken in combination, but exhibit antimicrobial effects independently (Reeves and Wilkinson, 1979; Hida et al., 2005; Tunali et al., 2012). GEM is a medication used to treat high blood pressure, but has been observed to increase the antimicrobial potency of some pharmaceuticals (Rudin et al., 1992; Bulatova and Darwish, 2008) and to have direct impacts on plant growth (D'Abrosca et al., 2008; Pino et al., 2016). These four CECs are found consistently in wastewater treatment plant effluent such as reclaimed water, and in soils irrigated with recycled water in the ng/L and μg/L range (Kinney et al., 2006; Batt et al., 2007; Erickson et al., 2014; Wu et al., 2015).

Extended periods of drought conditions are common in arid regions such as Southern California, which makes water conservation efforts such as reclaimed water use a necessity (Brown et al., 2013). Despite the presence of chemicals remaining in the treated wastewater, reclaimed water has done much to alleviate water demands and California plans to increase its use (California State Water Resources Control Board, 2010). Therefore, it is critical for us to understand the direct impacts CECs have on plants and

their associated soil microbiome to prevent elevation of plant losses from microorganisms. In our study we subject plants and microorganisms to concentrations similar to those found in treated wastewater effluent, or reclaimed water. A number of previous studies have found direct phytotoxic effects of various CECs, however these studies used concentrations that are higher than those found in typically treated wastewater effluent (D'Abrosca et al., 2008; Liu et al., 2009a; Pino et al., 2016; Madikizela et al., 2018). Some studies found phytotoxic effects to developing plants, but did not grow the plants in soil (D'Abrosca et al., 2008; Pino et al., 2016), which can impact plants uptake of chemicals through the roots (Pan and Chu, 2017). Use of higher concentrations of CECs and not growing the plants in soil means that the plants are exposed to unusually high concentrations of the compounds. This under states the potential severity of microbial community disturbances and phototoxic effects of *in situ* CEC concentrations found in recycled water. Therefore, the direct impacts of CECs on eggplants grown in soil were tested using concentrations within the range of CECs detected in recycled water or wastewater treatment plant effluent. The impacts of CECs on the growth of microorganisms—fungi, oomycetes, and bacteria that are known to be beneficial or deleterious to plants were also tested using CECs concentrations that are relative to *in situ* concentrations. We hypothesized that the CECs will not impact microbial growth, since we are using relatively low concentrations of CECs that are well below therapeutic doses. We also hypothesized that CECs would not have any impact on disease development in eggplants inoculated with the plant pathogen *Verticillium*

dahliae. Therefore, objective of this study was to determine the impact of CECs on microbial growth and disease development in eggplants.

Methods

Chemicals of emerging concern treatments

In total 4 CEC's -acetaminophen (APAP), trimethoprim (TMP), sulfamethoxazole (SMX), and gemfibrozil (GEM)—were evaluated to determine their impacts on the microbial growth and development of Verticillium wilt in eggplants. Each CEC was tested at a high concentration (H) and a low concentration (L) as specified in Table 1 below. The concentrations used mimic the range of concentrations of the respective CECs that have been found in the final effluent of wastewater treatment plants or in soils irrigated with reclaimed water (Vanderford and Snyder, 2006; Batt et al., 2007; Stackelberg et al., 2007; Dia-Cruz and Barcelo, 2008; Fram and Belitz, 2011). These compounds at the concentrations listed in Table 1 were used in all assays described below.

Media preparation

Czapek-dox, buffered 10% V-8, potato dextrose agar (PDA), and Luria Bertani (LB) broth were used to cultivate the different microorganisms. Czapek-Dox agar was prepared as the “originally proposed” version described in Thom (1930) and Smith (1941). Briefly, 30.0 g of sucrose, 3.0 g of sodium nitrate, 1.0 g of dipotassium phosphate, 0.5 g of magnesium sulfate, 0.5 g of potassium chloride, and 0.01 g of ferrous

sulfate per 1 L of sterile ddH_2O were used. The buffered 10% V-8 agar was made by adding 10% volume of V8 juice and 0.2 % (W/V) of calcium carbonate to sterile ddH_2O. The V8 calcium carbonate solution was clarified by centrifuging it at 3,000 × g for 10 min, and the supernatant was added to the required amount of ddH_2O to make a final concentration of 10% V8. To make solid media for petri dishes, 10% agar (W/V) was added. LB broth and PDA were prepared based on the manufacturer's instructions. PDA amended with 0.833 μM of bromocresol purple sodium salt (BCP) for a growth assay described below (Masachis et al., 2016).

Cultivation of organisms

The impacts of CECs on the growth of several microorganisms were tested. Organisms covering a wide range of classifications and common plant pathogens in regions where reclaimed water is used were also included in the study. Two *Verticillium dahliae* strains (0048 and 0049), two *Fusarium oxysporum lycopersici* strains (CS-3 and CS-5), *Phytophthora capsici*, *Piriformospora indica*, and *Bradyrhizobium japonicum* IRAT FA3 were tested. Two strains of *Verticillium* and *Fusarium* were used to assess the impacts of CECs on closely related organisms. *V. dahliae* strains were cultured on Czapek-Dox plates with 1% agar, *F. oxysporum* and *P. indica* were cultured on PDA and *B. japonicum* was cultured in LB broth. During growth rate assays, each organism was grown on their respective culturing media. The media was amended with the required volume of a given CEC working stock solution to reach the concentrations indicated in Table 1, after the base media was autoclaved and cooled, but before solidifying. An additional treatment

containing no CECs was used as a control for each organism. Two strains of *V. dahliae* and *F. oxysporum* were tested to find out the response of closely related organism to the same CECs.

Verification of organism identity

DNA from *V. dahliae* and *F. oxysporum* f. sp. *lycopersici* strains were extracted using the DNeasy Plant Mini Kit (Qiagen) following the manufacture's protocol. Extracted DNA was used in PCR assays to confirm the identity of the fungal isolates used in this study. *V. dahliae* isolates were confirmed following the procedure and commonly used primers described in Inderbitzin et al. (2013). Briefly, each PCR reactions consisted of 12.5 µL of 2 × Dream taq green master mix (Thermo Scientific), 1 µL of forward and reverse primer, 1 µL of extracted DNA (i.e., DNA template), and 9.5 µL of nuclease free water. All primers used in this assay were purchased from Integrated DNA Technologies. PCR reactions were carried out in a thermocycler with an initial denaturation step for 2 min (min) at 94°C. This was followed by 35 cycles of denaturation 94°C for 10 s (s), Annealing for 20 s, and elongation for 1 min at 72°C. Then, a final elongation step for 7 min at 72°C was used (Figure S2). Identification of *F. oxysporum* f. sp. *lycopersici* was confirmed in a similar fashion using a PCR approach described in Hirano and Arie (2006). The same PCR reaction mixture described above for identifying *V. dahliae*. To conduct the PCR, the mixtures went through 50 cycles of denaturation at 94°C for 1 min, Annealing at 62°C for 1 min, and elongation at 72 °C for 2 min. The *F. oxysporum* sample was screen using the Unif/r, Sp13f/r, Sp23f/r, and the sprlf/r primer sets described

in Hirano and Arie (2006) to positively identify the *F. oxysporum* strains used in this study (Figure S2).

Growth rate assay

Every fungal and oomycete growth assay had 5 replicates for each treatment, while bacterial growth assays had 3 replicates. For fungal organisms, a 5 mm diameter plug taken from the edge of an actively growing colony was placed in the center of the agar plate. Growth of the organism was monitored by measuring the diameter across the colony. Two diameter measurements were taken from two different angles and averaged together per replicate *V. dahliae* strains were measured 3, 7, 14, and 21 days post inoculation (dpi). *F. oxysporum* strains were measured 3, 4, 5, 6, and 7 dpi, *P. indica* was measured 3, 7, 11, 12, 14 dpi, and *P. capsici* was measured 1, 2, 3, and 4 dpi (Figures 1A–C). A growth assay carried out on PDA plates amended with the pH indicator bromocresol purple was used to detect if nutrients were consumed beyond the growing mycelium tips in the solid media (Figure 1A).

Cultivation of eggplants

The Patio Baby variety of eggplants from Johnny's seeds (Fairfiled, Maine, USA) was used for all eggplants in this study. A group of five eggplants ($n = 5$) were treated with a given CEC at a concentration specified in Table 1. An equivalent group of eggplants was treated in a similar fashion, except they were inoculated with *Verticillium dahliae* strain 0049 during transplanting (discussed below). The eggplant assay was carried out in two

separate sets. Set one (set I) consisted of the acetaminophen, trimethoprim, and associated no CEC control treatments. The sulfamethoxazole, gemfibrozil, and associated no CEC control treatments were carried out on the second set of eggplants (set II). Eggplant seeds were germinated in a growth room at 22°C. All plants were allowed to reach the 2–4 leaf stage before transplanting them into eggplant field soil obtained from farm lands in Bakersfield, California. This field is not irrigated with reclaimed water nor treated with fungicide because it is only used for organic farming. Eggplants were grown in this field, thus the soil was ideal for mimicking *in situ* agricultural soil conditions. During transplanting, the inoculated sets of eggplants were exposed to *V. dahliae* 0049 using the dipping method described by Bhat and Subbarao (1999). Plants were inoculated with 1.85×10^7 spores/ L. Tap water was used for the uninoculated controls. All seeds and plants were watered as needed using tap water for up to 1 week after transplanting to allow them to acclimatize. CEC treatments were applied to plants a week after transplanting. CEC were dissolved into the tap water to reach the final concentration listed in Table 1. The no CEC treatments were watered with tap water only. All treatments received 1L of the solution that contained the respective concentration of CEC per watering event. Watering was done 2–3 times a week as needed. All the plants for a given treatment ($n = 5$) were kept in the same tray. CEC solutions for the respective treatments were poured into the trays to ensure that all plants were receiving equal amounts of the solution (Figure 1D). This experiment was repeated 2 times.

Plant measurements and soil collection

Stem height measurements of plants and disease assessments were taken weekly starting the week of transplantation. Disease assessments relied on observations of external symptoms to avoid destructive sampling. Disease severity assessments were based on a 0–5 scale (Liu et al., 2009b). Briefly: 0 represented no wilted leaves; 1 ≤ 25% wilted leaves; 2 = 25–50% wilted leaves; 3 = 50–75% wilted leaves; 4 = 75–100% wilted leaves; and 5 = dead plant. Upon the final sampling, eggplants were cut at the base of the stem and the fresh weight of shoots was determined for each plant. Disease index was calculated using the disease severity assessment values as described in Liu et al. (2009b)

Statistical analyses

All parametric ANOVA and GLiM statistical analyses were done using SPSS software (ver. 24.0; SPSS; IBM, Somers, New York, USA). For the green house experiments repeated measures ANOVA was done to test for interactions between time and treatments. Repeated measures Friedman Ranks ANOVA were done using R software v. 3.5.3 (R Core Team, 2018) package npIntFactRep (Feys, 2015, 2016) when ANOVA assumptions could not be met. In both approaches, the Greenhouse-Geisser correction was applied when sphericity was not achieved. *Post-hoc* ANOVA were used when significant interactions with time were detected and the data satisfied all ANOVA assumptions. In cases where the assumptions could not be met, the best fitting generalized linear model (GLiM) was used as determined by the model with the lowest Akaike's information criterion (AIC). In all cases this model was found to be the normal

model with identity link function. Tukey was used for all *post-hoc* pairwise analyses when ANOVA was valid. In the cases where GLiM analyses were done, the *post-hoc*, pairwise analyses were carried out using the GLiM model with sequential Bonferroni correction for multiple comparisons. All *post-hoc* pairwise comparisons considered to be significantly different when $P < 0.05$. The two eggplant sets were treated as two separate experiments, in which statistical comparisons were only made within the respective sets. Comparisons to the no CEC controls or uninoculated plants were used to assess the impacts of CECs, disease, or the combination of both. The non-repeated measures ANOVA and GLiM procedures were also used to evaluate treatment effects in microbial growth assays.

Results

Identification of microorganisms

The PCR assay generated results that positively identified the *V. dahliae* and *F. oxysporum* strains. In both cases, positive amplification of their template DNA occurred with primer pairs specific to their respective identities (Figure S2). Specifically, we saw positive amplification for *V. dahliae* with the Df/Dr primer pair only, and positive amplification with the Unif/Unir and Sp13f/Sp13r primer sets for *F. oxysporum* f. sp. *lycopersici* (Hirano and Arie, 2006; Inderbitzin et al., 2013).

Impact of CECs on growth of microorganisms

Overall, colony diameter measurements of organisms grown on solid media demonstrated that the fungi tested were sensitive to most of the CECs used in this study (Figures S3A, S4). This is greatly exemplified with the allelopathic index (RI) calculations that are based on colony growth. The RI calculations indicate that some of these chemicals promoted growth, hindered growth or had no effect on a given organism (Figure 2 and Figure S3B). Positive RI values indicated that an organism's growth was promoted by the CECs, while a negative showed that its growth was inhibited. At 7 days post inoculation (dpi) *V. dahliae* strain 0049 grown with low concentrations of GEM (GEM-L) had a negative RI value that was significantly lower than the control (Figure 3B) (GLiM: χ^2_8 = 44.754, $P < 0.001$). By 14 dpi the inhibitory effects of CECs on *V. dahliae* 0049 growth were more apparent. Five treatments had significantly lower RI values than the control (Figure 3B) (GLiM: $\chi^2_8 = 247.137$, $P < 0.001$). GEM-L and high concentrations of SMX (SMX-H) treatments had the lowest RI values for 0049, which were significantly lower than all other treatments except for the high concentration of TMP (TMP-H) (GLiM: χ^2_8 = 247.137, $P < 0.001$). Colony diameter growth of the other *V. dahliae* strain 0048 (Figure S4A), did not follow the same trends. CECs either benefited or had no effect on *V. dahliae* 0048 mycelial growth. By 7 dpi three treatments had significantly higher RI values than the control (TMP-L, SMX-H, and APAP-H) (GLiM: $\chi^2_8 = 83.749$, $P <$ 0.001). By 14 dpi all treatments for *V. dahliae* 0048 had significantly higher RI values than the control (GLiM: $\chi^2_8 = 76.092$, $P < 0.001$) (Figure 2A).

The other plant pathogens examined, two *F oxysporum* strains, were also sensitive to the CECs tested. However, like *V. dahliae*, the CECs impacted the two *F. oxysporum* strains (CS-5 and CS-3) differently. The CECs appear to either have no impact, inhibit growth for one strain (CS-5), or enhance growth of the other strain (CS-3). By 3 dpi the RI values for all of the CEC treatments for *F. oxysporum* CS-5 were below the control, though, only 6 (APAP-L, TMP-H, TMP-L, SMX-L, GEM-H, and GEM–L) were significantly different (GLiM: $\chi^2_8 = 105.467$, $P < 0.001$) (Figure 3D). This trend continued into the following day at 4 dpi in which all treatments still had lower RI values than the control, though only 4 (APAP-L, TMP-H, GEM-H, and GEM-L) were significant (GLiM: $\chi^2_8 = 82.84$, $P < 0.001$). At this point the faster growing mycelia were approaching the edges of the agar plate. This most likely inhibited their growth, allowing slower growing mycelia to catch up and reduce differences found among treatments. Growth of *F. oxysporum* on PDA plates amended with BCP indicated that the fungal mycelia used up the nutrients in the media beyond the reach of the mycelia. This was shown by a color change in the media before the mycelia occupied it (Figure 1A). Treatment with high concentrations of TMP appeared to have the most dramatic impact on CS-5. At both time points TMP-H had the lowest RI value and at 4 dpi its RI value was significantly lower than all treatments (Figure 2D) (GLiM $\chi^2_8 = 82.84$, $P < 0.001$) except for APAP-L. Despite growth inhibiting effects seen for strain CS-5, growth promoting effects of CECs were observed on CS-3 (Figure 2C and Figure S4B). For instance, by 3 dpi the no CEC control had the smallest colony diameter (Figure S4B). The diameter was significantly smaller than all but two treatments, SMX-H and GEM-H

(GLiM: $\chi^2_8 = 66.502$, $P < 0.01$) (Figure S4B). By the next day at 4 dpi growth approached the edges of the plate and no significant differences in colony sizes were observed among any of the treatments. Growth of the plant pathogen *P. capsici* was inhibited by all of the CECs used in this study. At 2 dpi all CEC treatments had smaller diameter than the control, while only two of these treatments, SMX-L and GEM-H, were not significantly lower (Figure S4E) (ANOVA: $F_8 = 7.496$, $P < 0.001$). The RI values for each treatment were negative and significantly lower than the control (Figure 2E) (ANOVA: $F_8 = 20.796$, $P < 0.001$). The RI values were also much smaller than any of the other organisms tested, indicating that this strain is particularly sensitive. By 3 dpi only TMP-L treatment had a significantly smaller diameter than the control (Figure S4E) (GLiM: $\chi^2_8 = 35.136$, $P < 0.001$). TMP-L and GEM-L treatments had significantly lower RI values than the control at this time point as well (Figure 2E) (GLiM: $\chi^2_8 = 69.872$, $P < 0.001$). Again, this is most likely due to declining growth rates as the colonies approach the edge of the plate allowing the slower growing organisms time to catch up in size.

Testing the impacts of CECs on the growth of the plant symbiont, *Piriformospora indica*, yielded mixed results. In this case there were CECs that either had no impact, inhibited, or enhanced the growth of this organism (Figures S3A,B). At 8 dpi, for example, only one treatment had significantly less growth than the no CEC control, SMX-L (GLiM: $\chi^2_8 = 52.329$, $P < 0.001$), while the rest of the treatments were not significantly different from the control. At 10 dpi only the TMP-H treatment was significantly larger than the no CEC control (GLiM: $\chi^2_8 = 48.169$ $P < 0.001$). Furthermore, the RI values calculated using

colony diameter reinforce these trends (Figure S3B). At 8 dpi both the low and high concentrations of SMX had negative RI values that were below the no CEC control, but only the low concentration was significantly different (GLIM: $\chi^2_8 = 58.997$, $P < 0.001$). At 10 dpi the TMP-H treatment had a significantly higher RI value than the no CEC control, while none of the other treatments are significantly different (GLiM: $\chi^2 = 52.512$, $P < 0.001$). Overall, only two treatments were significantly different than the control, suggesting that this organism was not very sensitive to the CECs being tested.

Another plant beneficial organism, *B. japonicum*, was found to be insensitive to the CECs tested. This plant growth promoting rhizobacteria (PGPR) did not exhibit any signs of sensitivity to the CECs during its growth rate assay carried out in liquid media. *B. japonicum* remained in the exponential growth phase from 0 to about 24 h post inoculation (hpi) (Figures S3C–F). During this time, no significant differences in optical density were detected among different treatments within a given sampling time point. Therefore, it appears that *B. japonicum* is not sensitive to the CECs at the concentrations tested. To confirm this result an additional specific plate count assay of cells at 18 hpi was carried out. There were no significant differences among cell counts from different treatments (ANOVA: $F_8 = 1.203$; $P = 0.36$) (data not shown).

Besides impacting mycelial growth of fungal organisms, CECs impacted spore germination of *V. dahliae* 0049 and *F. oxysporum* CS-5 (Figure 3A). At 12 hpi, the *V. dahliae* no CEC control had significantly greater spore germination than all but two

treatments, APAP-H and SMX-L (GLIM: $\chi^2_8 = 95.648$, $P < 0.01$; *post-hoc* pairwise, $P < 0.05$). TMP-H treatment had the least number of germinated of spores compared to the other treatments (GLIM: $\chi^2_8 = 95.648$, $P < 0.001$). TMP also impacted spore germination of *F. oxysporum* CS-5. Both TMP treatments had lower spore germination than the control. TMP-L had the lowest spore germination out of all treatments and was significantly lower than the control and APAP-L (Figure 3B) (GLiM: $\chi^2_8 = 19.076$, $P = 0.014$).

Impact of CECs on plant growth and development

A greenhouse assay was carried out to evaluate the direct impacts of CECs on plant growth, monitor for changes in Verticillium wilt disease progression and severity using eggplants and *V. dahliae* strain 0049. Overall, the greenhouse assay indicated that the CECs studied did not have major effects on the growth and development of plants. Repeated ANOVA analyses showed that there was a significant interaction between time and treatments for stem heights. (set I: $F_{25.137,\ 111.721} = 3.901$, $P \leq 0.001$; set II: $F_{33.43,\ 148.577} = 9.704$, $P \leq 0.001$). As expected though, inoculation with *V. dahliae* proved to have drastic effects on plant growth. Major differences in stem height and other plant metrics discussed below occurred between inoculated and uninoculated eggplants. No significant differences in stem height occurred among plants treated with different CECs, or a different concentration of the same CEC within the set of inoculated or uninoculated plants ($P > 0.05$ for all *post hoc* comparisons). However, all of the inoculated plants had

significantly lower stem height than their uninoculated counter parts by week 6 and later (*P*'s < 0.05 for all *post hoc* tests) (Figures 1D, 4A–D).

Not surprisingly, a similar trend is revealed when measuring the number of leaves retained by the eggplants during the growing season (Figure 5A). There was a significant interaction between time and treatment for leaves remaining on plants (repeated measures Friedman Ranks ANOVA: setI: $F_{32.856, 146.027} = 2.207$, $P \leq 0.001$; set II: $F_{38.308, 170.257} = 8.825$, $P \leq 0.001$). Inoculation with *V. dahliae* led to higher leaf loss than the uninoculated controls in all treatments (Figures 1D, 5A). From the 6th week to the end of the experiment all of the inoculated plants retained significantly less leaves than their uninoculated counterparts (All *P*'s < 0.005). Some differences with leaf retention did occur between treatments and their respective controls, but only with inoculated plants. These differences occurred in weeks 4 (inoc APAP-H, inoc TMX-H, inoc SMX-L, and inoc GEM-L), 5 (inoc APAP-H and inoc SMX-L), 6 (inoc SMX-H), and 8 (inoc APAP-H) (*post hoc* comparisons all *P*'s < 0.05).

Shoot fresh weight followed the same trend as stem height and leaf loss discussed above except for 2 treatments. Among the uninoculated plants, both SMX-L and GEM-H had significantly higher shoot weight than their associated uninoculated no CEC control treatment (GLiM: $\chi^2_9 = 186.179$, $P < 0.001$). Major differences among inoculated and uninoculated samples were observed, with uninoculated samples having significantly

higher shoot fresh weight than their inoculated counterparts (Set I plants: ANOVA: F_9 = 20.281, $P < 0.001$; Set II plants: GLiM: $\chi^2_9 = 186.179$, $P < 0.001$ (Figure 5B).

Impact of CECs on disease severity

Inoculation of eggplants with *V. dahliae* caused Verticillium wilt disease. Disease severity was calculated based on the disease scoring and disease severity index according to Liu et al. (2009b) (Figure 6). There was a significant interaction of disease severity between treatments and time (repeated measures Friedman Ranks ANOVA: set I: $F_{29.304, 130.240} = 1.140$, $P \leq 0.001$; set II: $F_{30.609, 136.038} = 25.0123$, $P \leq 0.001$). Disease severity varied with the type of CEC and the growth phase of the plants. Most treatments reached their disease index maximum by 5 weeks and had the fastest increase between week 3 and 5. Some of the plant treatments still increased in severity after week 5, while most remained steady. Plants treated with TMP had a higher, but not significant disease severity index earlier in the season (between week 2 and 5) compared to the other CECs and the no CECs control (Figures 6A,B). However, SMX and GEM had lower disease index than the control between week 2 and 5, but after that their disease index was above the control although it was not significant (Figures 6C,D). Both inoculated TMP-H and inoculated no CECs had steady increases in disease severity after 5 weeks (Figures 6A,B). APAP-L and TMP-L plateaued at low disease severity between week 5 and 7, but TMP-L plants sharply increased in severity near the end of the experiment. APAP-L, however, maintained relatively low levels of disease severity to the end of the experiment, week 8. Plants treated with TMP (high or low) increased in disease severity

index between week 7 and 8, while the disease index of the control plants reduced in this time. APAP-L had significantly lower disease severity than the inoculated TMP-H and the inoculated no CEC treatment by week 8 (GLiM: $\chi^2_4 = 15.536$, $P = 0.004$) (Figure 6A). The disease severity of the eggplants was used to calculate the disease index. As expected, the disease index results paralleled the disease severity results (Figures 6B,D).

Discussion

In this study we investigated the impacts of CECs at concentrations found in reclaimed water on plant microbes and Verticillium wilt disease severity on eggplants. Microorganism growth assays done *in vitro* showed that the tested microorganisms were differentially affected by CECs. Even strains of the same fungus responded differently to the same CEC. In the greenhouse experiments, plant disease index varied with the stage of plant growth and the CEC that was applied. APAP and TMP had stronger impacts on disease development early in the season, while SMX and GEM appeared to have less impact on disease development early in the season. APAP and TMP had a stronger influence of disease development at higher concentrations than lower concentrations, but the concentrations of SMX and GEM used did not differently impact in disease development. Inoculation with the pathogen *V. dahliae* had the strongest impact on disease development than any CEC treatment.

Impact of CECs on microorganism growth

In this study we investigated the impact of CECs on growth of microorganism *in vitro*. We used growth rates to calculate the allelopathic index, which is normally used to evaluate true allelopathy of chemicals excreted by organisms directly into the environment (Williamson and Richardson, 1988; Liu et al., 2009b). By applying this concept to extrinsically derived chemicals we can compare the impacts of anthropogenic CECs to naturally formed compounds used by the producing organism to influence other organisms. Originally the concept of allelopathy was used to refer to any chemical involved with positive or negative plant-plant interactions (Patrick, 1986). Over time this concept has evolved to include chemicals involved between plant-plant or plant-microbe interactions, but only in the negative, or inhibitory sense (Patrick, 1986). Lower, negative RI values have been associated with reduction of disease severity in plants in some experiments (Liu et al., 2009a; Zhou et al., 2011). Although, in the study by Liu et al. (2009b) significantly lower RI values of root exudates did not always associate with reduced disease severity or incidence. Suggesting that growth can only partially explain disease progression or infectivity. It is likely that there are many other factors involved in the interaction between hosts, CECs, and pathogenicity. Environmental factors such as pH (Zimmermann and Curtis, 2017) or carbon substrate availability (Hida et al., 2005) can influence potency of some CECs. Environmental factors can also influence a microorganism's ability to infect a given host (Jarosz and Burdon, 1988). The interactions of CECs with plants and microbes are multifaceted.

Our microbial growth assays showed that nearly all the microbes tested were sensitive to the CECs used in this study. However, the specific effects of the CECs varied, among the organisms. CECs have been observed to exhibit growth promoting effects on microbes (Carvalho et al., 2010; Zimmermann and Curtis, 2017), while growth inhibition by these chemicals typically occurs at higher concentrations (Koch and Burchall, 1971; Kabbash et al., 2004; Argyropoulou et al., 2009; Al-Janabi, 2010). For these reasons we predicted that the microorganisms would not be affected the presence of CECs. We observed increased growth in the presence of CECs with two fungi, *V. dahliae* 0048 and *F. oxysporum* CS-3. Both of these strains exhibited significantly more growth and higher RI values compared to their controls, suggesting that these CECs have growth promoting effects on these particular organisms. In contrast, other strains of the same fungi *V. dahliae* 0049 and *F. oxysporum* CS-5 had nearly an exact opposite trend in which the CECs either had no effect or were inhibitory to mycelial growth, suggesting that our hypothesis was partially correct. Their RI values correspond well to values found for root exudate of resistant tomato plant roots (−0.155 to −0.020) (Liu et al., 2009b). Having similar RI values to root exudates that exhibit antimicrobial effects also suggests that the *in situ* levels of CECs are at biologically relevant concentrations. Our results concurred with another study that showed that closely related fungi can respond differently to some CECs, in particular, sulfamethoxazole (Hida et al., 2005).

Not all the microorganisms evaluated were equally sensitive to all the CECs. *P. indica* was only affected by two CEC treatments in opposing ways at two separate time points,

while the growth of the bacterium *B. japonicum* was not affected by any CEC. These CECs usually exhibit antimicrobial effects at equal or greater concentrations of those found in human blood plasma levels corresponding to therapeutic doses. Typical therapeutic plasma levels of these CECs fall within the ranges of 10,000–20,000 μg/L; 3,000–8,000 μg/L; 80,000–100,000 μg/L, and 19,000–45,000 μg/L for APAP, TMP, SMX, and GEM, respectively (Nolte and Buettner, 1974; Reeves and Wilkinson, 1979; Spence et al., 1995; Kyrklund et al., 2003; Niemi et al., 2003; Stuart et al., 2004; Clajus et al., 2013; Zimmermann and Curtis, 2017), which are about 10,000–100,000 times more than those used in our study (Table 1). However, these studies were conducted on human pathogens and not soilborne organisms. CECs have been shown to disrupt the functions of soilborne nitrogen cycling bacteria when tested at concentrations higher than therapeutic concentrations (Colloff et al., 2008) and at levels found in wastewater treatment plant effluent (Wang and Gunsch, 2011).

Taken together our results and those of others indicate that many more plant or soil associated organisms may be sensitive to CECs at the concentrations found in reclaimed water. Thus, additional studies on the impacts of CECs on free living and plant associated microorganisms are necessary to fully understand the impacts of these chemicals on disease severity especially in fields where reclaimed water has been used to irrigate plants for years. We observed that fungal strains were sensitive to CECs concentrations well below typical therapeutic doses. Our microbial growth rate assays demonstrated that

these particular CECs are capable of impacting microbial growth at *in situ* concentrations and suggesting the potential to disrupt plant- pathogen dynamics.

Spore germination of microorganisms

The spore germination assays demonstrated that the CECs used in this study can affect fungal spore germination in addition to mycelial growth discussed above. In spore germination assays we used sucrose solutions to decrease the osmotic potential of the solution to optimal spore germination conditions as described for *V. dahliae* in Ioannou et al. (1977). We used the same sucrose solutions for the *F. oxysporum* CS-5 assay to optimize spore germination as well. When we used sterile water, the germination rate was only 5%. This is similar to results obtained by Steinkellner et al. (2005), who also obtained 5% germination rate of *F. oxysporum* in sterile water. The control (sucrose only solution) for our *V. dahliae* spore germination assay reached over 80%, which is comparable to the amount of spore germination in a previous study that uses similar methods (Ioannou et al., 1977). All of the CEC treatments had lower spore germination than the control, although two (APAP-H and SMX-L) were not significantly lower. TMP-H treatment had the greatest impact on *V. dahliae* 0049 spore germination and had significantly less germination than all treatments. This combined with the results of the mycelium growth assay, discussed above, suggest that this particular strain of *V. dahliae* is sensitive to TMP. Although spore germination did not get as high in the *F. oxysporum* CS-5 assay, the sucrose solution was successful with increasing spore germination rates. The average spore germination for the *F. oxysporum* assay reached above 40% for the

controls, which is higher than the about 5% germination amounts generated in the water only (Steinkellner et al., 2005). Over all, spore germination of *F. oxysporum* CS-5 exhibited less sensitivity to the CECs, but TMP had an effect of decreasing spore germination. The effects of TMP in reducing spore germination and mycelial growth did not translate to reduced disease severity as discussed below. The combined results of our spore germination assay suggest that soilborne microbes may be altered by CECs found in reclaimed water, which may impact plant-microbe interactions and plant disease severity.

Impact of CECs on plant growth and severity of Verticillium wilt of eggplants

Eggplants were grown in a greenhouse at the University of California, Riverside to evaluate the impacts of CECs on host-pathogen dynamics. Plants were watered with the range of concentrations of the respective CECs to simulate agriculture conditions in which plants are being irrigated with reclaimed water (Table 1) (Kinney et al., 2006; Vanderford and Snyder, 2006; Batt et al., 2007; Fram and Belitz, 2011). We predicted that disease severity would increase because the CECs would most likely enhance *V. dahliae* growth (Carvalho et al., 2010). Antibacterial compounds can displace microbes that are antagonistic toward plant pathogens, allowing them to increase in numbers and therefore have a higher chance to establish an infection (Mulligan et al., 1982; De Vries-Hospers et al., 1991; Azevedo et al., 2015). Previous studies have shown that certain pharmaceutical products can have detrimental impacts on plants (Liu et al., 2009a; Madikizela et al., 2018). The impacts of CECs on disease severity or disease severity

index varied with the CEC: TMP-H had a higher severity index earlier in the season (week 2–4), while SMX at both concentrations had a greater impact later in the season (week 5–9). APAP-L and TMP-L had the least effects on disease index between week 5 and 7. However, these differences were not statistically significant from the associated controls. Our result indicated that APAP-L and TMP-L were beneficial to the eggplant in some capacity. However, the exact interaction or mechanism is not known and requires additional study.

Besides examining disease severity, plant growth metrics of stem height, leaf loss, and shoot fresh weight were used to assess if the CECs directly impacted plant growth, or if there were any interactions between plant growth, disease severity and CECs. As expected, both stem height and final shoot fresh weight were significantly lower in the *V. dahliae* inoculated plants than in the uninoculated ones. Inoculated plants also lost significantly more leaves. Uninoculated plants did not have any indications of Verticillium wilt. By the end of the experiment, there were no significant differences found for stem heights or percentage of leaf loss between the different CEC treatments for inoculated or uninoculated plants. Plant growth was not impacted in any visible way by the concentrations of the CECs we used. This fits well with other studies that indicate that larger, more complex organisms, such as developed plants, will not be impacted directly by CECs at low concentrations (Wu et al., 2013). Although, other studies indicate that plants take up these compounds and that these CECs can be found in tissues of plants that have been irrigated with water containing these CECs (Dodgen et al., 2013;

Wu et al., 2013, 2015). Most of these studies have only been conducted within a single growing season. However, these chemicals can accumulate into tissues over time (Wu et al., 2010) thus longer studies are needed given that these compounds are impacting microbial and insect life at low concentrations (Pennington et al., 2018). Other studies have also found direct impacts to plants by anthropogenic compounds. The studies by D'Abrosca et al. (2008) and Pino et al. (2016) observed growth inhibition of plants when they were exposed to low levels of anthropogenic chemicals, including gemfibrozil. Inhibition of seed germination by CECs was also observed in the study D'Abrosca et al. (2008). Together these studies suggest that seeds and seedlings are more vulnerable to CECs, and the chemicals may have direct impacts on developed plants once they reach higher concentrations. In our study the plants used were at the 4th leaf stage and therefore had time to develop beyond the seedling stage before being exposed to CECs, which partially explains why there was not a great impact to their stem growth, or leaf production. In addition, some studies found that not all plants are impacted equally by a given CEC (D'Abrosca et al., 2008), and some plant tissues such as roots, maybe more sensitive to CECs than other parts of the plant (Pino et al., 2016).

By the end of the experiment we observe that two treatments had effects on shoot fresh weight. The uninoculated SMX-L treatment had significantly greater biomass than the associated uninoculated control. This result suggests that at low concentrations SMX may be beneficial for plant growth. Perhaps the plants are able to utilize the sulfur component of sulfamethoxazole. We also saw significant shoot fresh weight in plants treated with

GEM-L than the associated uninocualted no CEC control. Other studies conducted on plants at the seed stage have indicated that even low concentrations of GEM can have deleterious impacts on growth (D'Abrosca et al., 2008; Pino et al., 2016). However, these studies were conducted on seeds. Developed plants appear to be more resistant to chemical interference. The study Wu et al. (2013) found no phytotoxic effects of the CECs they tested on mature plants at low concentrations. It has been observed that small amounts of harmful substances can actually stimulate plant growth in a concept known as hormesis (Pan and Chu, 2017). Hormesis may at least partially explain the increased shoot biomass observed in the SMX-L and GEM-L treatments (Pan and Chu, 2017). The two studies that found GEM to be harmful were also done in the absence of soil, while our plants were grown directly in soil. GEM was most likely broken down or partially absorbed by the soil, thus further reducing the amount of the chemical the eggplants were exposed to Pan and Chu (2017). A lower amount of GEM may not have phytotoxic effects and instead have stimulatory effects on a given plant due to hormesis. However, the concept of hormesis was not formally tested in this experiment and should be investigated further.

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Figures

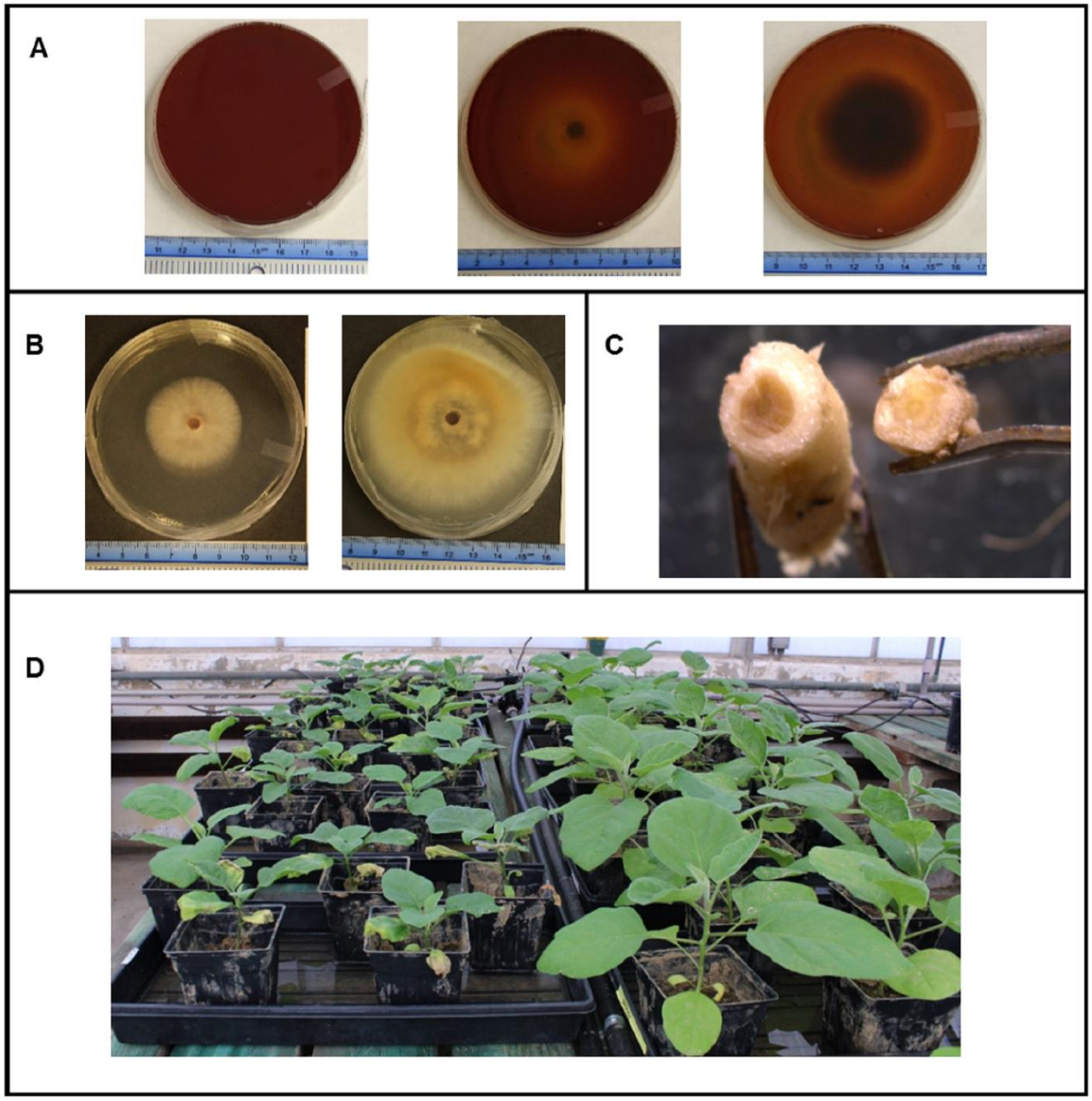


Figure 1.1 (A) *Fusarium oxysporum* grown on media containing the pH indicator bromocresol purple to indicate how far nutrients are being leached from the colony **(B)** Typical growth of *Verticillium dahliae* on Czepeck dox agar. **(C)** Stem cross section of an inoculated eggplant (left) and an uninoculated eggplant (right). **(D)** Side by side comparison of inoculated eggplants (left) to uninoculated eggplants (right).

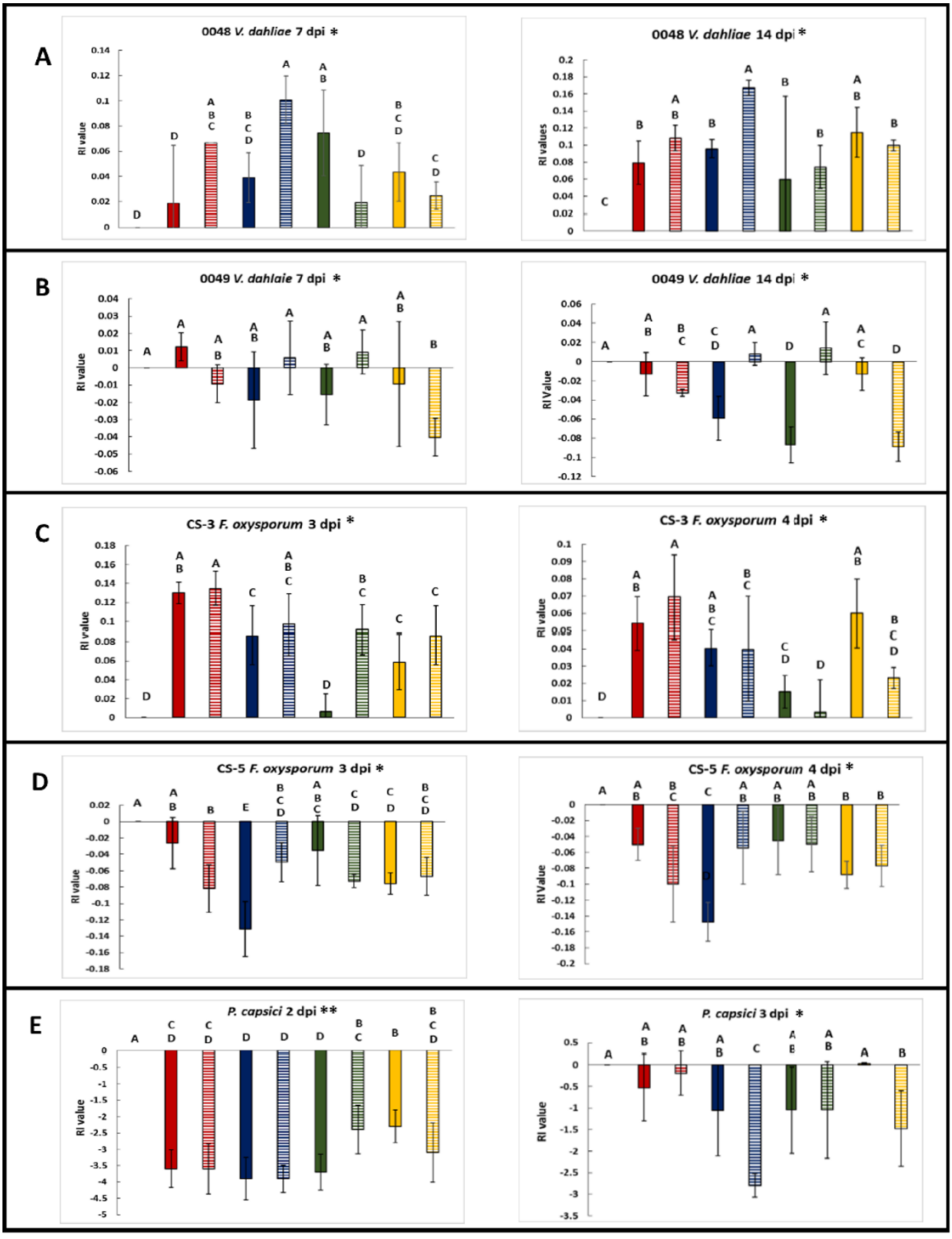

A
0048 V. dahliae 7 dpi *
RI value
0048 V. dahliae 14 dpi *
RI values
B
0049 V. dahlaie 7 dpi *
RI value
0049 V. dahliae 14 dpi *
RI Value
C
CS-3 F. oxysporum 3 dpi *
RI value
CS-3 F. oxysporum 4 dpi *
RI value
D
CS-5 F. oxysporum 3 dpi *
RI value
CS-5 F. oxysporum 4 dpi *
RI Value
E
P. capsici 2 dpi **
RI value
P. capsici 3 dpi *
RI value
No CECs
APAP-H
APAP-L
TMP-H
TMP-L
SMX-H
SMX-L
GEM-H
GEM-L

Figure 1.2 Comparing the Allelopathic index values (RI) of the plant pathogens used in this study. **(A)** *Verticillium dahliae* strain 0048 measured at 7 and 14 dpi, **(B)** *Verticillium dahliae* strain 0049 measured at 7 and 14 dpi. **(C)** *Fusarium oxysporum* f. sp. *lycopersici* strain CS-3 measured at 3 and 4 dpi. **(D)** *Fusarium oxysporum* f.sp *lycopersici* strain CS-5 measured at 3 and 4 dpi. **(E)** Phytopthora capsici measured at 2 and 3 dpi. *Samples compared statistically using GLiM with normal distribution and identity link function, pairwise analyses with sequential Bonferroni pairwise correction for multiple comparisons ($P < 0.05$). **ANOVA used for statistical analyses, post-hoc Tukey test used for pairwise analyses ($P < 0.05$).

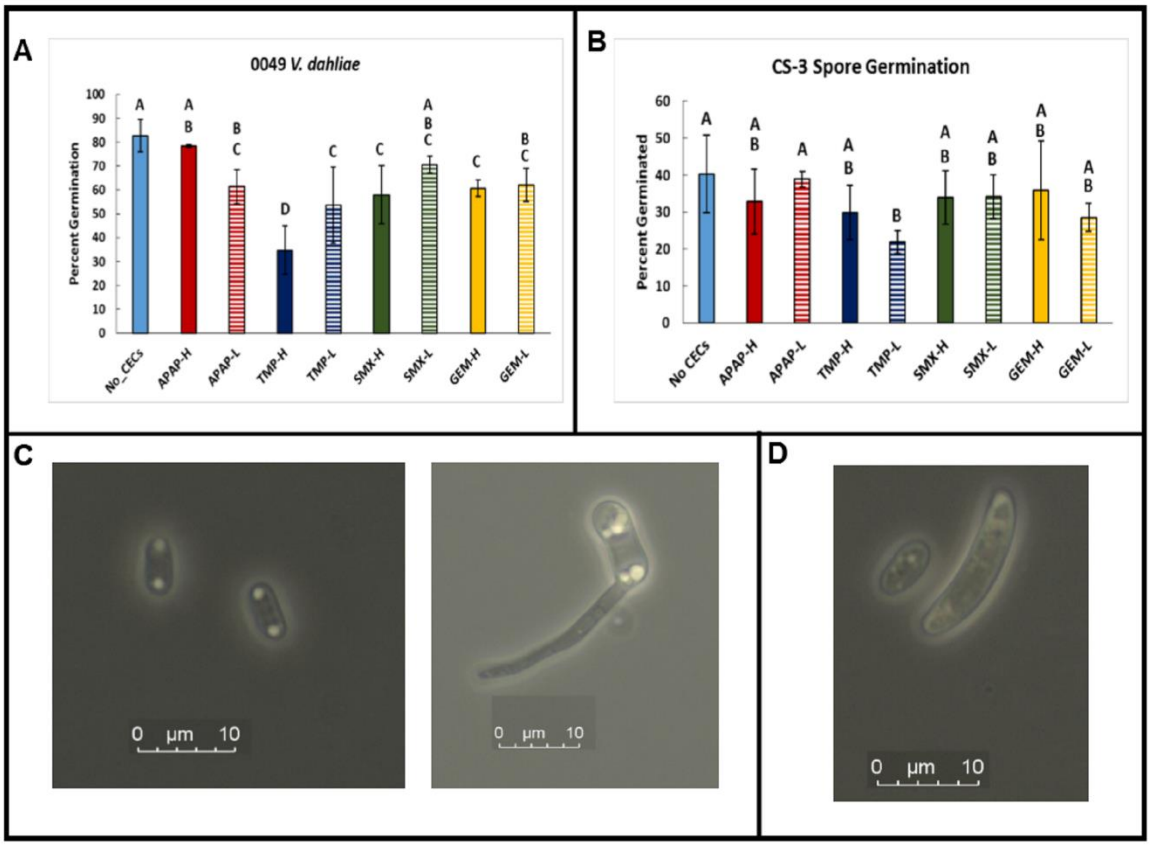


Figure 1.3 Spore germination assays done in a mild sucrose solution. GLiM with normal distribution and identity link function used for all statistical comparisons. Samples that do not share the same letter are significantly different as determined by a post-hoc, pairwise GLiM analyses done with sequential Bonferroni correction for multiple comparisons (all P's < 0.05). **(A)** Spore germination results for *Verticillium dahliae* after 12 h of incubation in a 0.085 M sucrose solution. **(B)** Spore germination results for *Fusarium oxysporum* f.sp *lycopersici* after 12 h of incubation in 0.085 M sucrose solution. **(C)** Photographs of *Verticillium dahliae* spores at 1,000× total magnification. Ungerminated spore (left), germinating spore (right) **(D)** Photograph of small and large *Fusarium oxysporum* f.sp *lycopersici* spores at 1,000× magnification.

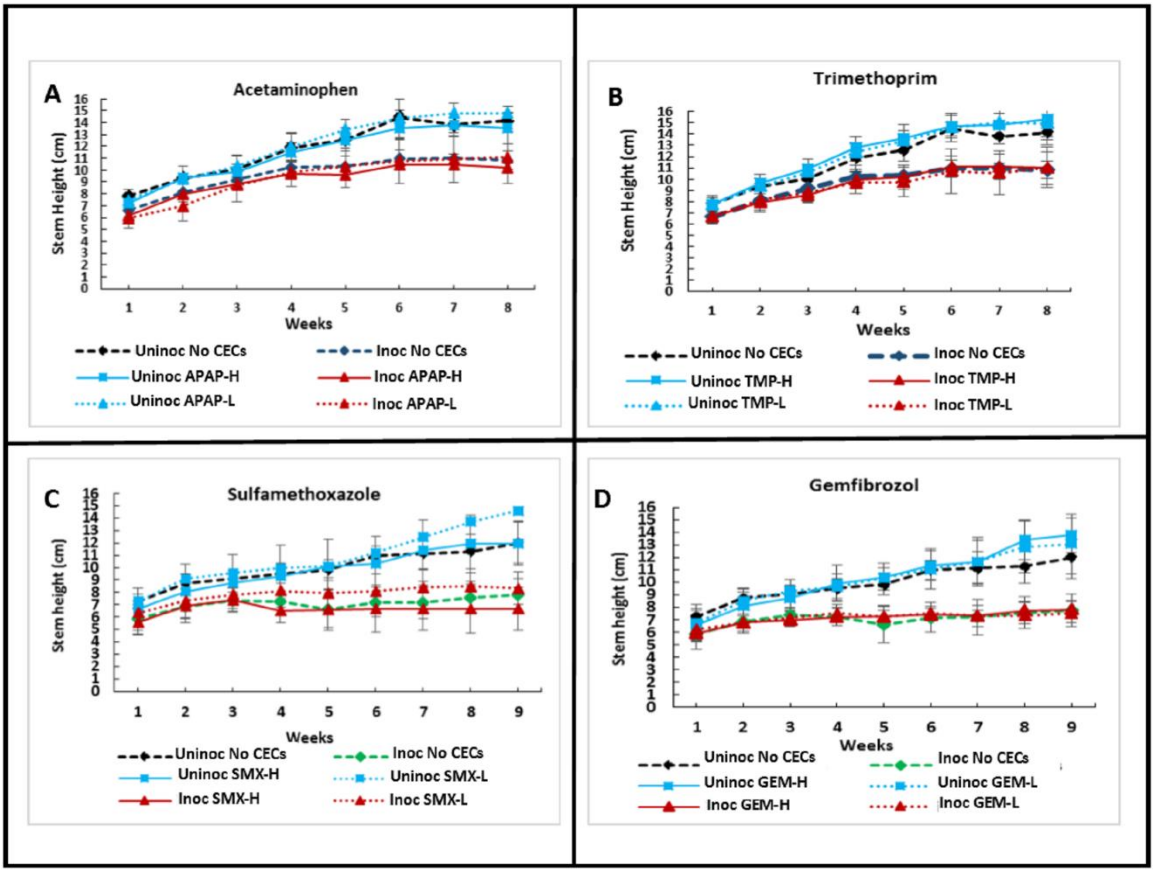


Figure 1.4 Effects of chemicals of emerging concern (CECs) overtime on stem height of inoculated and uninoculated eggplants grown in the greenhouse. Error bars represent standard deviation. **(A)** Eggplants treated with acetaminophen (APAP) and a no CEC control. **(B)** Eggplants treated with trimethoprim (TMP) and the no CEC control. **(C)** Eggplants treated with sulfamethoxazole (SMX) and a no CEC control. **(D)** Eggplants treated with gemfibrozil (GEM) and a no CEC control. The same no CEC controls were used for **(A,B)**, while **(C,D)** have the same no CEC controls. For each CEC two concentrations were used high (H) and low (L).

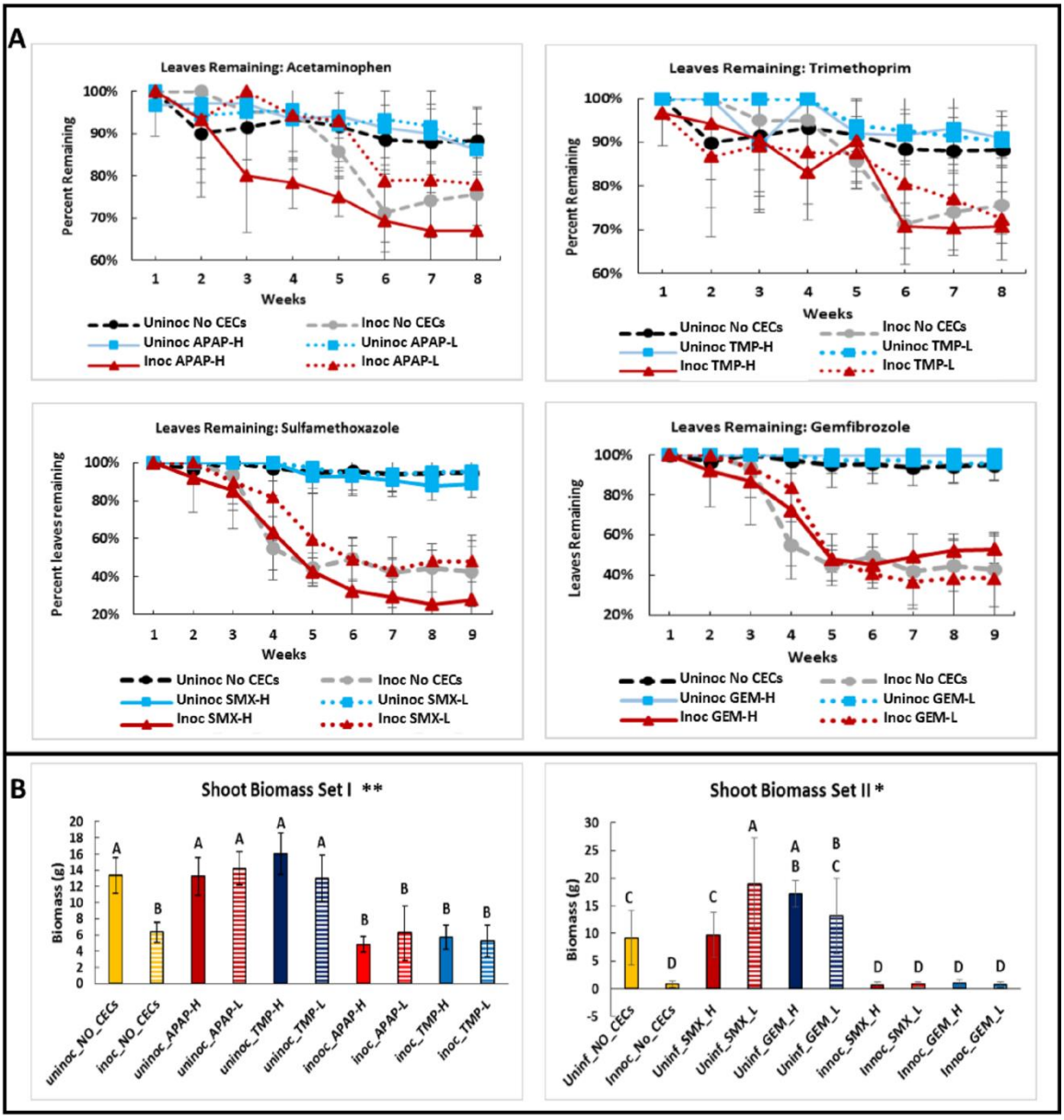

A
Leaves Remaining: Acetaminophen
Percent Remaining
Weeks
Uninoc No CECs
Inoc No CECs
Uninoc APAP-H
Uninoc APAP-L
Inoc APAP-H
Inoc APAP-L
Leaves Remaining: Trimethoprim
Percent Remaining
Weeks
Uninoc No CECs
Inoc No CECs
Uninoc TMP-H
Uninoc TMP-L
Inoc TMP-H
Inoc TMP-L
Leaves Remaining: Sulfamethoxazole
Percent leaves remaining
Weeks
Uninoc No CECs
Inoc No CECs
Uninoc SMX-H
Uninoc SMX-L
Inoc SMX-H
Inoc SMX-L
Leaves Remaining: Gemfibrozole
Leaves Remaining
Weeks
Uninoc No CECs
Inoc No CECs
Uninoc GEM-H
Uninoc GEM-L
Inoc GEM-H
Inoc GEM-L
B
Shoot Biomass Set I **
Biomass (g)
uninoc_NO_CECs
inoc_NO_CECs
uninoc_APAP-H
uninoc_APAP-L
uninoc_TMP-H
uninoc_TMP-L
inooc_APAP-H
inoc_APAP-L
inoc_TMP-H
inoc_TMP-L
Shoot Biomass Set II *
Biomass (g)
Uninf_NO_CECs
Innoc_No_CECs
Uninf_SMX_H
Uninf_SMX_L
Uninf_GEM_H
Uninf_GEM_L
innoc_SMX_H
Innoc_SMX_L
Innoc_GEM_H
Innoc_GEM_L

Figure 1.5 Comparisons of plant health metrics of eggplants grown in the green house among different chemicals of emerging concern (CECs) treatments. (A) Line graphs of leaves remaining on plants over time for each CEC. (B) Above ground biomass of both sets of eggplants at the end of the experiment. The CECs used were acetaminophen (APAP), trimethoprim (TMP), sulfamethoxazole (SMX) and gemfibrozil (GEM). For each CEC two concentrations were used high (H) and low (L). *Samples compared statistically using GLiM with normal distribution and identity link function, pairwise analyses with sequential Bonferroni pairwise correction for multiple comparisons ($P < 0.05$). **ANOVA used for statistical analyses, post-hoc Tukey test used for pairwise analyses ($P < 0.05$).

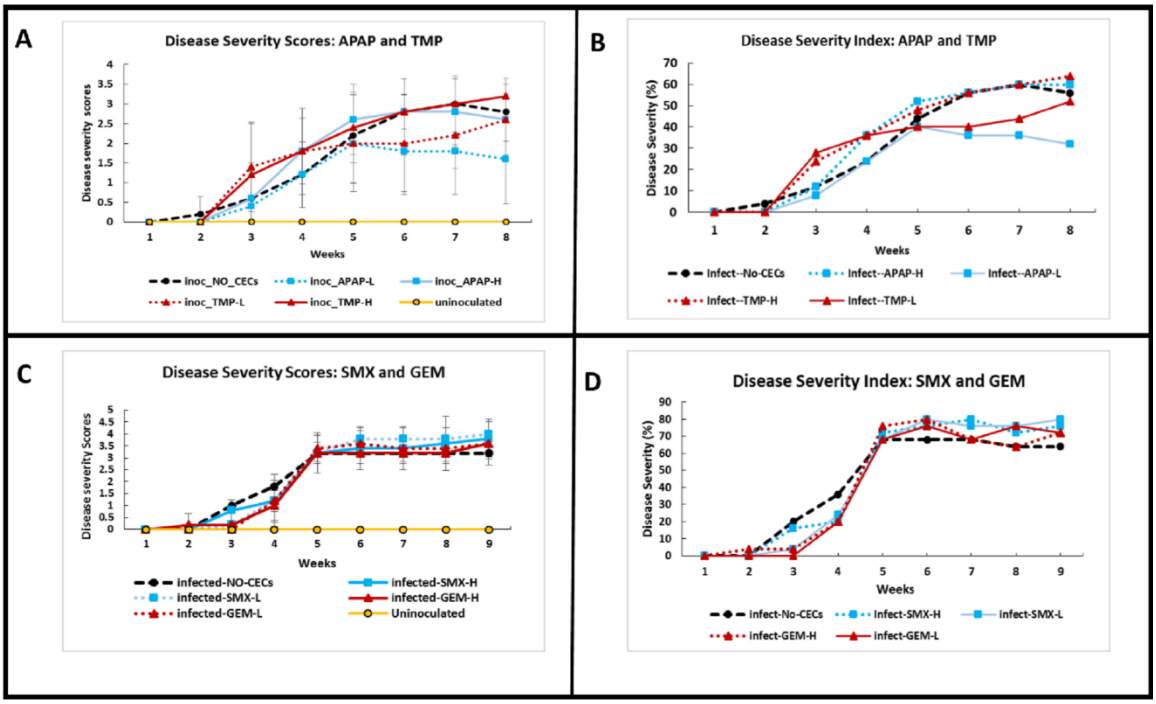


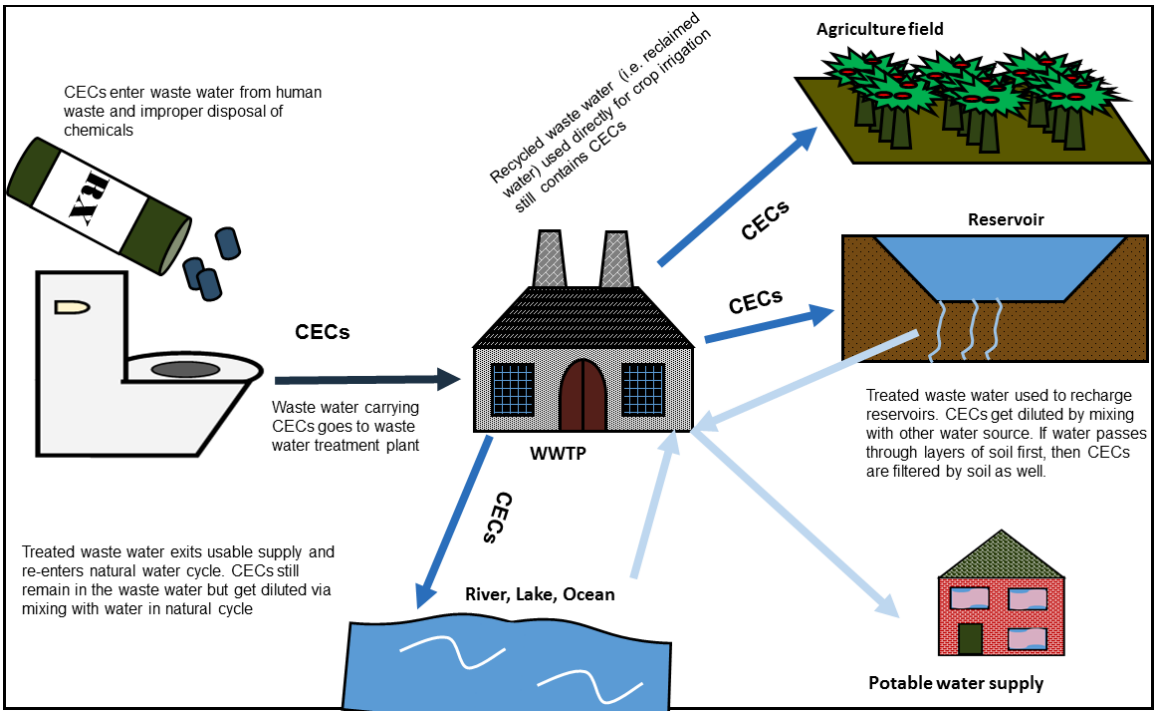
Figure 1.6 Comparison of disease severity and disease severity index of greenhouse grown eggplants inoculated with *Verticillium dahliae* and watered with or without the addition of chemicals of emerging concern. Calculations to determine the disease severity index (DSI) are described in Liu et al. (2009b) and Chiang et al. (2017). All error bars shown represent standard deviation. **(A)** Disease severity of inoculated and uninoculated eggplants for acetaminophen (APAP) and trimethoprim (TMP), treatments. **(B)** Comparing DSI values for APAP and TMP treatments over time. **(C)** Disease severity of inoculated and uninoculated eggplants for sulfamethoxazole (SMX) and gemfibrozil (GEM)treatments over time. **(D)** Comparing DSI values for SMX and GEM treatments over time.

Tables

Table 1.1 Concentrations of CECs used for all assays in this study.

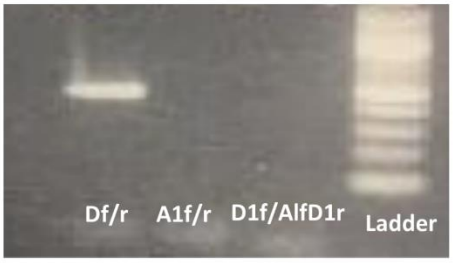
CEC	High concentration (H)	Low concentration (L)
Acetaminophen (APAP)	10 µg/L	5 µg/L
Trimethoprim (TMP)	2.5 µg/L	1 µg/L
Sulfamethoxazole (SMX)	2 µg/L	1 µg/L
Gemfibrozil (GEM)	10 µg/L	2 µg/L
No CEC control (No CECs)	0 µg/L	

Supplemental Figures

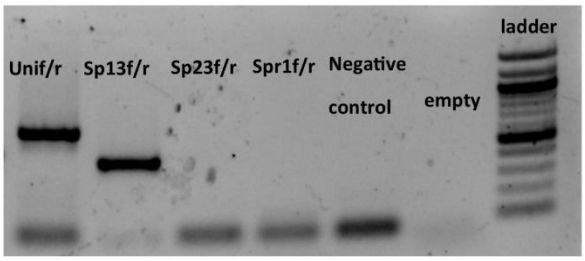


Supplemental Figure 1.1: Brief overview of wastewater cycle and fate of CECs. For more detailed descriptions of wastewater treatment, reclaimed water, and general cycle of the usable water supply see Warsinger et al., 2018

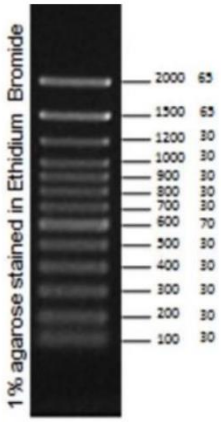
A



B



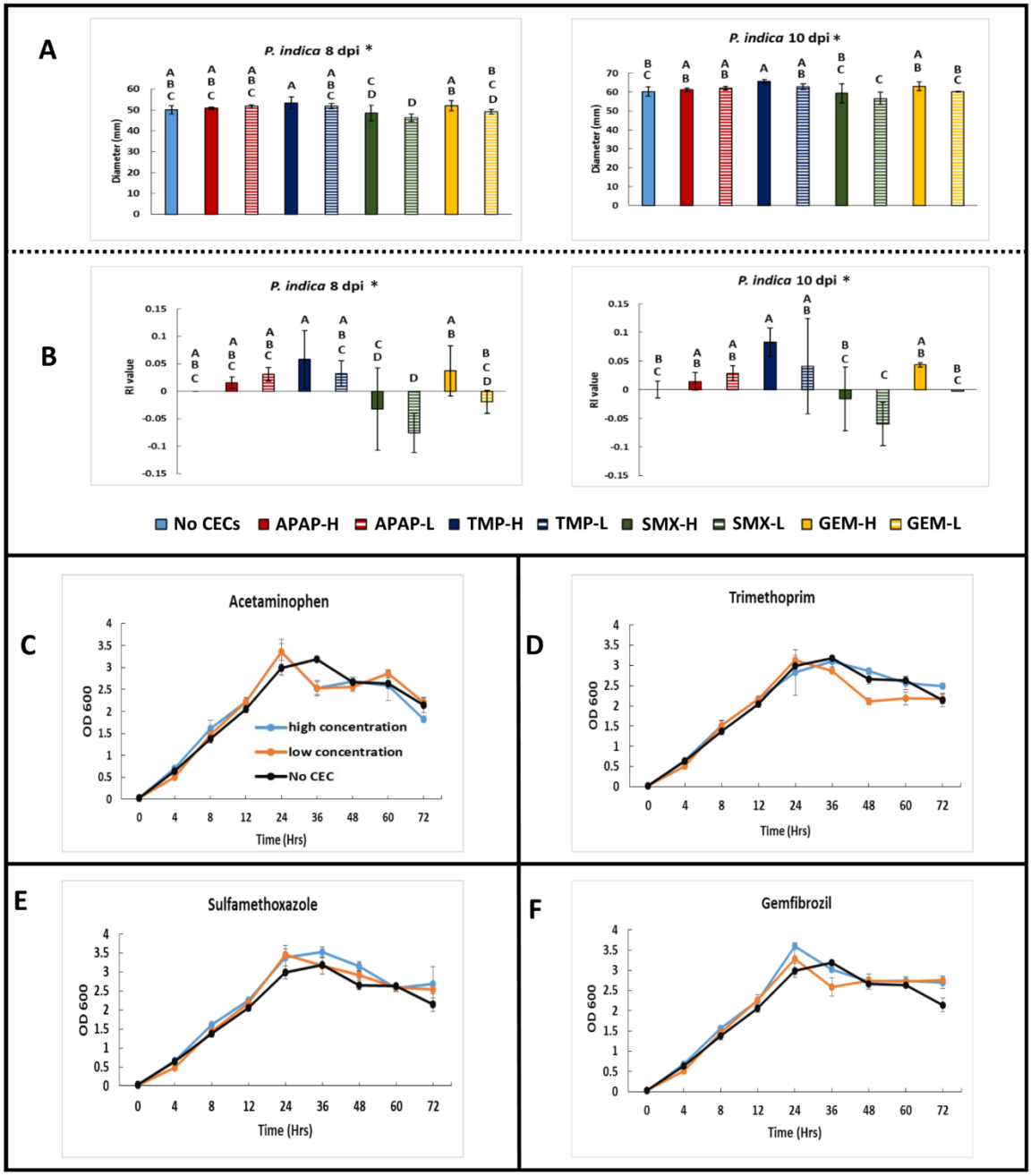
C



D

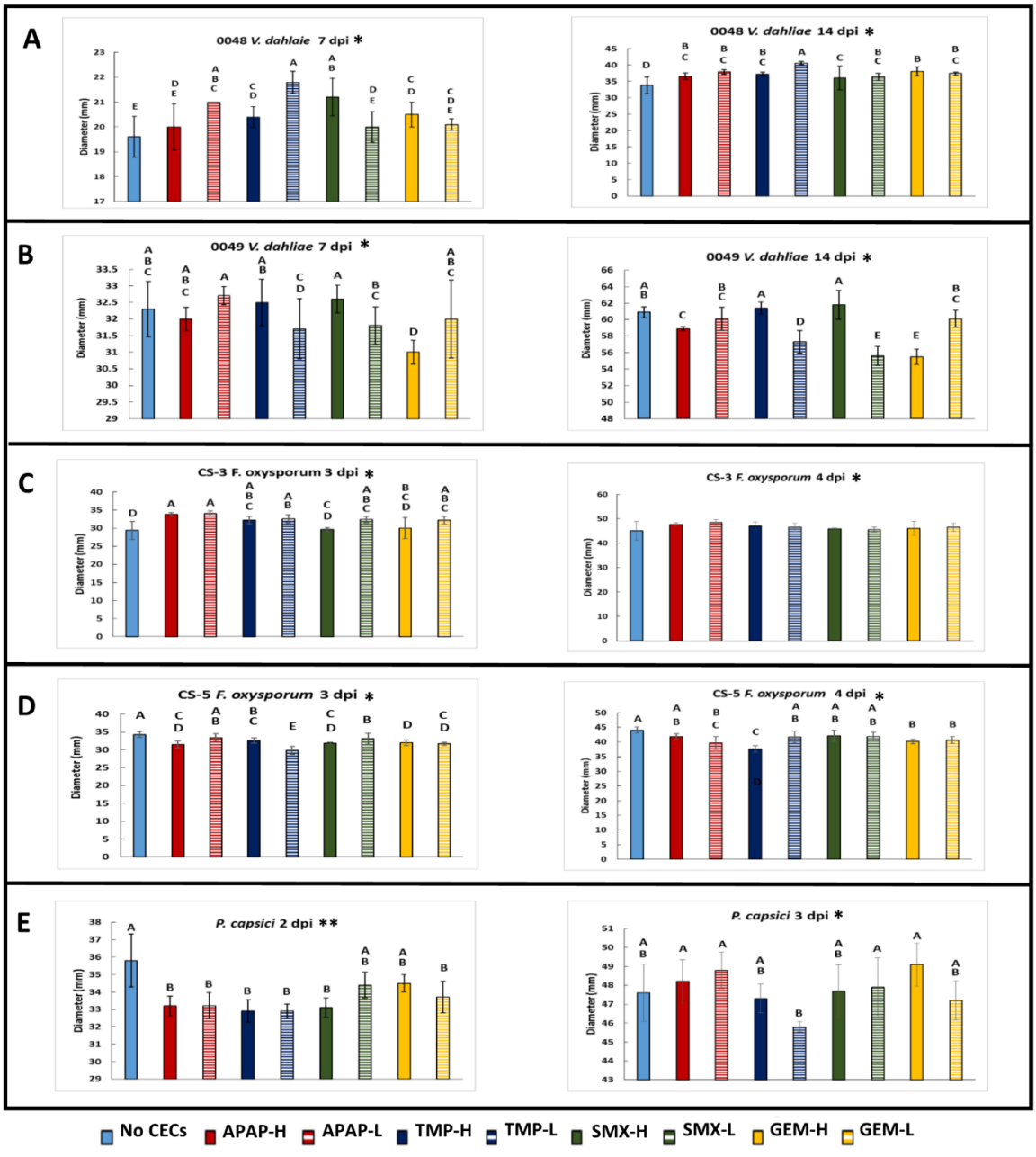
Primer pair	Organism DNA primers positively amplify	Source
Df/r	*Verticillium dahliae/ Verticillium longisporum*	Inderbitzin et al., 2013
A1f/r	*V. longisporum* species A1	Inderbitzin et al., 2013
D1f/AlfD1r	*V. longisporum* species D1	Inderbitzin et al., 2013
Unif/r	*Fusarium oxysporum* f. sp. *lycopersici* or f. sp. radices-lycopersici	Hirano and Arie, 2006
Sp13f/r	*F. oxysporum* f. sp. *lycopersici*	Hirano and Arie, 2006
Sp23f/r	*F. oxysporum* f. sp. *lycopersici*	Hirano and Arie, 2006
Spr1f/r	*F. oxysporum* f. sp. radices-lycopersici	Hirano and Arie, 2006

Supplemental figure 1.2 Results of PCR assay to identify *Verticillium dahliae* and *Fusarium oxysporum sp. lycopersici*. **(A)** Agarose gel showing results of amplifying *V. dahliae* with the given primer sets indicated on each lane. **(B)** Agarose gel showing results of amplifying *F. oxysporum* template DNA with the primer sets used over their given lane. **(C)** DNA ladder used to determine amplified DNA fragment size (picture not to scale with gels shown). **(D)** Key showing the organisms' DNA the primers are specific for and will amplify during a PCR.


A
P. indica 8 dpi *
Diameter (mm)
P. indica 10 dpi *
Diameter (mm)
B
P. indica 8 dpi *
RI value
P. indica 10 dpi *
RI value
No CECs
APAP-H
APAP-L
TMP-H
TMP-L
SMX-H
SMX-L
GEM-H
GEM-L
C
Acetaminophen
OD 600
high concentration
low concentration
No CEC
Time (Hrs)
D
Trimethoprim
OD 600
Time (Hrs)
E
Sulfamethoxazole
OD 600
Time (Hrs)
F
Gemfibrozil
OD 600
Time (Hrs)

Supplemental figure 1.3 Comparing colony diameter of plant pathogens in the presences of chemicals of emerging concern (CECs). **(A)** *Verticillium dahliae* strain 0048 measured at 7 and 14 dpi. **(B)** *V. dahliae* strain 0049 measured at 7 and 14 dpi. **(C)** *Fusarium oxysporum f. sp lycopersici* strain CS-3 measured at 3 and 4 dpi. The CECs used were acetaminophen (APAP), trimethoprim (TMP), sulfamethoxazole (SMX) and gemfibrozil (GEM). For each CEC two concentrations were used high (H) and low (L).

*Samples compared statistically using GLiM with normal distribution and identity link function, pairwise analyses with sequential Bonferroni pairwise correction for multiple comparisons ($P < 0.05$).


A
0048 V. dahlaie 7 dpi *
0048 V. dahliae 14 dpi *
B
0049 V. dahliae 7 dpi *
0049 V. dahliae 14 dpi *
C
CS-3 F. oxysporum 3 dpi *
CS-3 F. oxysporum 4 dpi *
D
CS-5 F. oxysporum 3 dpi *
CS-5 F. oxysporum 4 dpi *
E
P. capsici 2 dpi **
P. capsici 3 dpi *
Diameter (mm)
No CECs
APAP-H
APAP-L
TMP-H
TMP-L
SMX-H
SMX-L
GEM-H
GEM-L

Supplementary Figure 1.4 Comparing diameters of different fungal organisms in the presences of CECs. *V. dahliae* strain **(A)** 0048 and **(B)** 0049 measured at 7 and 14 dpi. *F. oxysporum* strain **(C)** CS-3 and **(D)** CS-5 measured at 3 and 4 dpi. **(E)** *P. capsici* measured at 3 and 4 dpi.

* Samples compared statistically using GLiM with normal distribution and identity link function, pairwise analyses with sequential Bonferroni pairwise correction for multiple comparisons ($P < 0.05$).

**ANOVA used for statistical analyses, post-hoc tukey test used for pairwise analyses ($P < 0.05$)

CHAPTER 2

Acetaminophen concentrations found in recycled wastewater alter soil microbial community structure and functional diversity

Previously published in the journal Microbial Ecology (McLain et al. 2022)

Abstract

The practice of using recycled wastewater (RWW) has been successfully adopted to address the growing demand for clean water. However, chemicals of emerging concern (CECs) including pharmaceutical products remain in the RWW even after additional cleaning. When RWW is used to irrigate crops or landscapes, these chemicals can enter these and adjacent environments. Unfortunately, the overall composition and concentrations of CECs found in different RWW sources vary, and even the same source can vary over time. Therefore, we selected one compound that is found frequently and in high concentrations in many RWW sources, acetaminophen (APAP), to use for our study. Using greenhouse grown eggplants treated with APAP concentrations within the ranges found in RWW effluents, we investigated the short-term impacts of APAP on the soil bacterial population under agricultural settings. Using Illumina sequencing-based approaches, we showed that APAP has the potential to cause shifts in the microbial community most likely by positively selecting for bacteria that are capable of metabolizing the breakdown products of APAP such as glycosides and carboxylic acids. Community-level physiological profiles of carbon metabolism were evaluated using

Biolog EcoPlate as a proxy for community functions. The Biolog plates indicated that the metabolism of amines, amino acids, carbohydrates, carboxylic acids, and polymers was significantly higher in the presence of APAP. Abundance of microorganisms of importance to plant health and productivity was altered by APAP. Our results indicate that the soil microbial community and functions could be altered by APAP at concentrations found in RWW. Our findings contribute to the knowledge base needed to guide policies regulating RWW reuse in agriculture and also highlight the need to further investigate the effects of CECs found in RWW on soil microbiomes.

Introduction

Potable water supplies are becoming scarce with the increasing world population. Changing climate factors such as rising temperatures and altered precipitation patterns limit the regeneration of these supplies (Pimentel et al. 1997; Misra 2014; Becerra-Castro et al. 2015). Conservation can only stretch water supplies so far and may not be enough to address the growing demands for clean water (Misra 2014). Alternative ways to generate usable water, such as recycling wastewater, are essential to help meet the rising demand (Misra 2014; Vo et al. 2014). Efforts to solve water shortage problems by importing water can impact non-arid regions and can cause the environment to suffer through reduction of habitat area and water availability for the biota (Pimentel et al. 2004). In addition, Importing water can be costly and it is not an ideal long-term solution (Stokes and Horvath 2006; Christian-Smith et al. 2010). Therefore, alternative methods for generating potable

water are becoming a necessity to meet rising water demands (Becerra-Castro et al. 2015; Parsons et al. 2010).

Use of recycled wastewater (RWW) to supplement potable water supplies has been very successful (Christian-Smith et al. 2010; Parsons et al. 2010; Warsinger et al. 2018) in arid regions, such as southern California. RWW is primarily used for agriculture and landscape irrigation (Parsons et al. 2010; Warsinger et al. 2018; Newton et al. 2011), allowing farmers in arid regions to maintain high agricultural outputs with less dependence on the potable water supply or having to increase water withdrawal from natural aquifers (Christian-Smith et al. 2010; Schulte 2011). Using RWW has been so successful that many water districts in California are planning on increasing their capacity for capturing and treating larger volumes of their wastewater (California State Water Resources Cont...). Despite the large conservation success of RWW, this water may pose risks to natural and agricultural environments. The wastewater treatment process is efficient at removing potentially disease-causing biological contaminants, but is less effective at removing chemical contaminants (Kolpin et al. 2002; Kinney et al. 2006; Batt et al. 2007; Gros et al. 2010). These contaminants include pharmaceuticals, personal care products, detergents, nanoparticles etc. that are collectively referred to as chemicals of emerging concern (CECs) (Kinney et al. 2006; Gros et al. 2010; Epa 2019). Processing of wastewater can reduce the levels of CECs by major proportions depending upon the RWW plant (for example: 74%, 71%, 67%, 91%, 99% for sulfamethoxazole, tetracycline, gemfibrozil, ibuprofen, and APAP respectively) (Kinney et al. 2006; Gros et al. 2010; Epa 2019). It was originally

believed that the final CEC concentrations in RWW effluent (typically in the μg to mg/L range) were too low to be biologically relevant (Boxall et al. 2006; Wu et al. 2013; Zimmermann and Curtis 2017). However, recent evidence suggests that the concentrations of CECs in RWW can impact microorganisms, insects, and plants (Wang and Gunsch 2011; Barra Caracciolo et al. 2015; Pennington et al. 2017; Pennington et al. 2018; Christou et al. 2018; McLain and Gachomo 2019). CECs accumulate in soils irrigated with RWW (Kinney et al. 2006), and they are taken up by plants inevitably accumulating in their tissues (Boxall et al. 2006; Wu et al. 2013; Bartha et al. 2010). Therefore, CECs pose a risk in the agricultural settings where RWW is primarily used, and RWW should be evaluated extensively to manage or reduce any potential hazards.

RWW sources vary in the concentration and composition of CECs temporally and spatially, which may be related to the sources and human activities that generate the wastewater (Sim et al. 2011). Additionally, it is more than likely that the different CECs will interact with each other and affect the behavior of different CECs that are present, possibly ameliorating or intensifying their effects. For example, gemfibrozil can increase the potency of the antifungal compound fluconazole (Bulatova and Darwish 2008), sulfamethoxazole can increase the antimicrobial effects of rifampicin (Macingwana et al. 2012), and APAP can induce β-lactamase activity and decrease the susceptibility of bacteria to certain antibiotics (Zimmermann and Curtis 2017). These combinations of factors make it difficult to distinguish the impacts of individual CECs in RWW on the plant associated microbial communities. Therefore, we investigated their impacts on plant-microbe interactions. In

this study, we investigated how APAP can alter the soil microbiome and consequently impact plant health which is correlated to productivity (Bais et al. 2006; Van der Heijden et al. 2008; Berendsen et al. 2012). Since CECs accumulate in soils irrigated with RWW, they can alter the plant associated soil microbiome (Pennington et al. 2017). The addition of CECs into a given soil environment has the potential to select for a specific group of organisms, possibly ones that can benefit directly from the compound (Aislabie et al. 2004). Since high usage of APAP is likely to continue, and it has been found to impact soil microorganisms and their functions, we decided to evaluate the short term (3 and 7 weeks post application) impacts directly on the soil microbial community of an important agricultural crop. We hypothesized that APAP at levels found in RWW will alter the soil bacterial community structure and function within a single growing season.

Methods

Eggplant Cultivation and Soil collection

Solanum melongena (eggplants, variety Patio Baby) were cultivated as described in supplementary material and methods (SI-1) and our previous study (McLain and Gachomo 2019). Treatments were applied by irrigating with water containing 10 μg/L or 5 μg/L of APAP (APAP-10 and APAP-5 respectively), and control plants (NO CEC) with tap water (Wu et al. 2012; Lapworth et al. 2012). Tap water sources are independent to the RWW system and previous observations have indicated that tap water contains a negligible amount of CECs (Lapworth et al. 2012). Given that the composition of RWW is very variable and that RWW contains a plethora of compounds that may impact the

plants or soil microbes directly, we decided to dilute APAP in tap water to reduce the number of factors that could contribute to the results obtained (Kinney et al. 2006; Gros et al. 2010; Epa 2019). Soil samples were collected before treatments (T0 time point), 3 and 7 weeks after beginning of treatments (T1 and T2 respectively). Push cores of 1 cm diameter and 3 cm deep of soil were collected at least 3 cm from the eggplant stem and 3 cm from the wall of the pot containing the soil. Please see the Supplementary Methods for more details

DNA extractions and Illumina sequencing library preparation

Total environmental DNA was extracted from 0.25 g of soil samples described above using the DNeasy Powersoil kit (Qiagen, Valencia, CA, USA), following manufacturer instructions, except 50 µL of solution C6 was used. DNA quality was checked using an Implen NanoPhotometer (Implen, Westlake Village,CA, USA). Amplicon libraries of the bacterial 16s rRNA gene were generated from the extracted DNA to characterize the bacterial community. A two-step PCR dual indexing inline barcoding procedure and primers were used to generate amplicons for Illumina sequencing (Pennington et al. 2017; Chelius and Triplett 2001; Kembel et al. 2014). Phusion High-Fidelity PCR master mix with HF buffer (Thermo Scientific) and 0.2 µM primers were used as PCR reagents with 1 uL of extracted DNA for the template. PCRs were carried out on the BioRad T100 thermal cycler as described by Kembel and colleagues (Pennington et al. 2017; Kembel et al. 2014) except we used 56.5 annealing temperature, 24 cycles, and final elongation time of 5 minutes. PCRs were screened for quality and fragment size using gel electrophoresis

with a 1% agarose gel. Amplicons from successful PCRs were purified using the Agencourt AMPure xp beads protocol (Beckman Coulter, Brea, CA, USA), except that SPRI beads (Beckman Coulter, Brea, CA, USA) were used and all ethanol washes were done using 80% ethanol. Cleaned DNA products were used as a template in a second PCR under similar conditions as described above except 0.3 µM HPLC purified PCR2F and PCR2R primers were used (Pennington et al. 2017; Kembel et al. 2014) and 7 cycles were used with an annealing temperature of 65 °C. PCRs were screened as described for the initial PCR. DNA concentrations were measured using the nanodrop spectrophotometer, and amplicons were pooled in equal molar concentrations of 5 nM for sequencing. The samples were submitted to the UCR genomics core facility where library quality was assessed using a 2100 Bioanalyzer (Agilent) and the libraries were sequenced using a MiSeq sequencer (Illumina) and Miseq Reagent kit version 3 (Illumina) with 2 x 150 cycles. The Raw sequences were submitted to NCBI and are under the accession numbers PRJNA808107.

Data analysis - Processing and quality filtering

The forward and reverse Illumina sequencing reads were joined together and quality filtered using default settings in QIIME1 (Caporaso et al. 2010). Joined sequences were demultiplexed using their unique barcode pairs in QIIME1. Demultiplexed samples were uploaded into QIIME2 with their associated quality scores (Estaki et al. 2020). Sequences were quality filtered further using the deblur method in QIIME2 (Estaki et al. 2020; Nearing et al. 2018; Caruso et al. 2019). Samples that contained less than 9,000

sequences were removed. The number of sequences per sample were rarefied down to match the sample with the lowest amount, 10,300 sequences (Estaki et al. 2020). Deblur classified these sequences into amplicon sequence variants (ASVs) that were taxonomically identified to the lowest possible level by matching to the Greengenes database (v 13.8) using QIIME2 default parameters (DeSantis et al. 2006). Negative controls were sequenced in parallel, any ASV's detected were filtered out from the data using QIIME2 before downstream analyses. Community α-diversity was measured using the Shannon Wiener index in QIIME2 and statistically compared using the best fitting generalized linear model (GLiM) (normal distribution and identity link function) as determined by the model with the lowest Akaike's Information Criterion (AIC) in SPSS (IBM, V. 27.0). Box plots of α-diversity metrics were generated in QIIME2. Community differences among all time points (**β**-diversity) were evaluated using PERMANOVA (Anderson and Walsh 2013; Navas-Molina et al. 2013) on Bray-Curtis distance matrices in QIIME2 (Bray and Curtis 1957; Wolsing and Priemé 2004). Boxplots of the **β**-diversity were plotted in QIIME2. Community data from QIIME2 was used in Paleontological Statistics (PAST) (Hammer et al. 2001) to generate PCA graphs showing the taxa that contributed to the most differences among communities. The group significance test in QIIME1, which uses pairwise Kruskal Wallis tests, was used to statistically compare the abundance of ASVs (Caporaso et al. 2010). Taxa were considered to be significantly different in relative abundance if $P < 0.05$, with an FDR value lower than 0.2. A conservative FDR value of less than 0.2, as described by Efron (2007), was used in order to obtain a more inclusive set of microbes that are potentially

impacted by APAP so that more bacterial taxa could be considered for additional study. A similar logic was used by Go et al. (2015) to screen for candidate metabolites, and the study Kong et al. (2019) used FDR < 0.2 to determine which microbes were significantly differentially abundant in the oral and gut microbiome of humans. Community data generated in QIIME2 was imported into PICRUSt2 (Douglas et al. 2020) to predict the potential bacterial metagenome present in the soil communities. The data was normalized by copy number and predictions were based on the Kyoto Encyclopedia of Genes and Genomes (KEGG orthologs) database. STAMP (Parks et al. 2014) was used to do initial ANOVA's on each predicted gene to screen for ones that were differentially abundant among all treatments. Genes that were found to be significantly differentially abundant ($P < 0.05$) with a high effect size (measured as eta-squared (η^2)), $\eta^2 > 0.40$, were kept for additional pairwise analyses described below to ensure that the differences were biologically relevant (Lakens 2013). Welch's t-test, in STAMP, was used for pairwise comparisons among all treatments for genes that passed this screening.

Evaluating changes in microbial functional diversity:

In order to verify PICRUSt2 predictions and determine changes in functional diversity, the utilization of different carbon sources for microbes in APAP-10 treated and untreated soils were evaluated using the Biolog EcoPlate (Liu et al. 2009; Liu et al. 2012). The Biolog EcoPlate contains 31 ecologically relevant carbon sources and water (control) in triplicates within a 96 well plate (Table 1). The same soil samples collected at 7 weeks after treatment with APAP-10 (T2), described above, were used in the Biolog procedure

described by Liu et al. (2009) with a few exceptions. To make soil suspensions, 1g soil was added to 10 mL of dH_2O, shaken at room temperature, added to the Biolog plates that were incubated for 6 days at 25 °C, and the absorbance at 590 nm was read at 12, 24, 48, 72, 96, and 120 hours post inoculation (hpi) using a Promega GloMax-Multi Detection System.

The absorbance of each well was standardized by subtracting the absorbance for the water control. Average well color development (AWCD) was used as a measure utilization of the carbon source in each well by the microbial community. The formula used to calculate AWCD was as follows:

$$\text{AWCD} = \Sigma \frac{(Optical\ Density\ in\ Carbon\ Source\ Well - Optical\ Density\ of\ Control)}{31}$$

A 3-way best fitting generalized linear model (GLiM) (Gamma distribution with log link function) as determined by the model with the lowest AIC was used to determine the interaction effect of APAP treatment and their respective impacts on AWCD. One way GLiMs were used to compare the effects of soil treatment among time points. GLiM, Post hoc pairwise comparisons were done using the least significant difference (LSD) to evaluate treatment effects within each time point. Biolog plate and community data from QIIME2 were imported into PAST to conduct canonical correspondence analysis (CCA).

Hydrolysis of fluorescein diacetate (FDA) was used as a proxy to measure microbial activity in soils treated with APAP-10 and without APAP. Soil similar to that used to grow eggplants as described above, was irrigated with APAP-10 in the greenhouse for three weeks, with no plants grown in it. Each treatment was replicated 4 times. The FDA assay and standard curve was carried out as described in Inbar et al. (1991), with the exception that 6.0 g of wet weight soil were incubated for 15 hours at 30 ℃. For the standard curve 50 mL acetone solutions containing 0 to 800 ug of FDA, in increments of 200 ug, were measured using spectrophotometry.

Results

Bacterial community

Illumina sequencing data indicated that APAP did not have an effect on community α-diversity. APAP-10 at T2 had the highest Shannon Wiener index value of 10.18, while the no CEC treatment at T2 had the lowest at 9.63 (Fig.1). The initial diversity present in the soil community at T0 was 10.04. These differences in diversity were not impactful, as no significant interactions nor differences were detected among treatments and time points (2-way GLiM: χ^2_2= 0.774, P = 0.679; χ^2_2= 1.874, P = 0.392; χ^2_2= 0.078, P = 0.780; respectively)

However, the community structure was altered by the addition of APAP. The initial overall PERMANOVA comparison did not detect significant differences among treatments (PERMANOVA pseudo-F = 1.85, pseudo-P = 0.149), while pairwise analyses

did. APAP-10-T1 soil community structure was significantly different from the T0 soil community (PERMANOVA pseudo-F = 1.743, pseudo-P = 0.047) (Fig. 2). At T2 the soil community structures treated with APAP-10 and APAP-5 were significantly different from the T0 soil community (PERMANOVA pseudo-F = 2.100, pseudo-P = 0.026; pseudo-F = 1.749, pseudo-P = 0.016; respectively). At T1 and T2, the untreated soil community structure was not significantly different from the T0 community (PERMANOVA all P's > 0.05). The relative abundance of different bacterial groups was impacted by the addition of APAP. A total of 748 ASVs were identified among all samples, and 247 of them were found to be significantly differentially abundant between T0 and the APAP-10 T2 communities (QIIME1 group significance Kruiskal-Wallist-test; all P's < 0.05, All FDR < 0.17. In all treatments, Proteobacteria were the most abundant in the soil with a relative abundance between 40-60% (Fig. 3). The relative abundance of the Chloroflexi phylum more than doubled in any soils treated with APAP, but decreased in the untreated soils between T1 and T2 (Fig. 3). The relative abundance of the Actinobacteria class increased from 6.3% and 8.6% at T1 to 9.8% and 11.0% at T2 for APAP-10 and APAP-5, respectively. This was lower than in the untreated soil that had relative abundance of 9.4% at T1 and 17.8% at T2 (Table 1). Bacteroidetes phylum abundance was lower in APAP treated soil than untreated soils by time point T2 with only 11.9% and 13.2% relative abundance for APAP-10 and -5 respectively, compared to 15.2% for the untreated soil community. Indicating an inverse relationship between abundance of the Bacteroidetes phylum and APAP concentration. At T2, the Gemmatimonadetes class had higher relative abundance in the APAP-10 and -5 treated

soil (8.3% and 6.5%, respectively) compared to the untreated soil (4.4%, Table 1). The relative abundance of Firmicutes did not change significantly with time or APAP treatment, and remained between 2.3% and 3.6%. However, the relative abundance of Acidobacteria decreased in all treatments compared to the original soil and the largest decrease was observed in the untreated soil (3.3% to 1.3%; T0 to T2 respectively) (Table 1).

The PCA plot of the sequencing data revealed 5 taxonomic groups that had a strong impact on causing community differences among the treatments (Fig. 4). The Actinobacteria class contained numerous lower divisions of microbial taxa, with the majority of their abundance being significantly lower in APAP-10 treated soils than the initial soil T0 (QIIME1 group significance Kruskal-Wallis; all P's < 0.05). There were two distinct groups of microbes in the Gemmatimonadetes phylum that decreased significantly in abundance from T0 to T2 in the APAP-10 treated soils (QIIME1 group significance Kruskal-Wallis; all P's < 0.05, all FDR < 0.13). The Pseudomonadaceae family makes up a large component of the vector representing the Gammaproteobacteria class (denoted with * in Fig. 4), and this family significantly decreased in abundance between T0 and T2 in APAP-10 (QIIME1 group significance Kruskal-Wallis; all P's < 0.05). The decrease in abundance of Xanthomonadaceae family within the Gammaproteobacteria class (denoted with ** in Fig. 4) after APAP-10 treatment was not significant (QIIME1 group significance Kruskal-Wallis; P > 0.05, FDR > 0.13; Fig. 4). However, a few individual organisms of agricultural importance in Xanthomonadaceae

and Pseudomonadaceae families increased with the addition of APAP, namely *Lysobacter* spp. and *Pseudomonas viridiflava* respectively, whose relative abundance was 0.064% and 0.26% higher in APAP-10 treated soil compared to the untreated control by T2.

Metagenome prediction

Interestingly, the PICRUSt2 metagenome analysis predicted there to be 7,393 potentially expressed genes among all soil bacterial communities in this study. The initial ANOVAs to screen for biologically relevant differences in gene abundance among treatments found 521 such genes. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, 202 of them were involved in metabolic pathways (Kanehisa et al. 2012) (ANOVAs, all P's <0.05; all $\eta^2 > 0.40$). At T2, APAP-10 had more genes predicted to be significantly differentially abundant than T0, No CEC or APAP-5 treatments (Fig. 5, Table 2). APAP-10 T2 had 47 predicted genes that were significantly greater in abundance compared to the initial soil community.

A diverse set of predicted metabolic genes had increased in abundance by T2 compared to T0. The majority were observed in APAP treated soil communities. APAP-10 T2 had many predicted upregulated genes related to amino acid, carbohydrate, energy, cofactors and vitamins, terpenoids and polyketides metabolism, biosynthesis of other secondary metabolites (Table 2), but the no CEC soil community had only one metabolic gene predicted to be increased. Additionally, 92% of the predicted genes for the metabolism of

terpenoids and polyketides were observed in APAP treated soil communities and about 70% of them were in the APAP-10 T2 soil community. Overall, APAP-10 T2 had the highest number of predicted genes to increase in abundance which were in more diverse metabolism categories compared to the other treatments (Table 2).

Evaluating changes in microbial functional diversity

The PICRUSt2 analysis of the expected metagenome predicted there to be a higher abundance of metabolic genes in the APAP-10 T2 soil community compared to the other treatments, suggesting there to be higher rates of metabolism in the APAP-10 soil community. Therefore, these predictions were confirmed by evaluating soil community functions using the Biolog Ecoplate assay. The breakdown of various carbon sources directly (measured as the average well color development, AWCD) serves as a proxy to measure soil community activity (Liu et al. 2012). Across all the time points, carbon sources and CEC treatments, carbon utilization (measured as AWCD) was significantly higher in APAP-10 treated soil compared to the control (3 way GLiM: $X^2 = 190.327$, $P \leq 0.001$). Carbon utilization was also significantly different among carbon types and timepoints (3 way GLiM: $X^2 = 86.067$, $P \leq 0.001$ & $X^2 = 3253.563$, $P \leq 0.001$; respectively). A significant 3-way interaction was detected between APAP treatment, carbon type, and time point (3-way GLiM: $X^2 = 54.522$, $P = 0.003$). Significant 2-way interactions were detected among CEC treatments and carbon type, CEC treatments and timepoints, and carbon type and timepoints (3-way GLiM: $X^2 = 40.705$, $P \leq 0.001$; $X^2 = 56.559$ $P \leq 0.001$; $X^2 = 182.62$, $P \leq 0.001$; respectively). From 24 hours post incubation

(hpi) to 144 hpi, the AWCD of APAP-10 treated soils was significantly higher than that of untreated soils (one way GLiM: $X^2 = 2544.759$, $P \leq 0.001$; all post hoc LSD comparisons < 0.05). (Fig. 6A). The AWCD of APAP treated soils were 1.3%,12.7%, 18.7%, 22.2%, 22.2%, 20.6% higher than untreated soils after 24, 48, 72, 96, 120, and 144 hpi, respectively (Fig. 6A). By 96 hpi, carbon utilization for amines, amino acids, carbohydrates, carboxylic acids, and polymers were significantly higher for APAP treated soils than untreated soils (One-way GLiM: $X^2 = 101.607$, $P \leq 0.001$; all post hoc LSD $P < 0.05$; Fig 6B).

The CCA analyses conducted to determine if substrate utilization could be a factor in shaping soil community differences indicated that amino acid, carbohydrate, carboxylic acids, and polymer metabolism contributed to community structural differences. These had the largest vectors on the CCA plot, indicating that differences in these metabolic pathways between treated and untreated soil communities had a large effect on influencing community structure (Fig. 7)

To confirm the results of the Biolog plates, the FDA hydrolysis assay was used as a proxy for soil community activity. The hydrolysis activity of the different treatments were, 169.94, 161.32, and 154.33 ug of FDA per g of dry soil, for APAP-10, APAP-5 and the untreated soil respectively. The amount of FDA hydrolyzed in the APAP-10 treated soil was significantly higher than in the untreated soil (ANOVA: $F_2 = 6.94$ $P = 0.018$;

Tukey pairwise comparisons $P < 0.05$). Thus, indicating higher microbial activity in APAP-10 treated soil. These results parallel well with the above Biolog Ecoplate results.

Discussion

The combined observations of the 16s rRNA data, predicted metagenome, Biolog EcoPlate assays and FDA analysis indicate that the bacterial communities in our soil samples were sensitive to the APAP concentration used in this study. Significant community differences were observed within 3 weeks of APAP treatment and significant differences in carbon metabolism were observed between treated and untreated samples collected 7 weeks after starting treatment. Taken together, our results show that APAP altered the soil bacterial communities and impacted community functions within a single growing season of eggplants.

In our study, APAP treatment did not change community **α**-diversity levels as previously observed in another study (Pino-Otín et al. 2017). This may be due to the fact that plants can stabilize their associated soil microbial communities (Berendsen et al. 2012; Hamonts et al. 2018). Given that microbes are not impacted equally by a disturbance (Colloff et al. 2008; Ding and He 2010), our results suggest that APAP did not completely displace many bacterial taxonomic groups, but caused a shift in the relative abundance of certain groups. Since we observed increases in microbial activity, (i.e. increased substrate utilization and FDA hydrolysis) it is possible that APAP was acting as a carbon source for a subset of soil community members and selected for microbes that can utilize it

directly, or indirectly (Bartha et al. 2010; Li et al. 2014; Huang et al. 2006; Huber et al. 2009; Palma et al. 2021). The differences in **β**-diversity between APAP treated soils and the initial sampling point suggests that the microbial communities were sensitive to APAP at the concentrations found in RWW, especially after 7 weeks of exposure. This is consistent with previous observations that indicated that pharmaceutical products, including APAP, can impact microbial communities, and hinder or disrupt key microbial functions (Zimmermann and Curtis 2017; Wang and Gunsch 2011; Barra Caracciolo et al. 2015; Alvarino et al. 2014). Therefore, our rationale is that since APAP is found in RWW as an intact active compound and is broken down into a glucoside by soil fungi or plants (Bartha et al. 2010; Huang et al. 2006; Huber et al. 2009) or into the carboxylic acid 2-hexenoic acid by soil microorganisms (Li et al. 2014), the intact active compound and breakdown products of APAP can be utilized as carbon sources by soil bacteria (Blackall et al. 1985; Chun et al. 1999; Mergaert et al. 2003). Bacterial groups that can utilize these carbon sources will most likely be selected for in APAP contaminated soils, thus altering the soil microbiome. Plant health is intimately related to its associated soil microbiome and its functions, thus any alterations to the microbiome could have negative impacts on plant productivity (Van der Heijden et al. 2008; Chaparro et al. 2012).

The APAP concentrations used in our study represent levels found in RWW effluent (Batt et al. 2007; Fram and Belitz 2011; Kibuye et al. 2019; Kasprzyk-Hordern et al. 2009; Dia-Cruz and Barcelo 2008). The exact concentrations of APAP in RWW effluent vary among regions and across seasons, and have been observed to reach concentrations up to 112.78 ug/L, with averages between 0.0081 ug/L (Kinney et al. 2006) and 11.73

ug/L (Kibuye et al. 2019; Kasprzyk-Hordern et al. 2009). Additionally, soils that are irrigated with RWW effluent can accumulate between 604% and 4860% of the APAP found in irrigation water. Our results demonstrated that these concentrations can impact soil microbial communities, especially with repeated exposure. Our results concur with previous findings showing that microbes in agricultural soils are sensitive to APAP present in RWW (Barra Caracciolo et al. 2015; Pino-Otín et al. 2017; Gielen et al. 2011). However, in these other studies the resolution for detecting specific microbial community members was limited because they relied on non-sequencing-based approaches to characterize changes in the soil microbial community. In contrast, our study was able to detect specific shifts in the microbial community and identify specific bacterial groups that were impacted by APAP treatment using Illumina sequencing-based approaches.

When the microbial community shifts, community functions may also change. Addition of APAP to soils disrupted key aspects of nitrogen cycling although the concentrations of APAP (50 to 1,000 mg/L) used in those studies were greater than those found in RWW effluent (Barra Caracciolo et al. 2015; Alvarino et al. 2014). Our Biolog assay showed altered microbial functions using concentrations within the range found in RWW effluent (10 ug/L). Besides lower APAP concentration, our study distinguishes itself from previous ones in a few other ways. Unlike previous studies that focused on nitrogen cycling (Colloff et al. 2008; Underwood et al. 2011), our study examined utilization of 31 ecologically relevant carbon sources (Table 1). This approach encompasses a much larger portion of the soil microbial community and was not limited to a specific set of

community members such as anammox bacteria (Alvarino et al. 2014), or bacteria that contain amoA, napaA, or nifH genes for nitrification, denitrification, or nitrogen fixation respectively (Colloff et al. 2008). Thus, by using various carbon sources we screened for a wide variety of bacterial groups that were impacted by APAP treatment. In addition, we employed secondary methods to identify the specific microbes responsible for the observed changes unlike these other studies that examined microbial community functions using Biolog plates (Liu et al. 2009; Pino-Otín et al. 2017; Gielen et al. 2011). Data obtained from the Biolog Ecoplates does not represent true, *in situ*, rates for soil bacteria community metabolism, because it only measures metabolism from a subset of organisms capable of growing under laboratory conditions and may not reflect *in situ* conditions. Despite this shortcoming, many studies have demonstrated that it is a great method to evaluate changes in soil community functions due to disturbances or changes in biotic and abiotic factors (Liu et al. 2012; Pino-Otín et al. 2017; Gielen et al. 2011; Adams et al. 2017; Du Plessis et al. 2005; Harris-Hellal et al. 2009).

In our results, APAP treated samples had significantly higher rates of carbon utilization in nearly every category measured by 96 hpi (amines, carbohydrates, amino acid, carboxylic acid, and polymer metabolism) compared to the controls. APAP may not be a carbon source for all organisms, therefore, its addition to the soil might have selected for microbes that metabolize it (Liu et al. 2012) demonstrated that APAP is broken down in non-sterilized soil, but not in sterilized soil, indicating that soil microbes metabolize APAP. Metabolomics analyses of APAP treated soil revealed that the microbes break

down APAP to 8 identifiable intermediates (Liu et al. 2012). The intermediate 2-hexenoic acid, a carboxylic acid, was the most abundant metabolite in the soil after APAP treatment (Li et al. 2014). Therefore, we hypothesized that APAP treatment in our study increased carboxylic acid content in the soil, which in turn led to an increase of microbes that metabolize carboxylic acids. The Biolog assay confirmed our hypothesis to be true by showing a significant increase in carboxylic acid metabolism in APAP treated soils.

Using PICRUSt2, we developed initial predictions on the expected impacts of APAP on microbial communities. The PICRUSt2 metagenome predictions paralleled the trend of increased carbon metabolism in APAP treated soil observed in the Biolog assay. The gene prediction data indicated that soil communities treated with APAP may increase in multiple genes for a variety of carbon metabolism pathways, most notably for amino acid and carbohydrate metabolism. This concurred with our Biolog plates data that indicated significantly higher utilization of amino acids and carbohydrates in APAP treated soils compared to the untreated controls. The CCA of the Biolog assay also indicated that amino acid and carbohydrate metabolism had a strong impact on community shifts between the APAP-10 and untreated soil microbial communities (Fig. 7). Previous studies have also indicated that additional carbon input led to increased soil microbial activity measured as respiration (Gallardo and Schlesinger 1994; Carney et al. 2007; Cleveland et al. 2007; García-Orenes et al. 2010), FDA dehydrogenase activity (García-Orenes et al. 2010; Friedel et al. 1994; Adak et al. 2014; Chander et al. 1998) or microbial biomass (Chander et al. 1998). Data from our Biolog assay was congruent with

the PICRUSt2 predictions and FDA hydrolysis which all showed increased carbon metabolism after APAP treatment. Therefore, we presume that APAP was acting as a carbon source, and thus stimulating microbial activity. However, additional studies are required to confirm this assumption.

PICRUSt2 predictions were based on functions linked to given 16s rRNA genes that were detected in our soil samples. Thus, shifts in the abundance of 16s rRNA genes may be interpreted as shifts in community functions, however since these are predictions based on the presence of 16s rRNA genes, these results should be confirmed using another method. By utilizing the Biolog plate assay we were able to examine changes in metabolic rates for specific substrates, and test these predictions. We observed that shifts in relative abundance of the soil microbial community members were consistent with the observed changes in the microbial community function determined in the Biolog assay. *Amycolatopsis thermoflava* and *Cellvibrio* spp., microbial groups that increased in relative abundance after APAP application, were major contributors to community differences among the soil communities. They are capable of metabolizing a diverse set of carbon substrates, including glycosides (Blackall et al. 1985; Chun et al. 1999; Mergaert et al. 2003). Glycosides are major breakdown products of APAP due to microbial activity in the soil (Huang et al. 2006; Huber et al. 2009) and plant detoxification (Bartha et al. 2010). Their accumulation in plant roots or in the soil probably led to the increase of glycoside metabolizing organisms like *Cellvibrio* bacteria. *Cellvibrio* is a genus of cellulolytic bacteria that are capable of degrading plant cell walls.

Some *Cellvibrios* can utilize many different carbohydrates including α- and β- glycosides (Blackall et al. 1985; Mergaert et al. 2003). These cellulolytic organisms can have major impacts on the soil community by degrading refractory cellulose, and thus making substrates available to other community members (Lynd et al. 2002; Štursová et al. 2012). *Cellvibrio* spp. can also utilize carboxylic acids, which are another major breakdown product of APAP (Li et al. 2014).

Additionally, the relative abundance of Acidobacteria was higher in APAP treated soils than in untreated controls. This concurs with other studies that observed higher relative abundance of Acidobacteria in the presence of a mixture of pharmaceuticals, including APAP (Rios-Miguel et al. 2021). Examples of Acidobacteria that followed this trend were *Candidatus Koribacter* and *Candidatus Solibacter*. *Candidatus* spp. have optimum growth at pH >6 (Koch et al. 2008; Sait et al. 2006) and pH plays a significant role in the growth of some Acidobacteria than other factors (Sait et al. 2006; Kielak et al. 2016). The pH of APAP in a saturated aqueous solution is about 6 (PubChem Compound Summary for CID 1983...), which may explain why Acidobacteria were more abundant in APAP treated soils than in the untreated controls. However, not all microbes were tolerant to APAP. For example, there was a decrease in the relative abundance of Actinobacteria in APAP treated soil compared to the untreated control. Several strains of *Actinomyces* (a genus in the class Actinobacteria) cannot metabolize APAP (Huang et al. 2006). This could explain the reduction in the relative abundance of Actinobacteria observed in our study. A group of bacteria identified to the Gemmatiomadetes phylum also decreased in

abundance in APAP treated soil. Two species in this phylum, *Gemmatimonas aurantiaca* and *G. phototrophica* are fastidious with carbon utilization, thus they may not be able to use APAP or its metabolites (Zhang et al. 2003; Zeng et al. 2015). Having particular carbon requirements may partially explain the decrease in Gemmatiomonadetes phylum members (DeBruyn et al. 2011). However, this needs to be evaluated further.

Irrigation with APAP impacted soil microbes of agricultural importance. For example, *Pseudomonas viridiflava* and *Lysobacter* spp. which increased in soils treated with APAP. *P. viridiflava* is pathogenic to approximately 30 plant species including eggplants, kiwis, tomato and melon (Young et al. 1988). This pathogen causes soft rot and subsequent browning of the stem or flowering parts, which leads to economic losses to the growers and predisposes the plants to fungal infections (Young et al. 1988; Everett and Henshall 1994). In this study, eggplants were grown in the soil irrigated with APAP, and we think that continued use of irrigation water containing APAP may favor infections by *P. viridiflava. Lysobacter* spp. are recognized for their potential as biological control agents of several plant diseases of economic importance such as *Fusarium* head blight of wheat, brown patch in turfgrass caused by *Rhizoctonia solani*, *Pythium* damping-off of sugarbeet and summer patch disease of Kentucky bluegrass caused by the root-infecting *Magnaporthe poae* (Jochum et al. 2006; Giesler and Yuen 1998; Kobayashi and Yuen 2005; Kobayashi et al. 2005). Therefore, irrigation with RWW containing APAP may cause the soils to be suppressive to several fungal diseases.

Our findings highlight the need to investigate the impacts of RWW on plant-microbe interactions. The fact that both plant pathogens and disease suppressive organisms increased in presence of APAP, underscores the complexity of soil systems and the impacts of APAP and other CECs found in RWW. The effects of using RWW are multifaceted and many more studies are needed to unravel this issue and to ensure that RWW can be used in a way that continues to persevere clean water supplies while facilitating the growth of healthy crops. Our study demonstrated that APAP concentrations found in RWW can alter soil microbial diversity and functions which may impact plant health and productivity. In spite of the benefits of RWW to agriculture, further investigation into effects of different CECs on soil microbes is needed in order to understand the risk that CECs may pose to natural and agricultural environments.

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Figures

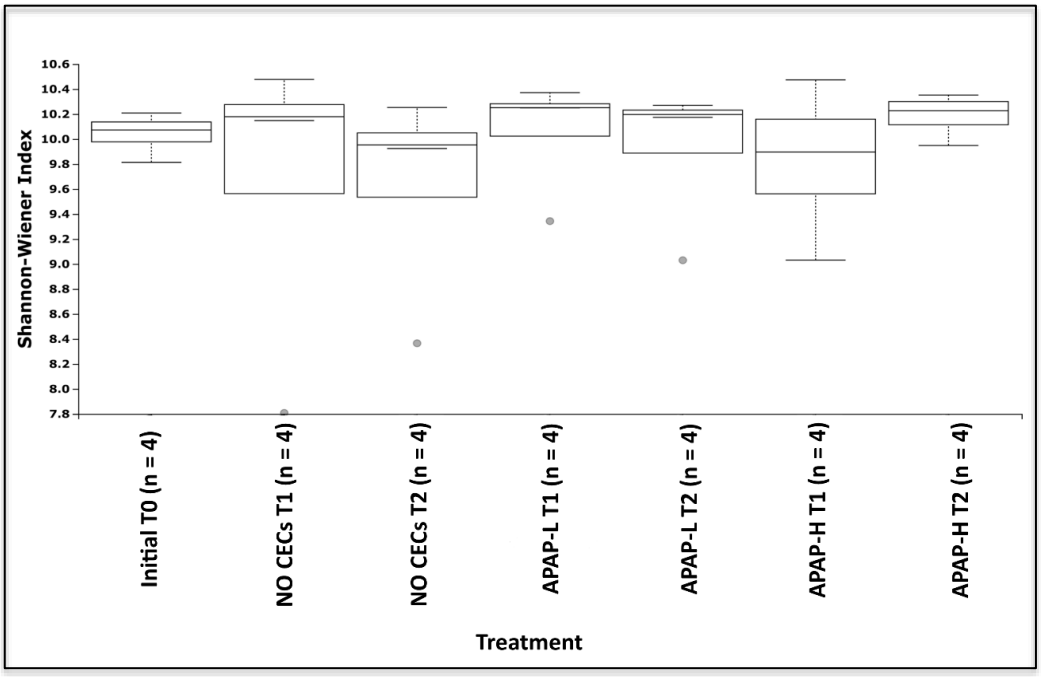


Figure 2.1 Box plots comparing the Shannon Wiener index of samples treated with 10 µg/L (APAP 10) or 5 µg/L of acetaminophen (APAP 5) and the untreated control (No CEC) collected at the beginning of the experiment, 3 and 7 weeks after start of treatment (T0, T1 and T2 respectively).

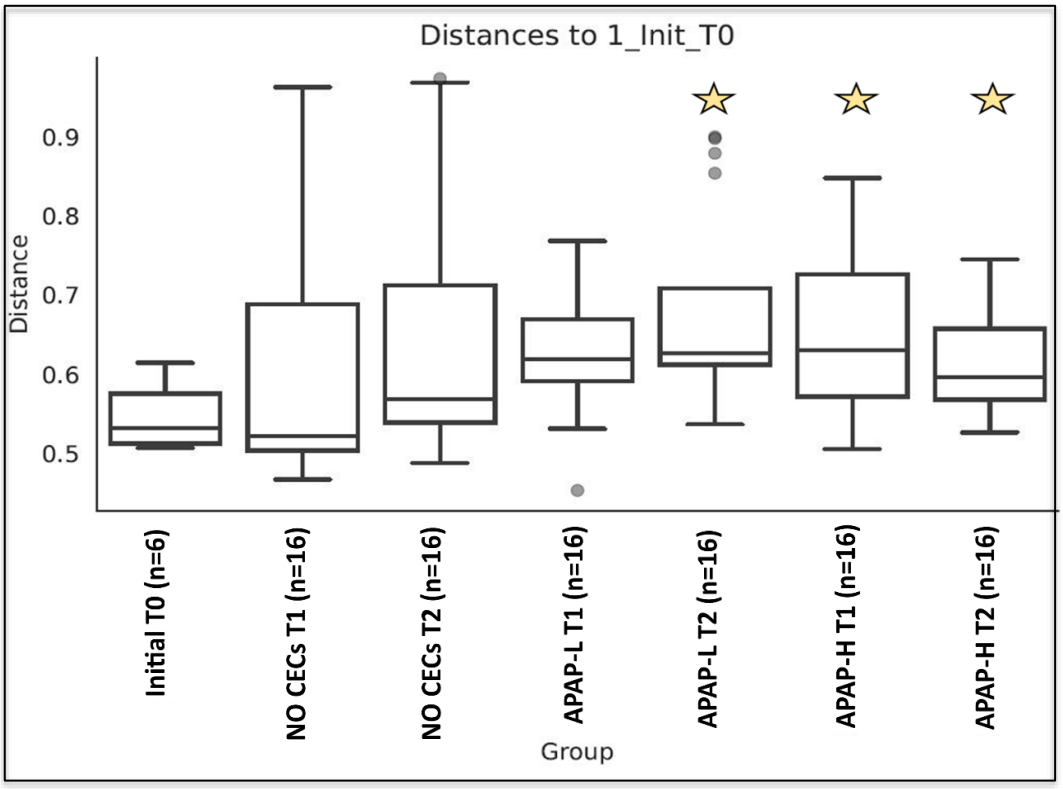


Figure 2.2 Box plot of Bray Curtis distance samples untreated and treated with 10 µg/L or 5 µg/L of acetaminophen (APAP 10 or APAP 5 respectively) and the untreated control (No CEC), and collected at the beginning of the experiment, 3 and 7 weeks after start of treatment (T0, T1 and T2 respectively). Star denotes samples that were significantly different from diversity values compared to the initial, T0, samples. Pairwise PERMANOVA all P's <0.05.

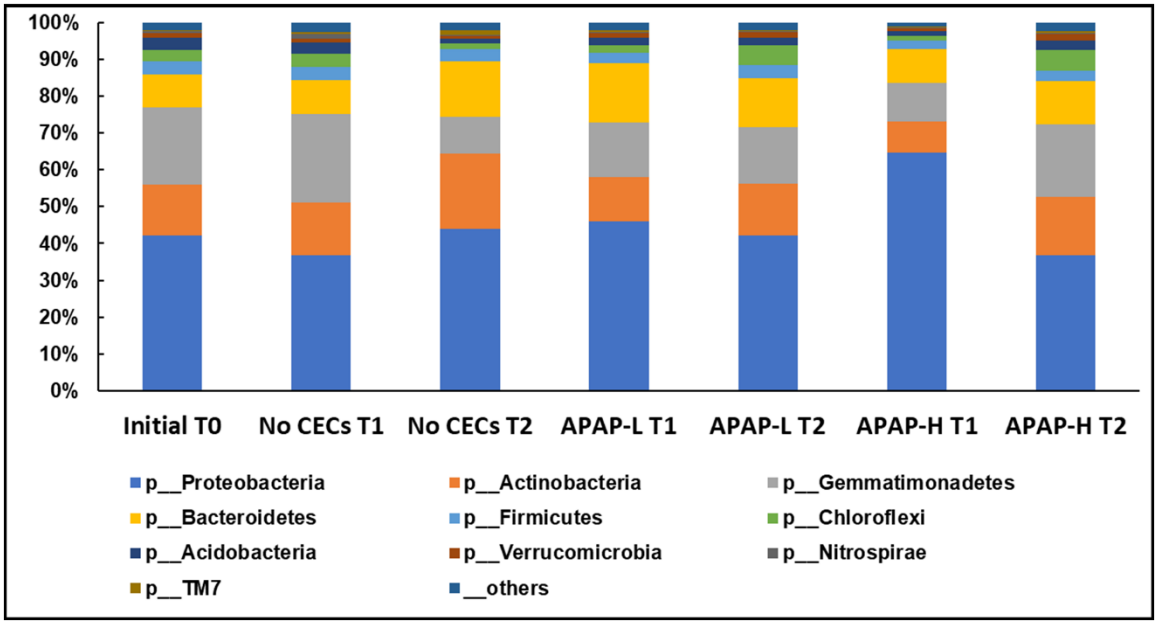


Figure 2.3 The relative abundance of bacteria in soil samples treated with 10 µg/L or 5 µg/L of acetaminophen (APAP 10 or APAP 5 respectively) and the untreated control (No CEC), and collected at the beginning of the experiment, 3 and 7 weeks after start of treatment (T0, T1 and T2 respectively). Relative abundance of the bacteria was determined at the phylum level only. P = phylum

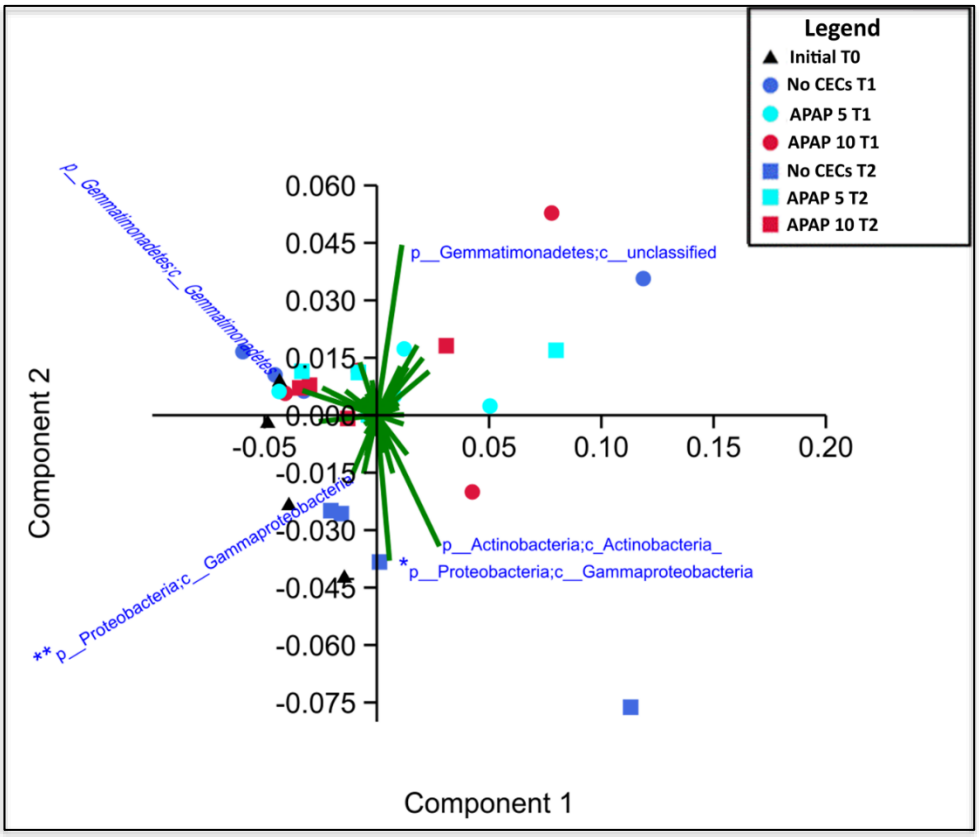


Figure 2.4 PCA graph of Illumina sequencing data with plotted vectors showing community members that contributed most to the variability in soil communities from soil treated with 10 µg/L or 5 µg/L of acetaminophen (APAP-10 and APAP-5 respectively) and the untreated control (No CEC), and collected at the beginning of the experiment, 3 and 7 weeks after start of treatment (T0, T1 and T2 respectively). * = Gammaproteobacteria class containing the Pseudomonadaceae family, ** = Gammaproteobacteria class containing the Xanthomonadaceae family

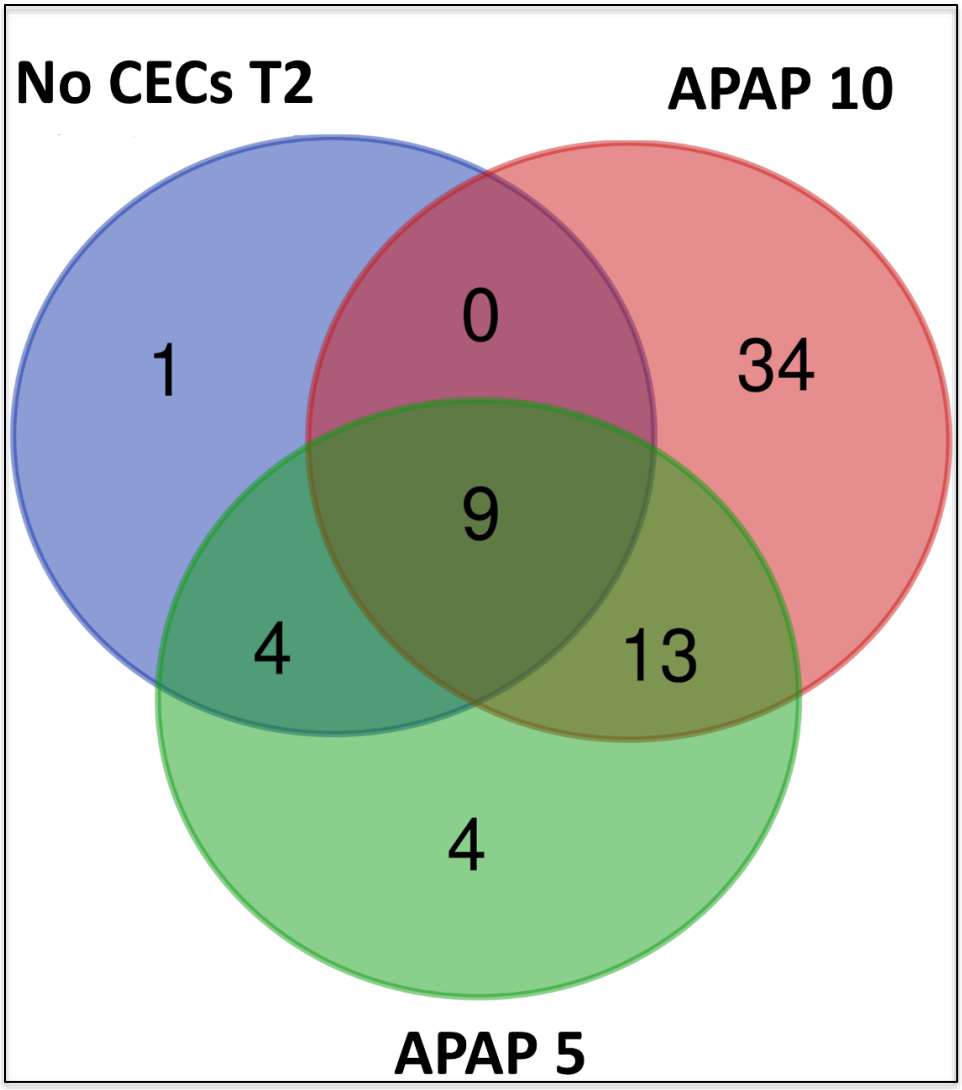
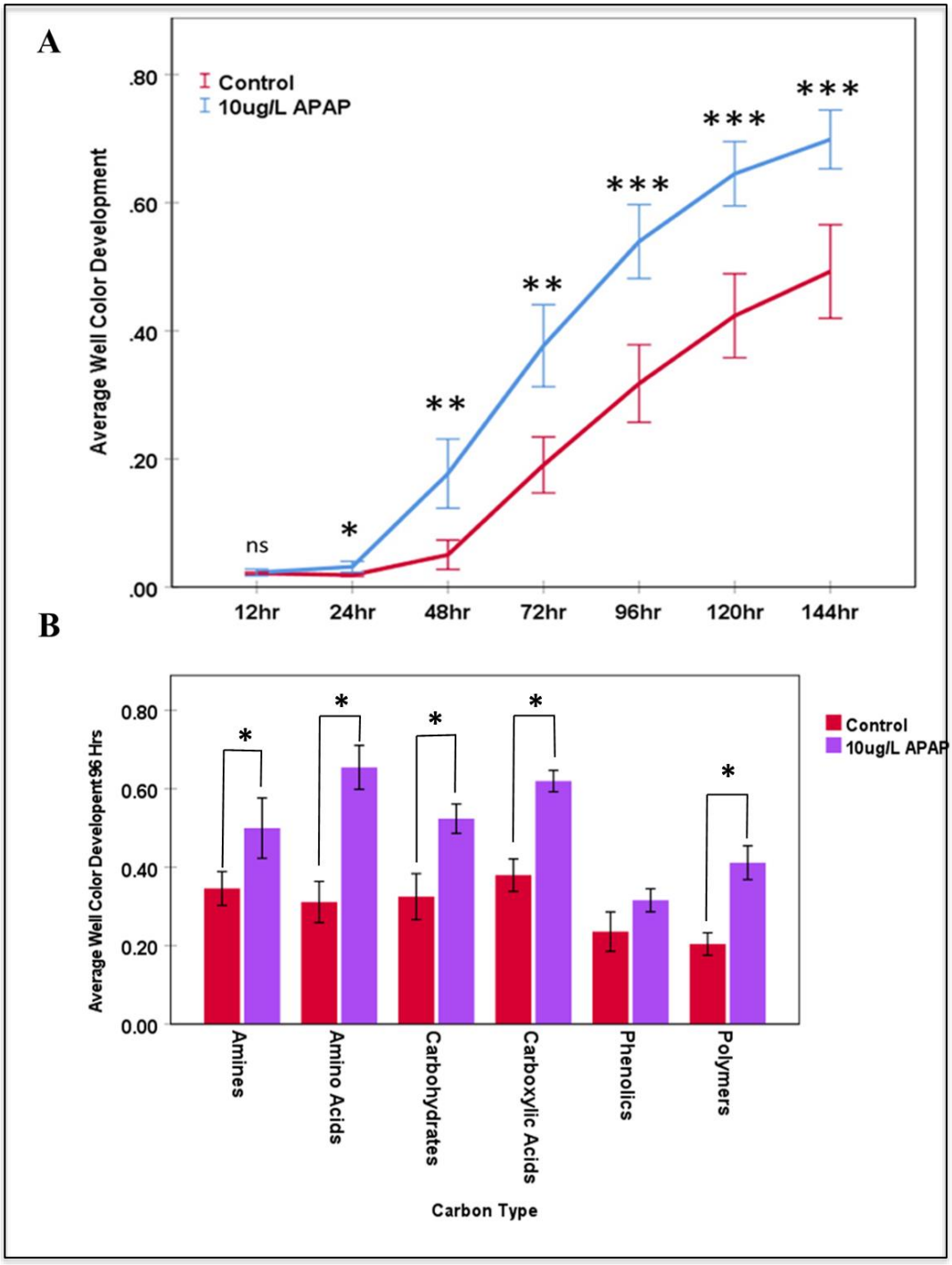


Figure 2.5 Venn diagram comparing genes predicted to be significantly more abundant in soil communities from soil treated with 10 μg/L or 5 μg/L of acetaminophen (APAP-10 and APAP-5 respectively) and the untreated control (No CEC), and collected at the beginning of the experiment and 7 weeks after start of treatment (T0 and T2 respectively) compared to the initial T0 communities (Welch's T test, all, all P's < 0.05)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0.20
0.00
Control
10ug/L APAP
Amines
Amino Acids
Carbohydrates
Carboxylic Acids
Phenolics
Polymers
Carbon Type

Figure 2.6 Average Well Color Development in Biolog EcoPlate wells containing samples from soil treated with 10 μg/L of acetaminophen (10 μg/L APAP) and the untreated control collected 7 weeks after start of treatment. A) Shows total Average Well Color Development for all carbon sources in treated and control samples over the course of 144 hours. B) Shows Average Well Color Development of each functional carbon group 96 hours after incubation. For all graphs, error bars represent the standard error. Lines between treatments represent GLiM post hoc LDS comparisons between control and 10μg/L APAP. Single asterisks represent a p-value <0.05, double asterisks represent a p-value < 0.01, and triple asterisks represent a p-value <0.001.

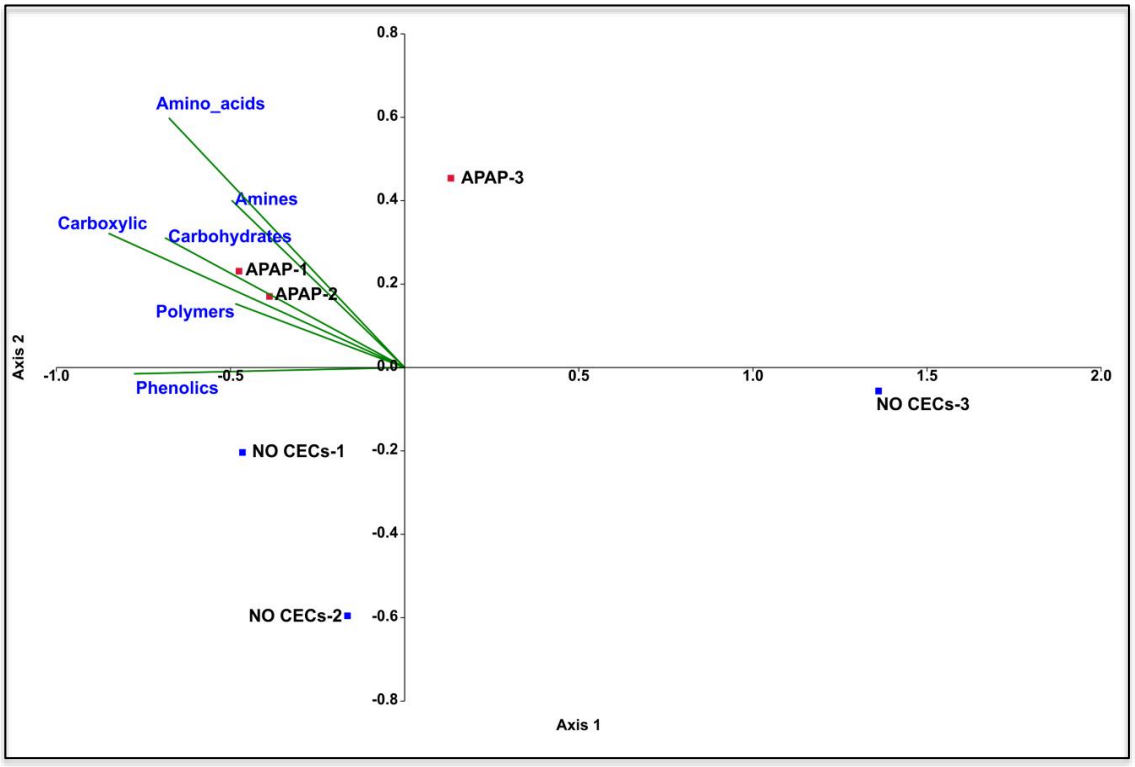

Figure 2.7 Canonical correspondence analysis in PAST of Biolog Ecoplates that were incubated for 96 hours with samples from soil treated with 10 µg/L of acetaminophen and the untreated control and collected 7 weeks (T2) after start of treatment. The figure shows the utilization of the 6 general carbon substrate groups. The vectors, in green, represent a given carbon substrate while vector length indicates the impact of the given factor on community differences. Each treatment had 3 replicates (n=3). APAP-1= APAP-10 T2 replicate 1, APAP-2= APAP-10 T2 replicate 2 and APAP-3= APAP-10 T2 replicate 3

Tables

Table 2.1 All 31 carbon substrates tested by the Biolog Ecoplates.

Substrate name	Category according to Adams	Category according to Adams	
Water (Blank)	**x**	x	
B-methyl-D-Glucoside	**carbohydrate**	carbohydrate	3
D-Galactonic Acid y-Lactone	**carbohydrate**		3
L-Arginine	**amino acid**		2
Pyruvic Acid Methyl Ester	**carboxylic acid**		4
D-Xylose	**carbohydrate**		3
D-Galacturonic Acid	**carboxylic acid**		4
L-Asparagine	**amino acid**		2
Tween40	**polymer**		6
i-Erythritol	**carbohydrate**		3
2-HydroxyBenzoicAcid	**phenolics**	also a carboxylic acid	5
L-Phenylalanine	**amino acid**		2
Tween80	**polymer**		6
D-Mannitol	**carbohydrate**		3
4-HydroxyBenzoicAcid	**phenolics**	also a carboxylic acid	5
L-Serine	**amino acid**		2
a-Cyclodextrin	**polymer**		6
N-Acetyl D-Glucosamine	**amine**		1
y-Hydroxybutyric Acid	**carboxylic acid**		4
L-Threonine	**amino acid**		2
Glycogen	**polymer**		6
D-Glucosaminic Acid	**carboxylic acid**		4
ItaconicAcid	**carboxylic acid**		4
Glycyl-L-GlutamicAcid	**carboxylic acid**		4
D-Cellobiose	**carbohydrate**		3
Glucose 1 Phosphate	**carbohydrate**		3
a-Ketobutytric Acid	**carboxylic acid**		4
Phenylethylamine	**amine**		1
a-D-Lactose	**carbohydrate**		3
D,L -a-Glycerol Phosphate	**carbohydrate**		3
D-Malic Acid	**carboxylic acid**		4
Putrescine	**amine**		1

Supplemental Table 2.2 The Differentially expressed metabolic genes that were predicted to be upregulated in each treatment after 7 weeks (T2)

Venn diagram category	KEGG ID	Definition	General pathway
T2 APAP-H only	K06001	tryptophan synthase beta chain	Amino acid metabolism
	K10793	D-proline reductase	Amino acid metabolism
	K12674	(carboxyethyl)arginine beta-lactam-synthase	Biosynthesis of other secondary metabolites
	K12675	Clavaminate synthase	Biosynthesis of other secondary metabolites
	K12673	N2-(2-carvoxyethyl)arginine synthease	Biosynthesis of other secondary metabolites
	K12676	Proclavaminate amidinohydrolase	Biosynthesis of other secondary metabolites
	K01452	Chitin deacetylase	Carbohydrate metabolism
	K19668	Cellulose 1,4-beta-cellobiosidease	Carbohydrate metabolism
	K07106	N-acetylmuramic acid 6-phosphate etherase	Carbohydrate metabolism
	K12449	UDP-apiose/xylose synthase	Carbohydrate metabolism
	K01233	chitosanase	Carbohydrate metabolism
	K13810	Transaldolase / glucose-6-phosphate isomerase	Carbohydrate metabolism
	K01179	endoglucanase	Carbohydrate metabolism
	K15916	Glucose/manose-6-phosphate isomerase	Carbohydrate metabolism
	K18649	Inositol -phosphate phostate / L-galactose 1-phospte phosphatase	Carbohydrate metabolism, Amino acid metabolism
	K00844	Hexokinase	Carbohydrate metabolism, Biosynthesis of other secondary metabolites
	K15052	propionyl	Energy metabolism
	K00387	Sulfite oxidase	Energy metabolism
	K12234	Coenzyme F420-0:L-glutamate ligase	Energy metabolism
	K11212	2-phospho-L-lactate transferase	Energy metabolism
	K08256	phosphatidyl-myo-inositol alpha-mannosyltransferase	Glycan biosynthesis and metabolism
	K06928	nucleoside-triphosphatase	Metabolism of cofactors and vitamins
	K01492	phosphoribosylglycinamide	Metabolism of cofactors and vitamins
	K03851	taurine-pyruvate aminotransferase	Metabolism of other amino acids
	K05553	minimal PKS acyl carrier protein	Metabolism of terpenoids and polyketides
	K13329	dTDP-4-dehydro-2,3,6-trideoxy-D-glucose 4-aminotransferase	Metabolism of terpenoids and polyketides
	K05554	aromatase	Metabolism of terpenoids and polyketides

	K13317	NDP-4-keto-2,6-dideoxyhexose 3-C-methyltransferase	Metabolism of terpenoids and polyketides
	K05552	tetracycline polyketide synthase	Metabolism of terpenoids and polyketides
	K14369	erythromycin 3''-O-methyltransferase	Metabolism of terpenoids and polyketides
	K16435	dTDP-4-dehydro-6-deoxy-alpha-D-glucopyranose 2,3-dehydratase	Metabolism of terpenoids and polyketides
	K05551	minimal PKS ketosynthase	Metabolism of terpenoids and polyketides
	K16004	narbonolide/10-deoxymethynolide desosaminyltransferase	Metabolism of terpenoids and polyketides
	K18445	diadenosine hexaphosphate hydrolase (ATP-forming)	Nucleotide metabolism
T2 APAP-L only	K07405	alpha-amylase	Carbohydrate metabolism
	K11645	fructose-bisphosphate aldolase, class I	Carbohydrate metabolism, Energy metabolism
	K00002	alcohol dehydrogenase (NADP+)	Carbohydrate metabolism, Lipid metabolism, Xenobiotics biodegradation and metabolism
	K02805	dTDP-4-amino-4,6-dideoxygalactose transaminase	Glycan biosynthesis and metabolism
T2 No CECs only	K00972	UDP-N-acetylglucosamine/UDP-N-acetylgalactosamine diphosphorylase	Carbohydrate metabolism
APAP-H and APAP-L	K00693	glycogen synthase	Carbohydrate metabolism
	K00033	6-phosphogluconate dehydrogenase	Carbohydrate metabolism, Metabolism of other amino acids
	K05979	2-phosphosulfolactate phosphatase	Energy Metabolism
	K02636	cytochrome b6-f complex iron-sulfur subunit	Energy Metabolism
	K00956	sulfate adenylyltransferase subunit 1	Energy Metabolism, Biosynthesis of other secondary metabolites, Nucleotide metabolism, Metabolism of other amino acids
	K02259	heme a synthase	Energy Metabolism, Metabolism of cofactors and vitamins
	K18660	malonyl-CoA/methylmalonyl-CoA synthetase	Lipid metabolism, Amino acid metabolism
	K00632	acetyl-CoA acyltransferase	Lipid metabolism, Amino acid metabolism, Metabolism of terpenoids and polyketides, Xenobiotics biodegradation and metabolism
	K00949	thiamine pyrophosphokinase	Metabolism of cofactors and vitamins

	K01674	carbonic anhydrase	Metabolism of cofactors and vitamins
	K01724	4a-hydroxytetrahydrobiopterin dehydratase	Metabolism of cofactors and vitamins
	K02361	isochorismate synthase	Metabolism of cofactors and vitamins, Metabolism of terpenoids and polyketides
	K16422	4-hydroxymandelate oxidase	Metabolism of terpenoids and polyketides, Biosynthesis of vancomycin group antibiotics, Biosynthesis of other secondary metabolites
APAP-L and No CECs	K00293	Saccharopine dehydrogenase (NADP+, L-glutamate forming)	Amino acid metabolism
	K14259	2-dehydro-3-deoxy-D-arabinonate dehydratase	Carbohydrate metabolism
	K01823	isopentenyl-diphosphate Delta-isomerase	Metabolism of terpenoids and polyketides
	K00757	uridine phosphorylase	Nucleotide metabolism, Xenobiotics biodegradation and metabolism
APAP-H, APAP-L, and No CECs	K16055	trehalose 6-phosphate synthase/phosphatase	Carbohydrate metabolism
	K01051	pectinesterase	Carbohydrate metabolism
	K07404	6-phosphogluconolactonase	Carbohydrate metabolism
	K18429	GDP/UDP-N,N'-diacetylbacillosamine 2-epimerase	Carbohydrate metabolism
	K13016	UDP-N-acetyl-2-amino-2-deoxyglucuronate dehydrogenase	Carbohydrate metabolism
	K18430	N,N'-diacetyllegionaminate synthase	Carbohydrate metabolism
	K08092	3-dehydro-L-gulonate 2-dehydrogenase	Carbohydrate metabolism
	K15862	cytochrome c oxidase cbb3-type subunit I/II	Energy metabolism
	K01113	alkaline phosphatase D	Metabolism of cofactors and vitamins

Supplemental Methods

Eggplant seedlings of the Patio Baby variety at the 2-4 leaf stage were transplanted into 2 liter pots containing soil collected from organic fields in Bakersfield, California. These are very deep poorly drained soils formed in stream alluvium derived from granitic rock (Bakersfield Series 2006) The soil did not have a history of irrigation with RWW. The plants for each treatment were maintained on trays and were irrigated 2-3 times a week using a liter of water spiked with either 5 or 10 μg/L of APAP, while the control plants were irrigated with tap water. The treatment started one week after transplanting and was carried out for 10 weeks. Soil cores of 1 cm diameter and 3 cm depth were collected weekly from the rhizosphere of the plants at least 3 cm from the stem and stored at -20 degrees Celsius. The treatment was replicated 4 times and each replicate had 5 plants. Soil samples collected before the onset of the treatment (T0), at 3 (T1) and 7 (T2) weeks after starting the treatment were chosen for further analysis. At the T1 and T2 the plants had been irrigated with APAP spiked water 7 and 15 times respectively. Therefore, a cumulative amount 35 μg and 70 μg of APAP had been added to 5 pots of the APAP-5 and APAP-10 treatments respectively at T1, while 75 μg and 150 μg had been added to 5 pots of APAP-5 and APAP-10 respectively at T2.

Supplemental References

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CHAPTER 3

Impacts of the fungicidal agents Ridomil Gold and SoilGard on the soil bacterial community associated with carrots inoculated with *Pythium*

Abstract

Protecting an efficient, high plant yield for crops is crucial for agriculture to be able to produce enough food to sustain the current and growing population. Preventing crop loss to plant pathogens also protects the agriculture industry from huge financial losses that can negatively impact the entire economy. Compounds such as chemical fungicides and biological control agents such as microorganisms that are antagonistic towards plant pathogens, have been deployed successfully to reduce the impacts of plant pathogens. Unfortunately, off-target effects of these chemicals have been observed in which the soil microbiome is altered in their presence. Alterations to the soil microbiome can negatively impact plant health and plant yields. For these reasons this study evaluates the impacts of the chemical fungicide Ridomil and the biofungicide SoilGard on the soil bacteria community associated with carrots. Our findings parallel the observations of other fungicidal agents that have been studied previously. The soil bacterial communities in this study were different in the presence of these agents, and the potential for soil community functions to be altered was also observed.

Introduction

A large part of California's economy relies on sales produced from crops. Unfortunately, many California crops are susceptible to at least one form of pathogen that is capable of reducing overall yields (Lu et al. 2012; Mahato et al. 2017; Manda et al. 2020). Growers address yield loss from pathogens by employing techniques such as crop rotation and the applications of cidal chemicals (such as fungicides) or mixtures of organisms that are antagonistic towards the pathogens (aka biofungicides) (Opender Koul 2012). However, off-target effects have been previously observed for fungicides such as altering the growth of growth promoting rhizobacteria (PGPR) (Mubeen et al. 2006; Gallori et al. 1991) and altering nitrogen cycling carried out by soil microorganisms (Mikael Pell et al. 1998; Gallori et al. 1991). Since plant health is intimately related to their associated soil microbiome, off target effects, especially those that impact PGPR's, can have detrimental impacts to the associated crops, reducing their yields (Ji et al. 2013; Deak and Malamy 2005; Chaparro et al. 2012; Van der Heijden et al. 2008).

Carrot (*Daucus carota)* production in California exemplifies the above scenario in which plants are treated with agents (fungicides or biofungicides) intended to target soil borne plant pathogens, *Pythium spp,* that cause carrot cavity spot (CCS). In 2019, about 95% of all carrot sales in the United States came from California (United States Department of Agriculture...), thus it is critical to protect the production of carrots in California to safeguard its agricultural economy and help sustain the global food supply. Ridomil Gold and SoilGard are two industry standard fungicides used to treat carrots to protect

them from *Pythium*. However, their effects may not be limited to just *Pythium* and they may have direct and indirect effects to the microorganisms that are present in the soil, thus altering the microbial community (Zhang et al. 2021; Gasoni et al. 2008; Ma et al. 2021; Al-Assiuty et al. 2014; Abbey et al. 2019). The altered soil microbiome may arrive at new community equilibriums with altered functions that are suboptimal for carrot growth and production (Barra Caracciolo et al. 2015). For these reasons, the off-target effects to the soil bacterial community associated with carrots treated with Ridomil (fungicide) and SoilGard (biofungicide), were evaluated in this study. We predicted that the applications of Ridomil or SoilGard would significantly alter the community structure and functions of the soil bacteria communities associated with carrots, compared to untreated controls.

Methods

Preparing Pythium inoculum and carrots

The *Pythium* used in this study to inoculate plants were grown up in 20 % V8 media following similar methodologies as described in McLain and Gachomo, 2019 and Vivoda et al., 1991. Carrots were inoculated with 4,000 CFUs per mL of *Pythium* and after 8 weeks, *Pythium*, was visible on all treated carrots. All carrots used in this study were grown in UC Soil Mix III in a greenhouse at UC, Riverside. Ridomil and soil Guard were added to their respective treatments 28 days after planting the carrots, using their recommended amounts (Table 1).

DNA extractions and Illumina library preparation

DNA was extracted from the soil samples collected at 2 and 8 weeks (T1 and T2 respectively) after *Pythium* inoculation using the DNeasy PowerSoil Kit (Qiagen, Valencia, CA, U.S.A) according to the manufacturer's instructions with the exception of an increase in vortexing to 15 minutes total during the lysis step and a final elution of 50 ul of the C6 buffer. There were 4 replicates per treatment.DNA concentration and purity was measured with an Implen NanoPhotometer (Implen, Westlake Village, CA, USA).

Sequencing of bacterial community

In order to determine the effects of Ridomil and SoilGard we sequenced the 16s rRNA gene of the bacterial community as done in (McLain et al. 2022). Briefly, in an initial PCR reaction (PCR I), individual DNA samples were amplified using Phusion High Fidelity Master Mix to amplify the V5-V6 region of the bacterial 16s rRNA gene. There were 3 technical replicates for each sample amplified. The reaction consisted of 10 ul 2x Phusion High Fidelity Master Mix (ThermoScientific, F531L) , 0.75 ul forward primer (10uM concentration), 0.75 ul reverse primer (10uM concentration), 1 ul DNA template and 12.5ul nuclease-free water. Each forward and reverse primer pair was unique to each sample. PCR I cycle conditions were as follows: 98 C for 30s; 23 cycles of 98 C for 10 s, 56.5 C for 30 s, and 72 C for 30 s , and a final elongation at 72 C for 5 min. Products were run on a 1% agarose gel for quality screening. 7 ul of each PCR I triplicate were

pooled into the same well of a 96 well plate and products of acceptable quality were cleaned up using the Agencourt AMPure xp beads protocol (Beckman Coulter, Brea, CA, USA) with SPRI beads (Beckman Coulter, Brea, CA, USA) and 80% ethanol washes used in place of the original protocol steps. A second PCR (PCR II) was performed on the cleaned PCR I products to add the final Illumina adapter sequence. The same reaction as PCR I was performed with new forward and reverse primers. Cycle conditions for PCR II were: 98 C for 30 s; 6 cycles of 98 C for 10 s, 65 C for 30 s, and 72 C for 30 s and a final elongation at 72 C for 5 min. All primers and barcode sequences are listed in Table 1. A second bead cleanup procedure as described above was performed. Concentrations for each sample were taken using a Nanophotometer (Implen, Westlake Village, CA, USA) and 5 nm of each sample was combined for Illumina MiSeq sequencing. The UCR genomics core facility performed library quality analysis using a 2100 Bioanalyzer (Agilent) and sequenced samples with a MiSeq sequencer (Illumina) and Miseq Reagent kit version 3 (Illumina) with 2 x 150 cycles.

Data analysis - Processing and quality filtering

The forward and reverse Illumina sequencing reads were joined together and quality filtered using default settings in QIIME1 (Caporaso et al. 2010). Joined sequences were demultiplexed using their unique barcode pairs in QIIME1. Demultiplexed samples were uploaded into QIIME2 with their associated quality scores (Estaki et al. 2020). Sequences were quality filtered further using the deblur method in QIIME2 (Estaki et al. 2020;

Nearing et al. 2018; Caruso et al. 2019). Samples that contained less than 9,000 sequences were removed. The number of sequences per sample were rarefied down to match the sample with the lowest amount, 10,300 sequences (Estaki et al. 2020). Deblur classified these sequences into amplicon sequence variants (ASVs) that were taxonomically identified to the lowest possible level by matching to the Greengenes database (v 13.8) using QIIME2 default parameters (DeSantis et al. 2006). Negative controls were sequenced in parallel, any ASV's detected were filtered out from the data using QIIME2 before downstream analyses. Community α-diversity was measured using the Shannon Wiener index in QIIME2 and statistically compared using the best fitting generalized linear model (GLiM) (normal distribution and identity link function) as determined by the model with the lowest Akaike's Information Criterion (AIC) in SPSS (IBM, V. 27.0). Box plots of α-diversity metrics were generated in QIIME2. Community differences among all time points (**β**-diversity) were evaluated using PERMANOVA (Anderson and Walsh 2013; Navas-Molina et al. 2013) on Bray-Curtis distance matrices in QIIME2 (Bray and Curtis 1957; Wolsing and Priemé 2004). Boxplots of the **β**-diversity were plotted in QIIME2. Community data from QIIME2 was used in Paleontological Statistics (PAST) (Hammer et al. 2001) to generate PCA graphs to visualize overall community differences. The group significance test in QIIME1, which uses pairwise Kruskal Wallis tests, was used to statistically compare the abundance of ASVs grouped into taxonomic groups at the order level. (Caporaso et al. 2010). Taxa were considered to be significantly different in relative abundance if $P < 0.05$, with an FDR value lower than 0.1. A conservative FDR value of less than 0.1, as described by

Efron (2007), was used in order to obtain a more inclusive set of microbes that are potentially impacted by APAP so that more bacterial taxa could be considered for additional study. A similar approach was used by Go et al. (2015) to screen for candidate metabolites, and the study Kong et al. (2019) used FDR < 0.2 to determine which microbes were significantly differentially abundant in the oral and gut microbiome of humans. Community data generated in QIIME2 was imported into PICRUSt2 (Douglas et al. 2020) to predict the potential bacterial metagenome present in the soil communities. The data was normalized by copy number and predictions were based on the Kyoto Encyclopedia of Genes and Genomes (KEGG orthologs) database. STAMP (Parks et al. 2014) was used to do initial ANOVA's on each predicted gene to screen for ones that were differentially abundant among all treatments. Genes that were found to be significantly differentially abundant ($P < 0.05$) with a high effect size (measured as eta-squared (η^2)), $\eta^2 > 0.40$, were kept for additional pairwise analyses described below to ensure that the differences were biologically relevant (Lakens 2013). Welch's t-test, in STAMP, was used for pairwise comparisons among all treatments for individual genes that passed this screening. Genes were binned into their respective pathways using the default settings in PICRUSt2 based on the MetaCyc database (Caspi et al. 2016; Douglas et al. 2020). The predicted pathway abundances were compared using PERMANOVA of Bray-Curtis distance matrices in PAST.

Results

Effects of fungicide and biofungicide on the soil bacterial community

Overall, both time and fungicide application had an impact on the soil bacterial community in this study. The initial soil microbial communities at T1 exhibited a high degree of community diversity (**α**-diversity) with averaged Shannon Wiener index values of 8.27, 8.37, and 8.18 for the control, Ridomil, and SoilGard treatments respectively (Fig. 1). Community diversity in all soil samples decreased by the second time point to Shannon Wiener values of 7.86, 6.80, 7.50 for the T2 control, Ridomil, and SoilGard treatments respectively. An overall significant impact of time was observed on the community **α**-diversity (2-way GLiM: $\chi^2_1 = 14.6$, $P < 0.001$; $\chi^2_2 = 2.84$, $P = 0.241$; time, treatments respectively, Fig. 1) and there was no significant interaction between time and treatments (2-way GLiM: $\chi^2_2 = 4.61$, $P = 0.10$). Pairwise analyses among individual treatments, separated by time points, found that Ridomil treated samples at T1 had significantly higher diversity than both the Ridomil and SoilGard treatments at T2 (GLiM: $\chi^2_5 = 22.05$, $P = 0.001$; pairwise LSD all P's < 0.05; Fig. 1). In fact, the Ridomil treatment at T2 had significantly lower Shannon Wiener values than any treatment at any time point (pairwise LSD, all P's < 0.05). Taken together, the **α**-diversity in all samples decreased over time, while the Ridomil treatment appears to have had the most impact to **α**-diversity over time.

Treatments and time had a robust impact on community structure (***β***-diversity) (Fig. 2). Overall, a significant impact of treatment and time were observed (2-way PERMANOVA Psuedo-$F_3 = 2.45$, $P = 0.003$; Psuedo-$F_1 = 20.11$, $P < 0.001$, respectively), while there

was a significant interaction between treatments and time (2-way PERMANOVA: Psuedo-F_3 = 0.92, P = 0.013). A comparison of all treatments separated by time points indicated that by T1 only Ridomil and SoilGard treated communities were significantly different to each other and not the control (PERMANOVA: Psuedo-F_5 = 7.605, P < 0.001; pairwise PERMANOVA P < 0.05). However, by T2, treatment affects became more apparent as both Ridomil and SoilGard treated soils had significantly different communities than the untreated T2 control soil (PERMANOVA: Psuedo-F_5 = 7.605, P < 0.001; pairwise PERMANOVA P < 0.05). Interestingly, T2 Ridomil and SoilGard treated communities were not significantly different, suggesting that their previous community differences diminished over time and they became more similar. An additional impact of time on the communities was also apparent in the observation that all 3 of the different treatments had significantly different communities than the T1 control sample (Fig. 2), and all of T2 treatments soil communities were significantly different than their respective T1 counter parts (PERMANOVA: Psuedo-F_5 = 7.605, P < 0.001; pairwise PERMANOVA all P's < 0.05). Taken all together, the data suggest that both time and agent treatment had an impact on the soil bacterial microbial community while treatment effects appear to have been more robust by T2.

Effects of fungicide and biofungicide on soil bacterial community taxonomy

As expected, the soil communities were very diverse, while bacteria from the orders Pseudomonadales, Flavobacteriales, Sphingomonadales, and Rhizobiales (aka

Hyphomicrobiales) comprised a major portion of the different soil communities (Fig. 3). Overall, the differences in relative abundance for Pseudomonadales, Flavobacteriales, and Rhizobiales orders were more robust than those found in the Sphingomonadales order. The group significance comparisons in Qiime1 determined that the relative abundances was significantly different among individual soil treatments separated by time for Pseudomonadales, Flavobacteriales, and Rhizobiales (Kruskall-Wallis, all P's < 0.05, all FDR < 0.10). The relative abundance of the Sphingomonadales order was not significantly different among treatments separated by time points.

The Pseudomonadales order ranged from having a relative abundance of 43.8 % to 8.2 % (T1 Ridomil and T1 SoilGard, respectively; Fig. 3). Interestingly, By T2, their relative abundance decreased drastically to 10.0% in the Ridomil treated soils. The relative abundance remained consistent from T1 and T2 in SoilGard treated samples (8.2 % and 11.5%; respectively) or in the untreated controls (21.9% to 20.8%; Respectively). The Flavobacteriales order increased over time from T1 to T2 control in soils (9.3% to 15.8%) and doubled in SoilGard treated soils (5.9% to 12.1%). In Ridomil treated soils, though, the relative abundance of bacteria from the Flavobacteriales order was lower but still increased over time (0.2% to 3.9%). The relative abundance for bacteria in the order Sphingomonadales remained pretty steady in untreated soil samples over time (9.3% and 8.8 %, T1 to T2 Respectively). However, the relative abundance increased in Ridomil treated samples (4.6 % to 8.2 %, T1 to T2 Respectively) but decreased slightly in SoilGard treated samples over time (8.4% to 6.8%, T1 to T2 Respectively). Ridomil

treated soil by T2 contained the most bacteria from the Rhizobiales order with 12.5% relative abundance, doubling from 5.4% at T1. In both SoilGard treated samples and untreated control samples the relative abundance increased slightly over time (7.5 % to 9.0% and 5.3% to 6.2%, T1 to T2, SoilGard and control treatments; respectively). Taken all together though, the differences in relative abundances in these major groups most likely attributed to the differences observed in both $\boldsymbol{\alpha}$- and $\boldsymbol{\beta}$-diversity of the soil communities discussed above.

Effects of fungicide application on differential gene expression

Similarly, to community structure, both time and compound treatments had potential impacts on overall bacterial community functions as determined through PICRUSt 2 gene predictions based on the 16s rRNA data. The initial ANOVA to screen for biologically relevant genes predicted there to be a total of 2,160 genes that were differentially present to a biologically relevant degree (ANOVA $P < 0.05$; $\eta^2 > 0.40$). The predicted community genomes for soil bacteria were significantly different among treatments and not across time, however a significant interaction effect of time and treatment was observed (2-way PERMANOVA: psudo$F_2 = 4.66$, $P = 0.0014$, pseudo$F_2 = 2.00$, $P = 0.082$; pseudoF2 = 1.65, P = 0.005; Respectively). Pairwise analyses also indicated that the Ridomil treated soil at T1 had a significantly different predicted community genome from the SoilGard treated soil at T1 and T2, and from the untreated soil at T2 (Pairwise PERMANOVA all P's < 0.05). Interestingly, by T2 the Ridomil treated soil's predicted genome was not significantly different from any other community genomes. SoilGard

treated soils, however, by T2 had a predicted community genome that was significantly different from T1 Ridomil and T2 control treatments (Pairwise PERMANOVA's all P's < 0.05). The clustering pattern exhibited on the NMDS plot (Fig. 4) exemplifies these trends, as the T1 Ridomil community appears to form a distinct cluster from all the other communities. The T2 Ridomil, T2 SoilGard, and T1 control communities appear to cluster together towards the center of the plot.

Upon binning the genes into their respective pathways, several of them were predicted to be significantly different in utilization among treatments including glycogen degradation, D-galacturonate degradation, glycolysis, mixed acid fermentation, and glucose degradation (Table 2). All together these observations indicate that both Ridomil and SoilGard have the potential to alter community functions in the soil. However, since these observations are based on predicted community genomes using the 16s rRNA data, this should be evaluated further using more direct methods.

Discussion

The results from this study indicate that the fungicide Ridomil and the biofungicide SoilGard can alter the diversity, community structure, and potentially the community functions of the soil microbiome associated with carrots. The data indicates that Ridomil may have more drastic effects, evident in the community diversity (**α**-diversity) observations in which only Ridomil treated soils by T2 had significantly lower diversity

than the untreated T2 controls. However, both agents had significant impacts on the microbial community structures (β-diversity), in which both treatments were significantly different than the T2 control. The observed shifts in community diversity and structure may cause differences in community functions to occur, which was predicted by the PICRUSt2 analysis. Changes in the overall predicted community genome composition suggest that carbon utilization may be altered in communities exposed to one of the agents. Ridomil appears to have an initial effect that may dissipate over time, while the impact of SoilGard appears to require more time to take effect. The predicted community genome for T2 Ridomil was not different from the control samples at T1 or T2. The predicted community genome for SoilGard treated samples, however, was found to be significantly different from the T2 control samples. Taxonomic shifts of some of the major groups of bacteria Pseudomonadales, Flavobacteriales, and Rhizobiales, were also observed. Major shifts to the relative abundance could alter overall community functions that are carried out, or that are supported by these groups.

Our Illumina sequencing approaches detected significant shifts in the diversity of our soil samples when exposed to the chemical fungicide Ridomil, and the biofungicide SoilGard. This is consistent with previous studies that found application of either a fungicide or biofungicide decreased soil bacterial populations and diversity (Zhang et al. 2021; Gasoni et al. 2008; Ma et al. 2021). Previous studies have observed increases in plant beneficial bacteria with the addition of fungicides or biofungicides (Chen et al. 2018; Xue et al. 2015; Mubeen et al. 2006). Many bacteria found in the Rhizobiales are considered

to be PGRPs, such as *Bradyrhizobium japonicum*. Several PGPRs were found to be sensitive towards chemical fungicides and they would either increase or decrease in abundance with a given compound present (Mubeen et al. 2006). Taken together, these observations are consistent with our findings that Ridomil and SoilGard treated soils had higher abundance of bacteria in the Rhizobiales order, a group that contains numerous plant beneficial bacteria, than in the control treatments by the second time point. The study Han et al. 2021 observed an increase in bacteria from the genus *Hyphomicrobium*, which is in the Rhizobiales order. This particular group of bacteria was able to degrade the fungicide used in their study. This suggests that some organisms found in the Rhizobalies order may be capable of degrading Ridomil allowing for their abundance to increase, but this requires further investigation. Han et al. 2021, found that there were different changes to the soil microbial community in different soil conditions. The finding that fungicidal agents have varying impacts to microbial communities with different soil conditions suggests that there are other factors that can influence how these compounds affect the soil microbial community and this warrants further investigation (Han et al. 2021). Numerous studies have also observed that fungicides and biofungicides have altered the soil bacterial community structures ($\boldsymbol{\beta}$-diversity), which is consistent with the findings of our Illumina sequencing results (Chen et al. 2018; Han et al. 2021; Ma et al. 2021; Xue et al. 2015). Shifts in the overall bacterial community structure could lead to changes in community functions. The PICRUSt2 gene prediction data did indicate that there was a potential that community functions did become altered in the presence of the bio- and chemical fungicides, which is also consistent with previous findings. Aspects

of nitrogen cycling were found to be disrupted in soils treated with fungicides (Mikael Pell et al. 1998; Gallori et al. 1991), while another study indicated that utilization of glycolysis pathways may be altered in soils in which a biofungicide is applied (Natsch et al. 1998). Though we did not detect any alterations to the nitrogen cycle using the gene prediction approach carried out in PICRUSt2, the potential for this exists and it warrants additional study. We did detect that different carbon metabolism pathways were predicted to be significantly differentially utilized, including glycolysis. Together this suggests that community functions can be altered by the presence of a fungicide or biofungicide. Since plant health can be directly impacted by the soil community processes (Sharma et al. 2011; Pieterse et al. 2014) , this warrants additional study that uses approaches such as RNAseq that can detect gene expression by the soil microbial community directly.

Taken together the results of this study contribute to the evidence that both bio- and chemical fungicides have off-target effects that impact the soil microbial community. The specific effects of each agent seem to vary depending on multiple factors including soil conditions, present plants and pathogens, and the agents themselves. Plant health and ultimately plant production is tightly coupled to soil conditions and the soil microbiome (Van der Heijden et al. 2008). Therefore, further investigations are required to study the specific effects of fungicidal agents, or pesticides in general, to ensure they are being used effectively to simultaneously protect plants and minimize deleterious impacts to them from off-target effects.

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Figures

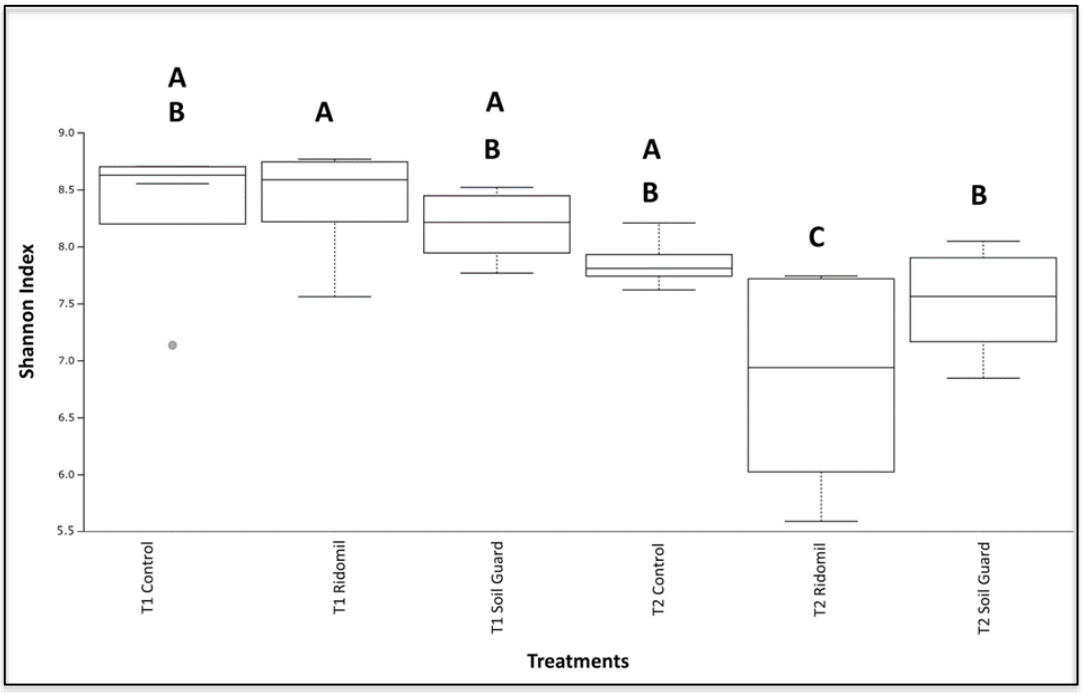

Figure 3.1 Box plots of the Shannon Wiener diversity (**α**-diversity) for each sample at both time points. Bars that do not share a letter were found to be significantly different by pairwise Kruskal-Wallis test comparisons (All P's < 0.05).

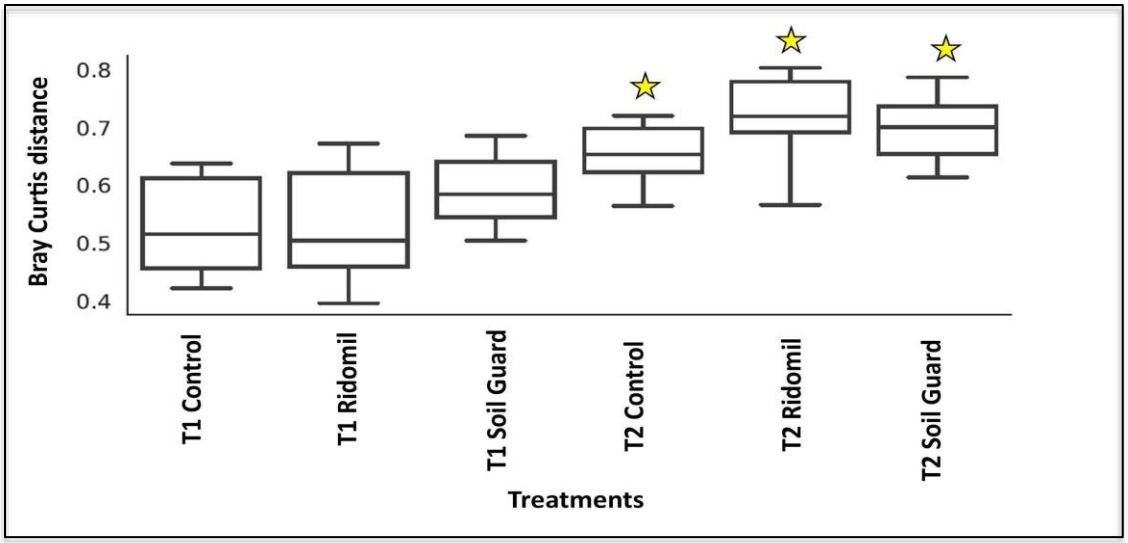

Figure 3.2 Showing the results of the distances as determined by a Bray-Curtis similarity matrix that were used to calculate differences in $\boldsymbol{\beta}$-diversity among all treatments. The Stars indicate which samples were found to be significantly different from the T1 control sample by pairwise PERMANOVA comparisons (all P's < 0.05).

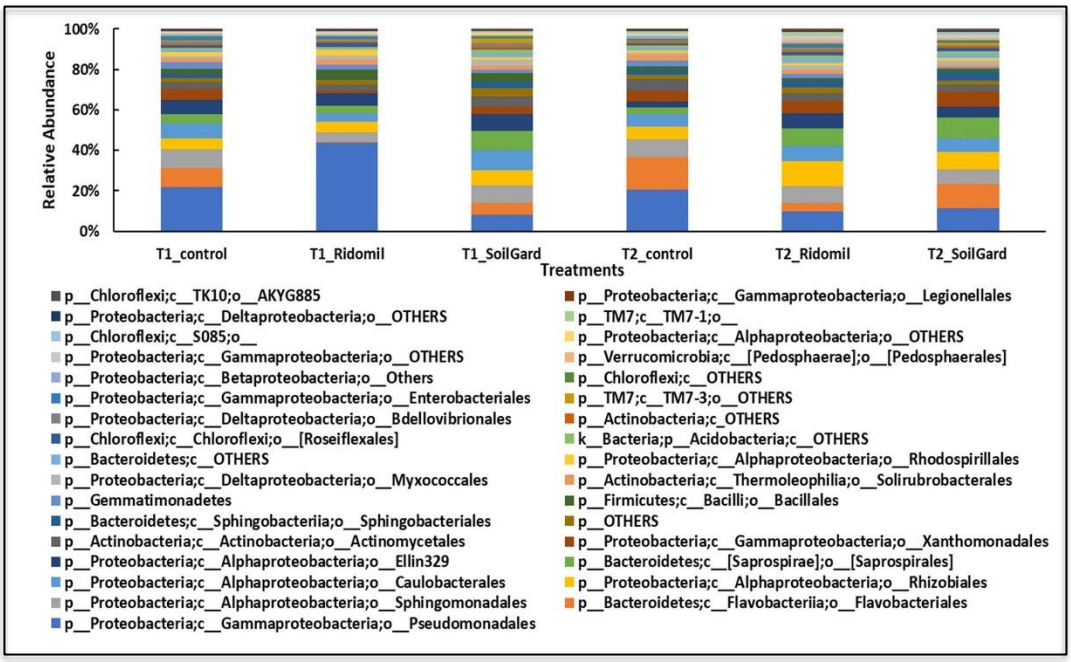

Figure 3.3 Bar Graph showing the relative abundance of bacteria detected using Illumina sequencing, averaged within each treatment.

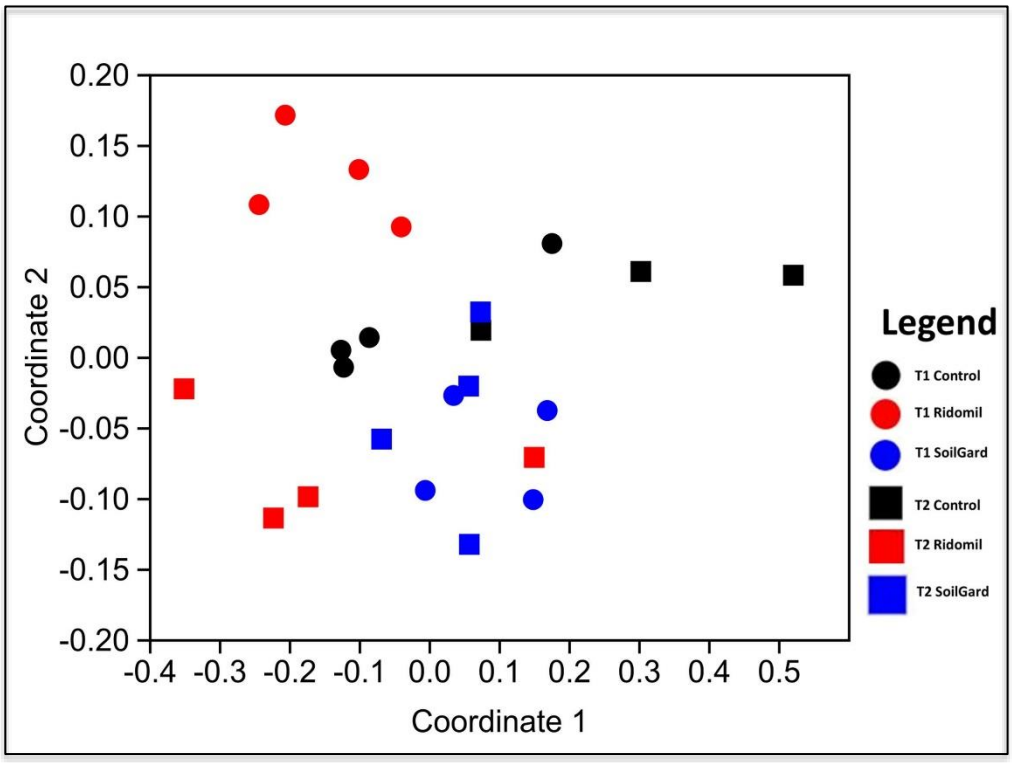

Figure 3.4 NMDS plot comparing the similarity of predicted genes present among the different soil communities at the different time points.

Tables

Table 3.1 The recommended amounts to use according to the manufactures for Ridomil and SoilGard. The amounts recommended for greenhouses (GH) were used when specified.

Name	**Company**	**Species Included**	**Recommended Application Rate for CCS**
SoilGard12G	Certis USA	*Gliocladium* (*Trichoderma*) *virens* strain GL-21 (1 x 10^6 CFU/g)	**For GH:** 1 to 2.0lbs per cubic yard **For Field**: 2 to 10lbs/acre
Ridomil Gold SL	Syngenta US	Chemical Fungicide: Mefenoxam (45% active ingredient)	0.5 to 1.3pt/acre

Table 3.2 A select set of pathways predicted to be significantly differentially used among all chemical treatments, including the control.

MetaCyc pathway	Name	P-value	Effect size
GLYCOCAT-PWY	Glycogen degradation	<0.001	0.85
GALACTUROCAT-PWY	D-galacturonate degradation	<0.001	0.82
ANAGLYCOLYSIS-PWY	Glycolysis III	<0.001	0.83
GLUCOSE1PMETAB-PWY	Glucose and glucose-1-phosphate degradation	<0.001	0.800
FERMENTATION-PWY	Mixed acid fermentation	<0.001	0.79
GLYCOLYSIS	Glycolysis	<0.001	0.77
NADSYN-PWY	NAD de novo biosynthesis II (from tryptophan	<0.001	0.73
PENTOSE-P-PWY	Pentose phosphate pathway	<0.001	0.68

CHAPTER 4

Bradyrhizobium japonicum IRAT FA3 Alters *Arabidopsis thaliana* Root Architecture

Publication summary of Schroeder et al., 2022, in which the dissertation author is a co-author of.

Abstract

Beneficial rhizobacteria can influence plant root development. Root system growth is mediated by multiple factors such as the distribution of the phytohormone auxin within root tissues. Auxin transporters help generate the auxin gradients required for normal root structure development. This study demonstrates that the plant-growth-promoting rhizobacteria strain *Bradyrhizobium japonicum* IRAT FA3 influences specific auxin efflux transporters to alter *Arabidopsis thaliana* root morphology. Upregulation of the auxin efflux genes *PIN2, PIN3, PIN7,* and *ABCB19* was observed from gene expression profiling of *B. japonicum* inoculated *A. thaliana,* accession Col-0, plants compared to nonincubated plants. The change in gene expression for these auxin efflux genes in the presence of *B. japonicum* indicate that auxin transportation is a major way in which the host plant interacts with the microbe. Overall, this study demonstrates that there are plant-microbe interactions that influence auxin transport, which influences plant root development and may help the plant cope with stress.

Introduction

The plant hormone auxin (predominantly existing in plants as indole-3-acetic acid (IAA)), plays a critical role in plant root development. However, for the phytohormone to function correctly, it needs to be transported to the correct tissues and cells using a network of transporters. Without functional auxin transporters the necessary concentrations of auxin required for different root cells will not be achieved preventing normal root development. Under normal conditions auxin transporter genes are expressed at optimal levels to ensure normal root development for growing seedling. However, auxin transporter gene expression can be altered or disrupted in a plant under stressful conditions such as drought, temperature, pH, salinity, and the presence of anthropogenic factors. However, the presence of plant-growth promoting rhizobacteria may help counter the effects of stress and promote optimal levels of auxin transporter gene expression for the plant. The (PGPR) strain *Bradyrhizobium japonicum* plays a role in regulating the expression of plant genes for Auxin transporters.

The impacts of *B. japonicum* on plant root development were examined by comparing 8 day old *Arabidopsis thaliana* Col-0 seedlings inoculated with *B. japonicum* IRAT FA3 (Gachomo et al. 2014) to noninoculated seedlings. The inoculated seedlings had significantly decreased primary root (PR) length while simultaneously increasing the amount of lateral root (LR) formation compared to noninoculated plants. These observations are consistent with auxin accumulation in plants that have been stimulated by rhizobacteria (Loper and Schroth 1986; Spaepen et al. 2007). It has also been found

previously that *B. japonicum* IRAT FA3 can increase plant biomass (Gachomo et al. 2014).

Methods – summarized from Schroeder et al., 2022

For details on how plants were grown, how plant material was collected, how bacteria was cultured and used to inoculate plants please see the publication (Schroeder et al. 2022).

RNA-seq and qRT-PCR –contributed by the author of this dissertation

Total RNA was extracted from 21-day-old *A. thaliana* seedling roots at 14 dpi, according to the manufacturer's protocol (GeneJET Plant RNA Purification Kit; Thermo Scientific, Madison,WI, U.S.A.).

For RNA-seq, the RNA was processed (74204 QIAGEN and AM1907 Invitrogen) and libraries were prepared with the NEBNext Ultra Directional RNA Library Prep Kit for Illumina by following the manufacturer's instruction (E7490S, E7335S, and E7420S; New England Biolabs).

Libraries were pooled and sequenced on an Illumina NextSeq 500 platform (Illumina, San Diego, CA, U.S.A.) at the University of California–Riverside Genomics Core Facility. The program Salmon was used to process raw RNA-seq data to quantify the transcript data from all samples (Patro et al. 2017). The quantified data were analyzed

using the R program ThreeDRNA (Calixto et al. 2018; Guo et al. 2020; R Core Team 2020). Briefly, quantified transcript data were uploaded into ThreeDRNA using the integrated tximport program (v. 1.18.0) and the default setting lengthScaledTPM method (Soneson et al. 2016). Low-quality expression data were filtered using the default count per million reads cutoff of 1 and sample number cutoff of 1. The processed data were used to generate a PCA graph using the RUVr method of removing batch effects from the data. Transcriptome data were normalized using the weighted trimmed mean of M values in ThreeDRNA. The data were statistically analyzed using the limma-voom pipeline recommended by ThreeDRNA, which balances outliers in the data that may occur from sample quality differences (Law et al. 2014; Ritchie et al. 2015). For downstream plots, the data were filtered to only include samples with P values < 0.05 and log2 FC ³ 1 between the noninoculated controls and samples inoculated with *B. japonicum*.

The filtered data were used to generate a heatmap using the program shinyheatmap (Khomtchouk et al. 2017). To identify auxin-related genes within our (adjusted P value < 0.05) RNAseq data for the heatmap, gene ontology (GO) terminology was compiled at the Panther Geneontology website with the annotation version GO database (released 2018-10-09), analysis type PANTHER Overrepresentation Test (released 2020-07-28), and test type GO biological process complete with Fisher's Exact false discovery rate (FDR) multiple test correction displaying only results with FDR < 0.05. For each qRT-PCR sample, genomic DNA was removed from 1 µg of total RNA prior to RT into first-strand cDNA (iScript Kit; Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's protocol. The first-strand cDNA was used for SYBR green monitored

qRT-PCR (Bio-Rad CFX). Primer sequences for PIN1, PIN2, PIN3, PIN7, ABCB4, and ABCB19 have been previously described (Terasaka et al. 2005; Wang et al. 2015; Xi et al. 2016; Yang et al. 2013). qRT-PCR analysis was performed using the Bio-Rad CFX Maestro Real-Time PCR System.

Results summary of Schroeder et al. 2022

Since auxin transportation is important for auxin functions, gene expression for specific auxin efflux carriers were examined using RNA-sequencing analyses. (RNA-seq). Over 70 differentially regulated genes related to auxin were identified after 14 dpi with *B. japonicum*. Auxin efflux transporter transcripts significantly increased in expression in *A. thaliana* roots inoculated with *B. japonicum*. For example, the genes for the auxin transporters PIN2, PIN7, and ABCB19 were significantly upregulated in inoculated plants compared to noninoculated controls. These RNA seq data were confirmed with quantitative real-time PCR (qRt-PCR) and the genes for PIN3, PIN7, and ABCB19 were found to be significantly upregulated compared to noninoculated control plants. accurate

B. japonicum increases PIN and ABCB gene expression – contributed by the author of this dissertation

The results of this study suggested that auxin efflux carriers play a role in *B. japonicum*-induced root architecture alterations. To demonstrate this, we investigated *B. japonicum*-stimulated gene expression modifications by performing RNA-sequencing (RNA-seq) analysis of Col-0 whole-root tissue. A general increase in auxin-related gene transcript

levels was evident in the *B. japonicum* inoculated roots (Fig 1), and more than 70 differentially regulated distinct auxin-related genes were identified at 14 dpi (Table 1). In the RNA-seq data, significant increases in transcripts of auxin efflux transporters PIN2 (1.44 log2-fold-change [FC]), PIN7 (4.15 FC), and ABCB19 (1.22 FC) were observed under *B. japonicum* cocultivation (Table 1). PIN3 transcripts increased (mean read counts were nearly 41% higher than control) but the data were not statistically significant. ABCB4 and PIN1 transcript levels did not show a clear change between inoculated and control roots. Other auxin-related genes upregulated by *B. japonicum* included ARF7 (6.60 FC), IAA14 (4.31FC), PILS7 (4.15 FC), ICR1 (3.68 FC), GH3.6 (2.41 FC), and ARF5 (2.27 FC), while among the downregulated genes were IAA28 (−1.08 FC), PILS3 (−1.10 FC), and BIG (−6.88 FC) (Table 1). Sequential activation of auxin response modules during LR development is a highly coordinated event (Lavenus et al. 2015). Our RNA-seq data are just an overview of *B. japonicum's* influence over host transcriptional reprogramming in the root system (Table 1; Fig. 1).

Quantitative real-time PCR (qRT-PCR) gene expression studies also illustrated transcriptional changes in auxin transporters after treatment with *B. japonicum*. We measured transcript levels of six major auxin efflux transporters in the roots of Col-0 plants at 12 h post inoculation (hpi). The 12-h time point was chosen to catch early transcriptional changes in response to *B. japonicum* cocultivation. *PIN3, PIN7*, and *ABCB19* were upregulated in *B. japonicum*-treated whole-root tissues (Fig. 2). *ABCB4* expression increased, but not significantly. There were no significant differences between

inoculated and noninoculated root transcript levels of *PIN1* or *PIN2* in the qRT-PCR data (Fig. 3). qRT-PCR and RNA-seq analyses detected significant increases in *PIN7* and ABCB19 expression with *B. japonicum*. RNA-seq analysis detected an increase in *PIN2* expression whereas *PIN3* upregulation was found to be significantly higher only in the qRT-PCR experiments ($P < 0.01$) in inoculated roots. Differential observations between the two assays reflect gene expression fluctuations over time with B. japonicum cocultivation

Discussion summary of Schroeder et al. 2022

Overall, this study demonstrates that auxin transport is critical for normal root development to occur. Increased auxin content responsiveness of plants inoculated with *B. japonicum* indicates that PGPR microbes are capable of interfering with the host plant auxin concentration and subsequent auxin-regulated gene expression. The RNA-seq analyses detected over 70 differentially expressed auxin-related genes in inoculated plants compared to the non-inoculated set of plants. Several of these genes have been previously shown to have a role in LR formation, PR length, or auxin transport. However, *ABCB4* and *PIN1* transcript levels did not change when plants were inoculated with *B. japonicum*, suggesting that these genes are not involved with plant-host interactions related to auxin transport.

Previous studies have demonstrated that plants treated with auxin have increased expression of *PIN3* and *PIN7*, which parallels the observations of the gene expression

assays of this study (Schroeder et al. 2022) of *A. thaliana* inoculated with *B. japonicum* that produces IAA (Vieten et al. 2005; Lewis et al. 2011). Furthermore, redundant cooperation of these two genes has not been established, despite their similar roles in root development (Friml et al. 2002; Paponov et al. 2005; Chen et al. 2015; Ruiz Rosquete et al. 2018; Zhou and Luo 2018)

Overall expression of *ABCB19, PIN3,* and *PIN7* increased in whole root tissues according to these RNA-seq and qRT-PCR assays with plants inoculated with *B. japonicum.* Therefore, the increased expression may be due to upregulation as a result of plants interactions with *B. japonicum*. *PIN2* expression was not observed to increase and LR number remained similar as compared to Col-0 plants when they were inoculated. Vieten et al. (2005) had previously indicated that *PIN2* played a role in PR length determination. They observed that *pin2* PR length was the same as *pin1* length which were both similar to the wild type plants. A different study observed that *Pin2* and the wild type PR length decreased while LR number increased in response to the PGPR *Bacillus megaterium* (López-Bucio et al. 2007). Despite their being variations of PR length and LR number of *pin2* mutants compared to wild type plants in different studies, many studies seem to indicate that the loss of *PIN2* function with differentially modulated root system architecture (Blilou et al. 2005; López-Bucio et al. 2007; Laskowski et al. 2008; Li et al. 2015; Qu et al. 2017). There are many species of PGPR that have been observed to stimulate root growth and affect auxin signaling (López-Bucio et al. 2007; Grunewald et al. 2009; Jiménez-Vázquez et al. 2020).

This study summarized here demonstrates that an IAA producing strain of *B. japonicum*- can reduce PR length by utilizing *PIN2* and *ABCB19.* The overall results of this study help elucidate the auxin pathways involved in the plant interactions with *B. japonicum* to alter root structure.

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Figures

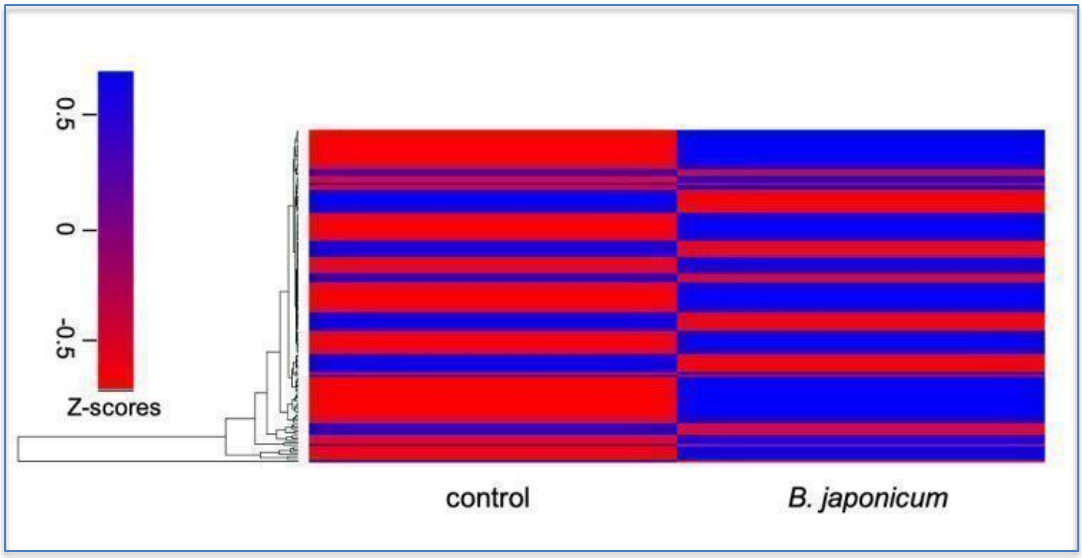

Figure 4.1 *B. japonicum* induced auxin-related gene expression in *A. thaliana* root tissue at 14 dpi. *A. thaliana* roots were treated with either *B. japonicum* or 10 mM MgSO4 (control) for 14 days. The heatmap displays auxin-related genes identified in the RNA-seq data that were significantly up-regulated (blue) or down-regulated (red) by *B. japonicum* relative to control. Genes were clustered based on Euclidean distance of expression values.

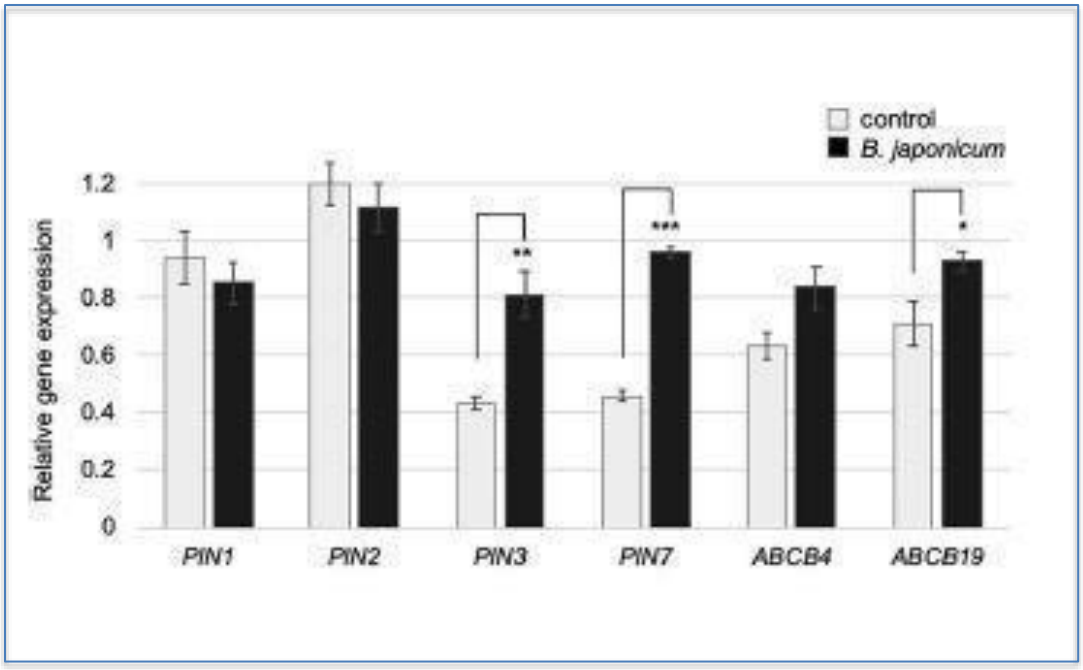

Figure 4.2 *Bradyrhizobium japonicum* induced *PIN3, PIN7,* and *ABCB19* gene expression in *Arabidopsis thaliana* root tissue at 12 h post inoculation. *A. thaliana* roots were treated with either *B. japonicum* or 10 mM MgSO4 (control) for 12 h. Quantitative reverse-transcription PCR expression data relative to the IPP2 reference gene showed significantly increased RNA transcripts in PIN7, PIN3, and ABCB19. Student's t test, *, **, and *** indicate P <0.05, 0.01, and 0.001, respectively

Tables

Table 4.1. RNA-sequencing differentially expressed auxin-related genes

AGI	Log2-fold change	Gene	Gene name and descriptive phrase[a]
AT5G05730.2	6.78	ASA1[b]	Anthranilate synthase a subunit 1; tryptophan synthesis, lateral root formation, response to bacterium
AT5G20730.1	6.6	ARF7	Auxin response factor 7; auxin-regulated transcriptional activator
AT1G28560.3	4.7	SRD2[b]	Shoot redifferentiation defective 2; organ morphogenesis, polar transport, cellular response to auxin stimulus, lateral root formation, meristem initiation
AT4G02570.1	4.7	AXR6	Auxin-resistant 6; mediating responses to auxin
AT1G14350.4	4.7	FLP	Four lips; auxin polar transport, regulation of lateral root development
AT4G13260.1	4.34	YUC2	Yucca 2; indole-3-pyruvic acid (IPA) to indole-3-acetic acid (IAA) conversion, auxin biosynthetic process
AT4G14550.2	4.31	SLR	Solitary root, (aka IAA14); lateral root development, expressed in stele and root tip epidermis
AT5G65980.1	4.15	PILS7	Pin-likes 7; auxin efflux carrier
AT3G23050.2	4.12	AXR2[b]	Auxin-resistant 2, (aka IAA7); repressor of auxin-inducible gene expression
AT5G62380.1	3.98	NAC101	NAC-domain protein 101; response to auxin
AT2G46370.1	3.95	JAR1[b]	Jasmonate-resistant 1; member of the GH3 family of proteins, cellular response to auxin stimulus
AT5G48070.1	3.72	XTH20	Xyloglucan endotransglycosylase/hydrolase 20; expressed in lateral root primordia, cell growth, cellular response to auxin stimulus
AT1G17140.2	3.68	ICR1	Interactor of constitutively active ROP1; regulation of auxin polar transport
AT1G25220.2	3.55	ASB1[b]	Anthranilate synthase b subunit 1, (aka WEI7); auxin biosynthetic process, lateral root formation, response to bacterium
AT5G63160.3	3.52	BT1	BTB and TAZ domain protein 1; response to auxin
AT2G23260.1	3.38	UGT84B1	UDP-glucosyltransferase 84 B 1; regulation of auxin metabolic process
AT2G01940.3	3.25	IDD15	Indeterminate (ID)-domain 15; regulation of auxin biosynthesis and transport
AT1G23080.3	2.89	PIN7	Pin-formed 7; auxin efflux, pattern specification during root development
AT1G25250.3	2.85	IDD16	Indeterminate (ID)-domain 16; regulation of auxin biosynthesis and transport
AT2G22330.1	2.69	CYP79B3	Cytochrome P450 family 79 subfamily B polypeptide 3; tryptophan metabolism
AT5G54490.1	2.46	PBP1	Pinoid-binding protein 1; upregulated by auxin
AT5G54510.1	2.41	GH3	Gretchen Hagen 3.6; auxin homeostasis, auxin-activated signaling pathway, response to auxin, unidimensional cell growth

AT1G28130.1	2.39	GH3	Gretchen Hagen 3.17; auxin homeostasis, response to auxin
AT1G19850.1	2.27	ARF5	Auxin response factor 5, (aka IAA24)
AT1G75580.1	2.15	SAUR51	Small auxin upregulated RNA 51; response to auxin
AT3G62100.1	2.15	IAA30	IAA inducible 30; response to auxin, root quiescent center
AT4G39950.1	1.84	CYP79B2	Cytochrome P450 family 79 subfamily B polypeptide 2; tryptophan metabolism, indoleacetic acid biosynthetic process, response to bacterium
AT1G68130.1	1.7	IDD14	Indeterminate (ID)-domain 14; regulation of auxin biosynthesis and transport
AT4G31500.1	1.69	SUR2	Super root 2; adventitious root development, defense response to bacterium, indoleacetic acid biosynthetic process, tryptophan biosynthetic process
AT4G37390.1	1.67	AUR3	Auxin-regulated 3; regulation of auxin activity
AT1G02335.1	1.67	PDGLP2	Plasmodesmal germin-like protein 2; primary root growth regulation by controlling phloem-mediated allocation of and lateral root meristems resources between the primary and lateral root meristems
AT5G05730.1	1.55	ASA1[b]	Anthranilate synthase a subunit 1; tryptophan synthesis, lateral root formation, response to bacterium
AT1G12140.1	1.46	DAO2	Dioxygenase for auxin oxidation 1; auxin homeostasis, expressed in root caps
AT2G22240.1	1.45	MIPS2[b]	Myo-inositol-1-phosphate synthase isoform 2; defense response to bacterium, response to auxin
AT5G57090.1	1.44	PIN2	Pin-formed 2, (aka EIR1); auxin efflux, auxin polar transport, response to auxin
AT1G59750.2	1.39	ARF1	Auxin response factor 1; mediator of auxin response gene expression, negative regulation of transcription, regulation of transcription
AT3G23050.1	1.36	AXR2[b]	Auxin-resistant 2, (aka IAA7); repressor of auxin-inducible gene expression
AT2G04160.1	1.35	AIR3	Auxin induced root cultures 3; lateral root morphogenesis, response to auxin
AT2G21050.1	1.24	LAX2	Like auxin-resistant 2; auxin influx carrier
AT4G14740.1	1.24	FL3	Forked-like 3; auxin-activated signaling pathway
AT1G48690.1	1.23	No	Auxin-responsive GH3 family protein
AT3G28860.1	1.22	ABCB19	ATP-binding cassette B19; auxin transport
AT2G37080.3	1.18	RIP2	ROP interactive partner 2; response to auxin
AT5G66700.1	1.16	HB53	Homeobox 53; auxin-inducible, root development
AT2G06850.1	1.14	XTH4	Xyloglucan endotransglucosylase/hydrolase 4; secondary cell wall biogenesis, response to auxin

[a] Minimal phrases representative of genes from the *Arabidopsis* Information Resource; aka = also known as.
[b] Splice variants are differentially regulated.

CHAPTER 5

Overall Conclusion

Drought conditions, pathogen attack, and abnormal root development due to stress are just a few of the major issues that can negatively impact plant growth and yields. Each of these issues must be addressed simultaneously to ensure efficient crop production occurs at rates high enough to support the global population. Therefore, this collection of studies examined the impacts of chemicals of emerging concern (CECs) that can deleteriously impact crops that are irrigated with recycled waste water (RWW). We also evaluated the impact to crops due to off-target effects of fungicidal agents used to address pathogen attacks. Lastly, *Bradyrhizobium japonicum* was examined to elucidate specific interactions in which it influences plant root development.

Examination of four commonly found CECs in recycled waste water used to irrigate crops (acetaminophen, trimethoprim, sulfamethoxazole, and gemfibrozil; APAP, TMX, SMX, GEM; respectively) indicated that the effects of these compounds were strain specific. Our results demonstrated that the growth of these particular plant pathogens can be impacted directly by CECs at concentrations found in RWW. The use of RWW has been very successful for water conservation, and its use should continue. When using it for crop irrigation growers should monitor their fields closely for any sign of exacerbation of diseases or increased growth of soil borne plant pathogens. Additionally, our results indicate that APAP can alter the soil microbiome associated with eggplants, and possibly other crops. Changes in soil community carbon substrate utilization were

also detected in the soil microbial communities treated with APAP. All together our observations demonstrate that CECs can alter the microbial community structures and functions, which could have direct impacts on plant production.

All together these studies contribute to the wealth of knowledge for some of the most prevalent sources of stress for crop plants. Our results show that CECs found in RWW may cause deleterious impacts to crop yields. Careful monitoring of crops and the soil for the presence of specific soil borne plant pathogens may offer solutions to this issue. This warrants additional study so that RWW can be used more effectively to combat drought. PGPRs also aid with optimal root development to cope with stressful conditions and pathogen attack. Proper implementation of PGPR could also help address root development issues caused by stress, but this would also require additional research. In short, the proper utilization of RWW and the use of PGPR's may help protect crop yields, but more research is needed to develop specific strategies.