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Effect of Low-Dose, Repeated Exposure of Contaminants of Emerging Concern on Plant Development and Hormone Homeostasis

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ABSTRACT OF THE THESIS

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by

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Master of Science, Graduate Program in Environmental Sciences
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Treated wastewater is increasingly being utilized to meet agriculture's water needs, however, treated wastewater contains numerous trace organic contaminants including constituents of emerging concern (CECs), such as pharmaceuticals and personal care products (PPCPs). With exposure and uptake of these compounds, phytotoxicity and health of crop plants is of concern, but is not well understood at present. This study simulated irrigation with treated wastewater and evaluated the effect of low-dose, chronic exposure to a mixture of 10 CECs, including 4 antibiotics, 3 analgesic anti-inflammatory drugs, 1 anti-epileptic, 1 beta-blocker, and 1 antimicrobial, on lettuce and cucumber plants, as well as the effect of the CEC mixture in conjunction with other abiotic stressors such as heat. The CEC mixture was added in nutrient media at

1-20X of their typical levels found in treated wastewater effluents. Relevant biological endpoints including germination, growth, phytohormone homeostasis, and CEC bioaccumulation into plant tissues were determined. Root length of lettuce seedlings significantly increased in a dose-dependent manner at the end of a 7 d germination study. However, a dose-dependent decrease in biomass was observed upon longer-term exposure (30 d) of cucumber seedlings to the CEC mixture, with the highest CEC treatment rate resulting in a relative percentage difference in average below-ground, above-ground, and total biomass of -51.2 ± 20.9, -26.3 ± 34.1, and -33.2 ± 41.7%, respectively. The response of selected phytohormones (auxins, jasmonic acid, and abscisic acid) were investigated to gain insight into the plant's physiological response to CEC exposure, as well as in conjunction with heat stress, due to the involvement of these phytohormones in growth, nutrient allocation, transpiration, and defense. Levels of abscisic acid, a stress response hormone involved in stomatal closure, were found to be significantly elevated ($p < 0.05$) in the leaves with increasing exposure rates of CECs, but, decreased significantly ($p < 0.05$) in the roots at environmentally relevant CEC concentrations. Elevated abscisic acid in the leaves may cause stomatal closure, anti-transpiration activity, and reduced leaf expansion leading to slowed plant growth and impaired ability to adapt to additional stressors, such as extreme temperatures or salinity. The dose-response of auxin, a phytohormone involved in cell elongation and meristematic tissue growth, was characterized by a hormesis effect. A significant 6-fold increase was observed in stem auxin concentrations at the low CEC treatment rate as compared to the control, followed by a gradual decrease to 2-fold the control at the

highest CEC treatment rate. Leaf auxin concentrations also significantly increased at the low CEC concentrations to 16-fold, followed by a decrease at the highest CEC treatment rate.

In nature, crops are simultaneously exposed to numerous biotic and abiotic stresses and therefore heat stress was chosen to investigate the interactive effects of simultaneous stresses on plants. Root auxin concentrations with heat, but no CEC exposure (59.8 ± 16.9 ng g^{-1}, f.w.), were significantly ($p < 0.05$) elevated compared to root auxin concentrations from CEC exposure, demonstrating that different types of stress may have opposing effects on plant hormone homeostasis. Stem auxin concentrations were decreased significantly ($p < 0.05$) in the multi-stressed plants (65.3 ± 14.0 ng g^{-1}, f.w.) as compared to those exposed only to the CEC mixture (132.6 ± 54.3 ng g^{-1}, f.w.). In contrast, no significant differences in leaf abscisic or jasmonic acid contents due to heat stress was observed within the same CEC treatment.

Evaluating the potential impacts of CEC mixtures in recycled wastewater, biosolids and animal wastes on growth and development of plants is important in ensuring food security and sustainability. The results of this study have implications for the beneficial reuse of treated wastewater, biosolids, and animal wastes in agriculture as they contribute to the promotion of safe reuse practices and improve agricultural sustainability.

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1. BACKGROUND

California uses approximately 60% of the state's freshwater sources for agricultural production (USGS, 2015). With changing climate conditions, a growing population, and increasing water scarcity, there is a great need to utilize recycled wastewater to augment agricultural irrigation. In the 2015 Estimated Use of Water in the U.S., total irrigation withdrawals in the U.S. from surface and groundwater rose by 2% from 2010 (Dieter *et al.*, 2018). Surface water withdrawals decreased by 8% while ground water withdrawals increased by 16% from 2010 to 2015, demonstrating the urgency to identify alternative water sources, such as reclaimed wastewater. Ten states reported utilization of reclaimed wastewater as an irrigation source, although accounting for only about 1% of the total irrigation water use. However, reclaimed wastewater use rose from 472 Mgal d^{-1} in 2010 to 669 Mgal d^{-1} in 2015 (a 42% increase), demonstrating its future relevance and feasibility of adapting and using this water source to meet alleviate the pressure on our water demands.

Reclaimed wastewater is the treated product from a wastewater treatment plant (WWTP) where the treatment is specifically designed to meet standards for metals, solids, microbial pollutants, and biological oxygen demand. Consequently, most WWTPs are not efficient in the removal of other trace organic contaminants, such as many contaminants of emerging concern (CECs), including pharmaceuticals and personal care products (PPCPs) (Archer *et al.*, 2017; Boyd *et al.*, 2003; Kolpin *et al.*, 2002). In a study that analyzed 247 targeted CECs across 25 WWTPs, 148 CECs were detected in the source water, and 121 detected at least once in the treated water with concentrations in

the ng-μg L^{-1} range (Furlong *et al*., 2017; Glassmeyer *et al*., 2017). The reuse of treated wastewater, as well as application of biosolids and animal manures (Christou *et al*., 2018; Holling *et al*., 2012; Jones *et al.*, 2007; Wu *et al.*, 2015), introduces numerous CECs and their metabolites into the agro-environment (Christou *et al*., 2016; Kositch *et al*., 2014), and some of the more persistent CECs may reach concentrations in the range of μg-g kg^{-1} (Du and Liu, 2012). Wu *et al.* (2009) investigated the occurrence of 18 pharmaceuticals in the Lake Erie basin, an area that is highly impacted by agricultural runoff. They found caffeine, carbamazepine, and paraxanthine to be most frequently detected at maximum concentrations of 4.2, 2.8, and 1.8 μg L^{-1}, respectively. Due to differences in sources (e.g., hospitals, residential, industrial) and treatment techniques, each WWTP produces a treated product that is unique in chemical composition and concentration. These mixtures are dynamic and ever-changing due to the introduction of new sources, chemicals, and seasonal variations (Daughton and Ternes, 1999).

Pharmaceuticals are designed to induce a biological response at low levels, raising concerns for effects that may be unanticipated when non-target organisms are exposed to these trace contaminants in the environment (Burns *et al*., 2017). Acute toxicity data are available for some CECs, however, it may be argued that the acute effects are not of the greatest concern with regard to the irrigation of crops. In the agro-environment, chronic exposures are likely of greater interest because of the continual addition of CECs, at low levels, into the environment through repeated irrigation and biosolid applications. Information on chronic effects of chemical mixtures is particularly lacking, but such interest is important because it reflects the more realistic contamination scenarios in the

agro-food system. A combination of multiple CECs that act on specific modes may cause significant effects through additive or synergistic interactions (Daughton and Ternes, 1999). For example, Kurelec (1992) added verapamil, an MXR inhibitor, and 2-aminoanthracene, to river waters at 1 mg L^{-1}, concentrations that were previously found to have no effect on fish individually in toxicity studies. However, when in combination, exposure to verapamil and 2-aminoanthracene made the fish attempt dramatic escapes, demonstrating the importance of chemical mixture effects. A CEC mixture of low concentrations (ng-µg L^{-1}) can have subtle effects on behavior or cause alterations in gene expression that can manifest in significant physiological changes over extended periods of time. These causes of subtle behavioral or genetic modifications are extremely difficult to pinpoint because they may be overlooked as part of evolution or the result of other environmental factors (Daughton and Ternes, 1999). Irrigation with reclaimed wastewater and amendment of soil with biosolids or animal wastes represent typical chronic exposures to CEC mixtures. Therefore, the chronic effects of low-dose CEC mixtures are a significant concern for crops because of recurring exposures through irrigation with reclaimed wastewater and biosolid application, as well as the growth duration of food crops, which can range from months to years (for perennials such as fruit trees).

Environmental risk assessments (ERA) for chemical mixtures is typically conducted in one of two ways. One approach involves testing the effects of the mixture as a whole using *in vivo* or *in vitro* systems. This provides a measure of ecotoxicity without knowledge of the composition. The second method involves using ecotoxicological data

from individual chemicals and combining that information with analytical concentration data and other information regarding their modes of action and synergistic effects (Kienzler *et al.*, 2016). However, this component-based approach is more data and labor intensive, requiring the screening of, for example, wastewater effluent. Screening wastewater effluent and the receiving waters is not always effective because only targeted compounds are detected and potential effects on non-target organisms are not considered. The data gap regarding toxicological effects from pharmaceuticals, cosmetics, and other CECs on terrestrial organisms also presents a roadblock to using the second approach for assessing effects of CEC mixtures. Tang *et al*. (2014) analyzed 299 organic compounds in recycled water, all with concentrations below the Australian Guidelines for Water Recycling, suggesting an absence of health risk. However, cell-based bioassays designed to mimic these detected concentrations of the targeted compounds showed that the known chemicals explained only 3% of the observed cytotoxicity and less than 1% of the oxidative stress response as compared to the environmental recycled water sample. This implies that a large proportion of the elicited toxicity was due to unknown/un-targeted stressors, emphasizing the limits of the component approach due to lack of information on bio-active metabolites, non-targeted contaminants, and synergistic effects.

CECs are ubiquitous in agroecosystems in some regions due to their continual addition through irrigation, and application of biosolids or animal wastes (Kinney *et al.*, 2006; Prosser and Sibley, 2015). However, relatively little is known about how the complex and dynamic mixtures of CECs would affect plant growth and fitness. Chronic toxicity data for mixtures of low-dose CECs is largely lacking for food crops in the

literature. In this study, we simulated irrigation with reclaimed wastewater and evaluated the effect that low-dose, repeated exposure to a mixture of CECs had on young cucumber plants by measuring relevant biological endpoints including germination, growth, and hormone homeostasis. We also assessed bioaccumulation of CECs into cucumber tissues over a range of concentrations of the same mixture to examine the connection between the observed effects and uptake of CECs by the plant.

2. EFFECT OF LOW-DOSE, REPEATED EXPOSURE OF CONTAMINANTS OF EMERGING CONCERN ON CUCUMBER PLANT BIOLOGICAL ENDPOINTS AND HORMONE HOMEOSTASIS

2.1 Introduction

2.1.1. CECs effect on biological endpoints

CECs, including pharmaceuticals, have been shown to have adverse effects in aquatic non-target organisms (Kidd *et al*., 2007). Although several studies have documented the uptake of pharmaceuticals and CECs into plant systems (Wu *et al*., 2014; Wu *et al.*, 2015), ecotoxicological assessment, such as phytotoxicity, from this exposure is less prevalent. Phytotoxicity and health of the plant due to CEC exposure among agricultural crops that come into contact with CECs may affect their productivity and is therefore a food security and sustainability concern.

Ecotoxicological studies have shown that exposure to CECs can cause deleterious effects to non-target organisms, including plants (Batchelder, 1982; Hillis *et al.*, 2011; Sun *et al.*, 2018). For example, pinto beans (*Phaseolus vulgaris*) were adversely affected when grown in manure contaminated with chlortetracycline and oxytetracycline as reflected in reduced weight, nodulation, and uptake of nutrients (Batchelder, 1982). However, when the same experimental set-up was performed with corn, no effect was observed indicating that the type and extent of effect that CECs have on plants differs between plant species and chemicals, likely due to different modes of action or mechanisms of mitigating stress.

At present, it is extremely challenging to assess the ecotoxicity of chemical mixtures because there is no standardized method to capture all the mechanisms of action that a mixture of CECs may act upon. Research on phytotoxicological effects due to CEC exposure is still in its infancy, as evident from the use of a variety of biological endpoints, such as biomass, germination, oxidative stress, chlorophyll content, and arbitrary measurement methods. The USEPA has developed three OCSPP test guidelines for terrestrial plants under Title 40 of the Code of Federal Regulations (CFR) to test individual pollutants submitted to the EPA under the Toxic Substances Control Act (TSCA). However, these tests were designed to assess toxicity and lethal effects of individual xenobiotics, such as herbicides and pesticides. These tests use multiple plant species (four monocots, six dicots, one root crop) and expose plants to different concentrations of the test substance in hydroponic or soil media. The USEPA Seedling Emergence and Seedling Growth Test (OCSPP 850.4100) measures endpoints such as germination (embryogenesis, cell elongation, and organ emergence), root elongation, and the root to shoot ratio as a function of biomass. Early Seedling Growth Toxicity Test (OCSPP 850.4230), exposes plants through a hydroponic medium for 14 days to the test chemical/substance and parameters such as survival, length and weight of whole plants, shoot length, shoot dry weight, root length and root dry weight, and observed phytotoxicity. The Vegetative Vigor Test (OCSPP 850.4150) evaluates the effect of a 21-d exposure of a test substance on growth by measuring survival, plant height, and plant biomass, in addition to qualitative phytotoxic effects, such as chlorosis, necrosis, pigmentation, leaf curling, and wilting,

The above protocols are typically used to assess the acute effects and environmental risks of individual chemicals, generally considering a single source. Some studies have adapted these protocols to assess the environmental risk of CECs on plant biological endpoints (Migliore *et al*., 2010; Schmidt and Redshaw, 2015). Germination rates and specific root length has been a commonly used measure of phytotoxicity. For example, tetracycline and sulfonamide antibiotics had a negative effect on rice (*Oryza sativa*), cucumber (*Cucumis sativus*), and sweet oat (*Cichaorium endivia*) seed germination with sweet oat being most sensitive to sulfamethoxazole at an EC_{10} of 0.1 mg L^{-1} and cucumber seeds being less sensitive to all antibiotics (Liu *et al*., 2009). Lettuce was found to be the most sensitive test plant species for germination studies involving gemfibrozil, atorvastatin, and tamoxifen, with 50% inhibition of germination at concentrations of 25, 56, and 37 μg L^{-1}, respectively (D'Abrosca *et al*., 2008). Xie *et al*. (2010) observed a hormetic response where low concentrations (0.0625-0.5 mg L^{-1}) of chlortetracycline stimulated wheat (*Triticum aestivum*) germination, cell division and growth, whereas higher concentrations (50-300 mg L^{-1}) significantly decreased bud length, germination, and root length.

Root elongation has been shown to correlate with environmental change, metal induced stress, and nutrient availability (Cheng *et al.*, 2005; Ostonen *et al.*, 2007) reflecting the economic functions of the root system (Schmidt and Redshaw, 2015). This endpoint has been frequently applied in agronomy and forestry and is known to be reasonably constant within a species (Van Noordwijk and Brouwer, 1991). Increased specific root length is indicative of greater ability for root proliferation, allowing for

greater absorption and nutrients. Schmidt and Redshaw (2015) observed the effects of environmentally relevant concentrations of NSAIDS on germination (embryogenesis, cell elongation, and organ emergence) and primary root length on radish and lettuce plants. They found a significant increase in *R. sativus* root length when exposed to tolfenamic acid (10 µg L^{-1}) and a significant enhancement of *L. sativa* primary root development in comparison when exposed to ibuprofen (10 µg L^{-1}). In contrast, D'Abrosca *et al*. (2008) observed inhibition of *L. sativa* root elongation in the presence of lipid regulators, anticancer, estrogen, and vasodilator drugs. These opposing effects demonstrate that phytotoxicological response is specific to the environmental conditions, test compounds, and test species and shows that an observed effect on a single endpoint is not sufficient or reflective of total effect on a specific tissue/organism. Nevertheless, the above studies have also established the sensitivity of root-based endpoints and their usefulness in studying the effects of CEC exposure in higher plants.

Measurement of biomass reflects the cumulative effect of metabolic activities in plants. For crop growers specifically, a reduction in biomass can mean a reduction in yields and profits. Exposure to CECs has been shown to cause alterations in biomass through both hydroponic and soil exposures (Batchelder, 1982; Boxall *et al*., 2006; Jjemba, 2002, Li *et al.*, 2011). Lettuce and carrot growth decreased when cultivated in soils containing phenylbutazone, oxytetracycline, and enrofloxacin (Boxall *et al.*, 2006), which was in agreement with previous findings on the growth of lettuce exposed to enrofloxacin in a hydroponic medium (Migliore *et al*., 2003). Li et al. (2011) found that oxytetracycline exposure (10 µmol L^{-1}) significantly decreased the total length (24.17%

difference from the control) and volume of wheat roots when grown in hydroponic solution. They also noted that oxytetracycline had a greater impact on root biomass than shoot biomass, with root and shoot biomasses decreasing by 73.2% and 51.1%, respectively, when the plant was cultivated in a solution containing oxytetracycline at 80 μmol L^{-1} (Li *et al*., 2011).

The root to shoot ratio as a function of biomass provides a measure of photosynthetic investment in plant tissues. Schmidt and Redshaw (2015) observed a significant decrease ($p < 0.05$) in the root to shoot ratio of *R. sativus* when exposed to diclofenac (10 μg L^{-1}). A lower root to shoot ratio, meaning more biomass in aerial tissues, indicates either greater investment in photosynthetic tissues due to interferences with photosynthetic mechanisms therefore requiring increased photosynthetic tissues to overcome the gap in energy requirements, or, inhibition of root function causing a decrease in nutrient uptake and growth of aerial tissues (Schmidt and Redshaw, 2015). Interestingly, they noted that this effect was not evident in the germination study, indicating that length of exposure and/or the stage of development may be important in the effects observed. A greater length of exposure would enable the plant to uptake, translocate, and accumulate significantly more chemicals to reach the threshold to elicit a physiological response.

The published studies investigating the effects of CECs on plant toxicity have shown that the biological endpoints discussed are inclusive of general plant health and productivity. Therefore, endpoints such as germination, specific root length, and biomass measurements were chosen in this study to evaluate the effects of repeated, low-dose exposure to a mixture of CECs in cucumber plants.

2.1.2. Plant response to stresses

Environmental risk assessments are used to collect information about how chemicals perturb biological systems in order to predict which chemicals are likely to cause adverse effects. Measurements of oxidative stress, chlorophyll content, lipid peroxidation, and metabolic enzymes are examples of adopted methods of measuring phytotoxicity in plants exposed to CECs (Li *et al*., 2011; Sun *et al*., 2018a).

When plants take up contaminants such as pharmaceuticals, they can be metabolized to aid in their inactivation or detoxification (Dudley *et al*., 2018; Huber *et al*., 2012; Wu *et al.*, 2016). Exposure to xenobiotics may also result in the generation of reactive oxygen species (ROS) that can cause oxidative stress and damage to cells. Recent studies have used ROS measurements as a way of quantifying stress to cells and organisms (Christou *et al*., 2016; Liu *et al.*, 2009; Sun *et al*., 2018a; Sun *et al.*, 2018b). For example, alfalfa plants exposed to sulfamethoxazole and EE2 exhibited significantly higher concentrations of H_2O_2 and NO (oxidative species) in the roots than treatments with diclofenac or trimethoprim (Christou *et al*., 2016). Interestingly, plants exposed to the mixture of the four CECs showed the highest H_2O_2 content in leaves as compared to exposures to individual compounds. In addition, the mixed CEC treatment caused a 2-fold increase in MDA content in the roots, indicating significant cell membrane damage (Christou *et al.*, 2016). Sun *et al*. (2018a) investigated oxidative stress in cucumbers exposed to a mixture of 17 PPCPs and found changes in ROS levels at much lower concentrations than that of morphological effects, indicating the sensitivity of this measurement. Similar to Christou *et al.* (2016), ROS production was found to coincide

with an increase in membrane damage and lipid peroxidation in cucumber plants, suggesting the connection between ROS and its consequential damage to cell membranes (Sun *et al.,* 2018a). The results that mixtures further enhanced oxidative stress and membrane damage suggest that studies using individual PPCPs might underestimate the impact of CECs on plant growth and points to the need to consider mixtures of low concentration CECs that are more reflective of treated wastewater effluents.

Decreased concentrations of chlorophylls and carotenoids of lettuce seedlings cultivated in environmentally relevant concentrations of several pharmaceuticals were observed by D'Abrosca *et al.* (2008). The authors hypothesized that the pharmaceuticals interfered with photosynthetic processes. The inhibition of photosynthesis correlated with reductions in fatty acids, sugars, and phenols, compounds important for taste (as a consumed crop) and defense from pests. Increasing concentrations of oxytetracycline (0-80 μmol L^{-1}) caused decreases in chlorophyll *a*, chlorophyll *b*, and total chlorophyll in wheat, with statistically significant differences occurring as concentrations as low as 10 μmol L^{-1} (Li *et al.*, 2011). This observation corresponded to a decrease in photosynthetic rate, stomatal conductance and transpiration rates, indicating that oxytetracycline was toxic to higher plants. Sun *et al*. (2018a) also observed decreases in chlorophyll a and b when cucumber plants were exposed to a mixture of 17 PPCPs at 5 and 50 μg L^{-1} when grown in hydroponic solutions.

Measurements and endpoints that give insight to alterations in an organisms' survivability, growth, behavior, and reproduction provide the ultimate assessment of ecological risk, including potential impacts to plants upon CEC exposure. Evaluations

such as oxidative damage, photosynthetic pigment content, and metabolic enzymes are valuable in that they indicate the onset of adverse effects and provide mechanistic understanding for the ecotoxicological effects of xenobiotics such as CECs.

2.1.3. Phytohormones

A group of signaling molecules, known as phytohormones, regulate a variety of cellular processes within plants. These hormones play critical roles in plant acclimatization by mediating seed dormancy, nutrient allocation, defense, and flowering (Wani *et al*., 2016), thereby acting as indicators of a plants' vitality, growth, behavior, and fecundity. Phytohormones, which include auxins, abscisic acid, and jasmonates, are produced at low concentrations and work as chemical messengers to communicate biotic and abiotic stressors (Wani *et al.*, 2016). For example, when a plant experiences water stressed conditions, abscisic acid levels increase to trigger the closing of the stomata to conserve water (Carter *et al*., 2015). Several studies have explored plant hormone homeostasis in response to environmental stressors such as high-saline and water-stressed conditions to understand observed changes in growth, transpiration activity, and fruiting (Anderson *et al.*, 2004; Ramegowda and Senthil-Kumar, 2015). Observations in phytohormone homeostasis could give insight into the cellular mechanisms and signaling that lead to more long-term, visual phytotoxic responses such as reduced biomass (Carter *et al.*, 2015).

Auxins (IAA) play a role in cell division and root elongation and have been shown to be involved in plant adaptation to stressful conditions by coordinating root and shoot

growth (Ahammed *et al*., 2016). Auxins are involved in the heat stress response by enhancing elongation of photosynthetic tissues away from heat-absorbing soil surface and elongating stems to capitalize on the cooling effect of moving air (Gray *et al*., 1998).

Abscisic acid (ABA) is pivotal to several plant physiological and developmental processes including cell turgor, fruit and leaf abscission, and stomatal closure (Speirs *et al*., 2013). ABA is considered the "stress hormone" in plants owing to its adaptive response and role in stress tolerance. ABA is involved in expression of numerous stress-responsive genes, transcriptionally regulating up to 10% of protein-encoding genes (Nemhauser *et al*., 2006). ABA plays a vital role in signaling water stressed conditions from the roots to the shoots to initiate stomatal closure resulting in water-saving, anti-transpirational activity (Wani *et al.*, 2016; Wilkinson *et al.*, 2012). The defense response is also partially mediated by ABA, with ABA playing both negative and positive roles. For example, exogenous application of ABA on *Arabidopsis* plants increased its susceptibility to fungus, *Fusarium oxysporum* (causal agent of wilt), by suppressing the transcription of defense genes (Fujita *et al*., 2006). In contrast, ABA plays a more positive role in pre-invasive defense response against pathoPgens by initiating the closure of stomata, therefore increasing penetration resistance (Melotto *et al.*, 2006). Several studies have considered ABA to gain a mechanistic understanding of how environmental stressors effect the physiological response to a plant (Carter et al., 2015; Ramegowda and Senthil-Kumar, 2015), and has also been a target of manipulation from a bioengineering standpoint to increase a plant's tolerance to environmental stressors such as drought or disease (Ramegowda and Senthil-Kumar, 2015).

Jasmonic acids (JA) are multifunctional signaling compounds involved in senescence, tendril coiling, and reproductive processes such as flowering and fruiting (Ahammed *et al*., 2016; Creelman and Mullet, 1995; Fahad *et al*., 2015). JA levels differ amongst plant tissues and stage of development, with highest levels being found in flowers and reproductive tissues (Creelman and Mullet, 1995). JA is involved in response to environmental stressors such as salinity, drought, and low temperature (Seo *et al.*, 2011), as well as plant response to pathogens by inducing genes that enhance production of compounds used in plant defense (Pauwels *et al.*, 2009). In response to salinity stress, an increase in endogenous JA levels in the roots reportedly alleviated the deleterious effects of salinity stress (Wang *et al*., 2001). This observation was in agreement with Yoon *et al.* (2009) who observed that exogenous application of a jasmonic acid metabolite decreased the adverse effects of salinity stress in soybean seedlings.

Since several studies have linked exposure of CECs to phytotoxicity and oxidative stress (Christou *et al*., 2016; Sun *et al.*, 2018a), it may be hypothesized that phytohormone homeostasis is also influenced by CECs to mediate a plant's stress response to such xenobiotics. Carter *et al*. (2015) observed a significant decrease in leaf ABA concentrations in response to verapamil and carbamazepine exposure in soil (0.005-10 mg kg^{-1}). IAA concentrations significantly increased in response to verapamil treatments, however, a hormesis effect was found in the carbamazepine treatments, where IAA concentrations were elevated from the controls at treatment concentrations from 0 to 2 mg kg^{-1}, followed by a rapid decrease at carbamazepine concentrations of 4-10 mg kg^{-1}. This work was one of the first to link uptake of CECs such as pharmaceuticals to effects

on plant hormone homeostasis, which at high concentrations, resulted in reductions in biomass (Carter *et al*., 2015).

Several studies have effectively utilized a phytohormone profile to explore a plant's response to environmental stressors, such as salinity and drought. Since the phytohormones discussed above have active roles in multiple areas of the physiological response, it is believed that their response to chronic exposure of a mixture of CECs would help elucidate the stress response to CECs and mechanistically explain the visual phytotoxicity that is measured in endpoints such as root length, germination, and biomass. Therefore, recognizing how low-dose, repeated exposure to CEC mixtures would add greatly to our understanding of how beneficial reuses of treated wastewater, biosolids, and animal wastes may affect crop productivity and food security.

2.1.4. Interactive effects of simultaneous stresses on plants

In nature, crops are often simultaneously exposed to diverse biotic and abiotic stresses. Controlled laboratory and greenhouse experiments focusing on only single stresses are often not reflective of true environmental conditions due to combinational stresses that occur in the field (Wani *et al*., 2016).

Extreme temperatures affect several biochemical processes and the stability of proteins, RNAs, membranes, and cytoskeletal structures (Dobrá *et al.*, 2015). Heat stress disrupts cellular homeostasis, respiration, and photosynthesis leading to decreased plant growth and productivity (Ahammed *et al.*, 2016; Kotak *et al.*, 2007). However, plants have developed sophisticated mechanisms that allow plant cells to sense changes in

temperature and activate defense mechanisms to protect against damage imposed by heat stress, known as the heat stress response (HSR).

Phytohormones, including ABA, JA, and IAA, have been reported to play pivotal roles in plant response to heat stress (de Wit and Frankhauser, 2014; Dobrá *et al.*, 2015; Gray *et al*., 1998; Mittler *et al.*, 2012). For example, exogenous application of ABA increased thermotolerance of *Agrostis stolonifera* (Larkindale *et al.*, 2005) likely by controlling water movement via stomatal closure and inducing the expression of protective compounds and proteins to heat stress. Dobrá et al. (2015) observed a significant increase in ABA leaf content for 30 min exposure to heat stress (40 °C) after a 30 min delay, followed by a significant decrease from the initial levels after being under heat stress for over 1 h. A decrease in leaf ABA content increases the transpiration rate and consequently decreases leaf temperature to alleviate heat stress. However, water deficits can occur upon prolonged enhanced transpiration rates, therefore forcing stomatal closure as to not impose water-stress to the plant (Macková *et al*., 2013). JA plays a positive regulatory role in basal thermotolerance (Ahammed *et al.*, 2016) and has been shown to improve heat tolerance in wild-type *Arabidopsis thaliana* (Clarke *et al*., 2009).

With a changing climate and projected increases in water scarcity, it is expected that irrigation with TWW will be adapted to meet the needs of agriculture. The effect of trace contaminants present in TWW can have negative (susceptibility) or positive (tolerance) impacts on a plant's physiological response to other natural environmental stressors, such as heat. Extreme high temperatures are one of the most frequent abiotic stresses experienced by plants (Dobrá *et al.*, 2015). With a rate of warming of 0.05 °C per decade

(1998-2012) (Stocker, 2014), the frequency and magnitude of temperature change will be a future environmental stressor that can affect crop productivity. Battisti and Naylor (2009) predicted that every 1 °C increase in seasonal temperature would cause 2.5-16% loss in direct crop yields. Therefore, heat was chosen as the combinational stressor in this study because arid and semi-arid environments (e.g., southern California) that are more prone to use TWW for irrigation are the same areas that typically experience extreme temperatures and excessive heat.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

A total of 10 CECs were selected based on their detection frequency and concentrations in treated wastewater. These CECs included 4 antibiotics, 3 analgesic anti-inflammatory drugs, 1 anti-epileptic, 1 beta-blocker, and 1 antimicrobial. Standards of naproxen, diclofenac, atenolol, trimethoprim, and tetracycline were purchased from Sigma Aldrich (St. Louis, MO). Sulfamethoxazole was obtained from MP Biomedicals (Solon, OH). Ibuprofen and triclosan were purchased from Alfa Aesar (Ward Hill, MA). Azithromycin was purchased from Tokyo Chemical Industry (Tokyo, Japan). Isotope labeled standards azithromycin-*d5*, diclofenac-*d4*, and trimethoprim-*d9* were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Triclosan-*d3*, atenolol-*d7*, sulfamethoxazole-*d4*, and carbamazepine-*d10* were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). Hormone standards indole-3-acetic acid and jasmonic acid were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA) and abscisic

acid was purchased from Chem-Impex International (Wood Dale, IL). Abscisic acid-*d6* was purchased from Toronto Research Chemicals and indole-3-acetic acid-*d7* was purchased from Cambridge Isotope Laboratories. Stock solutions were prepared in 25% DMSO or HPLC grade methanol and stored at -20 °C before use. HPLC grade acetonitrile and methanol were used for extraction. Ultrapure water (18 MΩ) was obtained from a Milli-Q system (Millipore, Carrigtwohill, Cork, Ireland). Solvents for UPLC analysis including methanol and formic acid were Ultima grade (Fisher Scientific, Fair Lawn, NJ).

2.2.2 CEC mixture treatments

Ten CECs were selected based on their occurrence and recalcitrance to wastewater treatment. A "1X" concentration was used to approximate the median concentration of each CEC in treated wastewater effluents, as determined by literature analysis (Archer *et al.*, 2017; Lishman *et al*., 2006; Miege *et al*., 2009). A dose response curve was generated by exposing seeds and plants to the CEC mixture at multiples of the 1X Median concentration (i.e., 2, 5, 10, 20-X). Selected chemical properties and concentrations of the 10 CECs are found in Tables 1 and 2. Control treatments and solvent control treatments (0.02% DMSO) were used in parallel.

2.2.3. Lettuce seed germination bioassay

Seed germination and primary development were assessed using adapted procedures from USEPA Ecological Effects Test Guidelines for seedling emergence and seedling growth (OCSPP 850.4200). Lettuce (*Lactuca sativa*) seeds from Botanical Interests (Broomfield, Colorado;

Lot # 12955) were sterilized with 1% (v/v) NaClO for 20 min and thoroughly rinsed with DI water. Lettuce seeds (n=10 seeds per dish, 4 dishes per treatment) were placed in a 10-cm petri dish containing Whatman filter paper with 5 mL of ¼-strength nutrient solution (Oasis® 16-4-17 hydroponic fertilizer 0.8 g L^{-1}, pH 5.5-6) amended with the respective CECs at different rates. Controls, solvent controls (0.02% DMSO), and CEC treated seeds covered in the petri dishes and incubated at 22 °C. After 7 d incubation, germinated seeds were counted, dissected into root and shoot, documented for length, and pooled to record the wet weight of roots or shoots. In addition, stages of development, including primary root, hypocotyl elongation, and cotyledon emergence were monitored daily. Each treatment was carried out in triplicate with one petri dish being equal to one replicate.

2.2.4. Cucumber low-dose, long-term bioassay

Cucumber (*Cucumis sativus*) seeds from Fisher Scientific (Fair Lawn, NJ) were started in an organic soil mixture and then transferred to hydroponic solution (Oasis® 16-4-17 hydroponic fertilizer 3.2 g L^{-1}, pH 5.5-6) after the first true leaves appeared. Hydroponic solutions were spiked with CEC mixtures at different doses (1, 2, 5, 10, 20-X). Controls, solvent controls (0.02% DMSO), and negative controls (CEC spiked nutrient solution with no plants) were set-up simultaneously. Plants were grown in a growth chamber (14 h / 22 °C day, 10 h/ 20 °C night cycle, relative humidity 50-60%). Hydroponic solutions (containing CECs at the respective levels) were renewed every 3-4 d as needed. After 30 d cultivation, cucumber plants were dissected into roots and shoots

and the biomass was recorded. New leaves, old leaves, stems, and roots were sub-sampled for analysis of bioaccumulation of CECs and measurement of hormones.

2.2.5 Cucumber long-term multiple stress bioassay

Cucumber plants were cultivated in the same manner as described above. On day 26, plants were transferred to a greenhouse with full sunlight where the temperature ranged between 22-41 °C between day and night, with a relative humidity between 10-57%. Plants were sampled midday 4 days later when temperature was about 32 °C for a minimum of 1 h, ensuring that the plant was under heat-stress.

2.2.6. Plant hormone extraction and analysis

After 30-d cultivation, cucumber plant tissues (0.02-0.05 g) were extracted and analyzed for various hormones. Plant tissues (roots, stems, leaves, flowers) were immediately frozen using liquid nitrogen upon sampling and then ground to a fine powder using a mortar and pestle. The samples were extracted with 2 mL of 80% methanol with 0.1% formic acid containing 50 ng of labeled standards as recovery surrogates. The extract was transferred to 2 mL microcentrifuge tubes and kept at -20 °C for 12 h, after which the sample extract was vortexed, and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was filtered through a 0.22 µm polytetrafluoroethylene (PTFE) membrane (Millipore, Carrigtwohill, Cork, Ireland), transferred to a clean 2 mL microcentrifuge tube and evaporated to near dryness on ice under nitrogen. The samples were resuspended in 200 µL of 50% methanol and centrifuged at 10,000 g for 3 min. Supernatants were transferred to 300 µL inserts housed in 2 mL glass vials and stored at -20 °C until analysis.

2.2.7. Extraction of CECs from plant and hydroponic samples

Freeze dried plant tissue samples (roots, old leaves, new leaves, flowers) were extracted and analyzed following a previously published method (Wu *et al*., 2014). Briefly, approximately 0.2 g of plant tissue was added to 50-mL polypropylene centrifuge tubes and spiked with deuterated standards of the CECs as recovery surrogates. Methyl tert-butyl ether (MTBE) (20 mL) was added to each sample, vortexed for 30 s, and placed in an ultrasonic water bath (50/60 Hz, Fisher) for 20 min. The samples were centrifuged at 3000 rpm for 20 minutes and the supernatant decanted into a 40 mL glass vial. Samples were re-extracted with methanol (20 mL) following the above procedure and their resulting supernatants combined. The extracts were evaporated under a steady flow of nitrogen until near dryness, resuspended in 1 mL of methanol, and diluted with DI water to a volume of 20 mL. Clean-up was performed by loading the sample on 150-mg Oasis© HLB cartridges (Waters, Milford, MA) pre-conditioned with 7 mL methanol and 14 mL DI water. After loading, the cartridge was dried on a vacuum manifold and eluted with 20 mL methanol under gravity. Eluates were dried under nitrogen and reconstituted in 1.5 mL of methanol-water (1:1, v/v). Samples were filtered through 0.22 μm polytetrafluoroethylene (PTFE) filters into 2 mL glass vials and stored at -20 °C until instrumental analysis. Freshly prepared nutrient solution containing CECs at different levels were extracted to determine the initial concentrations of CECs in the medium. Additional spiked nutrient solutions were exposed to the plant growth conditions for 3 d without plants and others with plants to determine the dissipation of CECs between nutrient solution renewal. The nutrient solution volume was recorded and loaded on to

pre-conditioned 150 mg Oasis HLB cartridges and prepared as described above. Samples were stored in 2 mL glass vials at -20 °C until instrumental analysis.

2.2.8. Instrumental analysis

Hormone and CECs in sample extracts were quantified on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) combined with a Waters Micromass Triple Quadrupole (TQD) mass spectrometer equipped with electrospray ionization (ESI-MS/MS) interface (Waters, Milford, MA.) Separation was achieved on an ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm particle size, Waters) at 40 °C using a 5 µL injection volume. Hormones and CECs analyzed in the ESI+ mode were separated using mobile phase A (100% water with 0.1% formic acid and mobile phase B (methanol with 0.1% formic acid). Separation of CECs in the ESI- mode was carried out using mobile phase A (100% water) and mobile phase B (100% methanol). For hormone separation, the following mobile phase gradient was used with respect to mobile phase A: 0-0.5 min, 95%; 0.5-1 min, 60%; 1-2 min, 10%; 2-4 min, 95%; 4-5 min, 95%. For CECs, the following mobile phase program was used with respect to mobile phase A: 0-0.5 min, 95%, 0.5-3 min, 10%; 3-6.5 min, 95%; 6.5-7 min, 95%. The flow rate was 0.3 mL min^{-1}. The TQD parameters were as follows: source temperature, 120 °C; desolvation temperature, 350 °C; capillary voltage, 3.0 kV; cone voltage, 20 V; desolvation gas flow, 600 L hr^{-1}; cone gas flow, 50 L hr^{-1}. Quantitative analysis was performed in the multiple reaction monitoring mode (MRM). Details on monitored ions and their respective collision energies can be found in Table 3. All data were processed using MassLynx 4.1 software (Waters, Milford, MA).

2.2.9. Data analysis and quality control

All plant and hydroponic treatments were set-up in triplicate. Controls, solvent controls, and negative controls were included with each sample extraction. A methanol-water (1:1, v/v) blank was run between treatments during analysis on the UPLC-MS/MS to assess and eliminate contamination. Labeled surrogate standards were used in all sample extraction and analysis to estimate analyte recovery and account for matrix effects during instrumental analysis. Surrogate recoveries can be found in Table 4. Calibration curves (ranging from 1 to 50 ng mL^{-1}) were used for quantification with r^2 values of at least 0.98 for all analytes. Limits of detection (LOD) and limits of quantitation (LOQ) can be found in Table 5 and were defined as the concentration at which a signal to noise ratio of 3 and 10 was achieved, respectively. Statistical analysis of data including means, standard deviations, t-tests, and ANOVA with post hoc Scheffe's test were performed using SPSS Statistics (version 24.0; IBM SPSS Statistics, Chicago, IL). Data are represented as mean ± standard deviation.

2.3. RESULTS AND DISCUSSION

2.3.1. Effects of CEC mixture exposure on lettuce germination and development

To assess the effect on germination, lettuce seeds were exposed to the mixture of ten CECs at concentrations 1X of that in treated wastewater up to 20X. DMSO was used as a carrier solvent to solubilize the CECs. To ensure that the observed effects were attributed to the presence of CECs, two types of controls (water, and 0.02% (v/v) DMSO) were included. No significant differences between controls and solvent controls were

observed. Germination, root length, and biomass were used as biological endpoints for the germination bioassay. No significant differences were observed in germination rate across all treatments.

At the end of the 7 d incubation, root growth of lettuce seedlings was found to increase with increasing rates of the CEC mixture with significant differences observed at the 2X, 10X, and 20X concentration (Figure 1a, $p < 0.05$). Compared to the control, root length was found to increase by 16 ± 3, 24 ± 6, and 32 ± 8 % at the 2X, 10X, and 20X CEC concentration levels, respectively. This was in contrast with studies that showed negative root length effects when plants were exposed to other CECs such as tetracyclines and sulfonamides (Batchelder, 1982; Liu *et al*., 2009). However, in those studies, high concentrations of a single CEC were generally considered. For example, Liu *et al*. (2009) observed inhibition of root growth in oats, rice, and cucumbers when the seedlings were exposed to oxytetracycline concentrations at 5-10 mg L^{-1}. Chemical mixtures may involve more complex interactions, where various chemicals may have different but related targets that can have an additive or nonadditive effect (Carpenter *et al.*, 2002). This may be the reason for the observed stimulatory effect by the CEC mixtures at environmentally relevant concentrations in this study. It could also be the result of a biphasic response where a favorable biological response at low dose and inhibition at high dose is observed, a phenomenon known as hormesis (Novak *et al.*, 2011). Primary root length after 7 d germination is indicative of the plant's ability to establish itself and obtain nutrients during this critical period of development. The stimulatory effect on root length observed in response to the low-dose exposure of a

mixture of CECs in this study suggested that a low-dose mixture may help the plant establish itself better and increase its ability to obtain water and nutrients during the beginning stages of growth. However, it must be noted that only a small set of CECs were considered in this study, and a similar response may not necessarily occur for other CECs or for these CECs with a different species.

Roots, stems, and leaves each maintains their own dynamic balance in biomass that is indicative of the relative above-ground resources (CO_2 and light) and below-ground resources (water and nutrients). The root to shoot biomass ratio provides insight into the overall health of the plant. A change in this ratio from the control suggests a change in the overall health of a plant. A lower root to shoot ratio suggests greater investment in above-ground tissues possibly due to interference with photosynthetic mechanisms or interference in root functioning, resulting in reduced nutrient uptake and therefore growth (Rogers *et al*., 1995). A greater root to shoot ratio is typically influenced by below-ground conditions, suggesting reduced water and nutrient availability. Although no significant differences in root to shoot ratio with respect to biomass during the 7 d study, a positive correlation between CEC treatment levels and root to shoot ratio was observed (Figure 1b), suggesting a possible interference with nutrient or water uptake by the roots. This was in agreement with Carter *et al*. (2015), who found that carbamazepine and verapamil exposure caused changes in sodium and calcium ion flow regulation in zucchini plants, demonstrating the influence of CECs on nutrient transport.

2.3.2. Uptake and translocation of CECs in cucumber plants

Since toxicity can only be elicited when a chemical has reached its target site, we monitored bioaccumulation of the target CECs into various cucumber tissues. The starting concentrations and their dissipation in the nutrient solution after 3 d with and without plants are found in Table 6. The 20X CEC treatment was used because the higher concentrations facilitated qualitative evaluation of CEC bioaccumulation and translocation. Among the various cucumber tissues, only one flower sample per treatment was collected due to the limited growth duration and plant tissue. Samples of flowers had to be pooled from replicates for each treatment, and therefore some standard deviations could not be calculated for the CEC concentrations in flower samples.

Concentrations of CECs in plant tissues increased with increasing concentrations in the hydroponic solution (Figure 2). All CECs except triclosan were detected in the roots (Figure 3). The absence of triclosan in the root samples could be due to its relatively high quantification limit of triclosan (LOQ = 20 µg L^{-1}), active metabolism (Macherius *et al.*, 2012; Macherius *et al*., 2014), or suppressed uptake of triclosan in the presence of other CECs. The bioconcentration factor (BCF) was calculated by dividing the concentration in the respected plant tissue after 30 d of cultivation by the initial concentration present in the hydroponic solution:

$$BCF\ (L\ kg^{-1}) = \frac{Concentration\ in\ plant\ tissue\ (ng\ g^{-1})}{Concentration\ in\ hydroponic\ solution\ (ng\ mL^{-1})} \qquad \text{(Eq. 1)}$$

The translocation factor (TF) of CECs in cucumber tissues was calculated as the concentration in above ground tissue divided by that in the root tissue:

$$TF = \frac{Concentration\ in\ above\ ground\ tissue\ (ng\ g^{-1})}{Concentration\ in\ root\ itssue\ (ng\ g^{-1})} \qquad \text{(Eq. 2)}$$

In general, BCF values in root tissues were greater than those of above-ground tissues, except for tetracycline and sulfamethoxazole. In this study, tetracycline had the lowest BCF in the root tissues (0.2 ± 5.2), however, tetracycline BCF's in above ground tissues ranged from 3.5 to 5.6 (Figure 3), demonstrating its high translocation potential (TF = 0.39-0.58, Figure 4). Carbamazepine had the highest BCF in all cucumber tissues with $BCF_{new\ leaves}$ of 53.9 ± 19.1 and BCF_{roots} 344.8 ± 94.7. The BCF_{roots} for carbamazepine was much greater than that found in previous studies (Herkholtz *et al*., 2010; Wu *et al.,* 2013), and this difference could be due to different test species used, stage of plant development, and experimental setup. In contrast, TF values of carbamazepine from this study (0.6, 0.6, and 1.5 for new leaves, flowers, and old leaves, respectively) were in close agreement with carbamazepine TF values in Wu *et al.* (2013). Sulfamethoxazole had similar bioconcentrations in the roots (7.12 ± 2.04) as above-ground tissues (3.97-8.88). Carbamazepine and sulfamethoxazole had the highest TF values in old leaves of 1.5 and 1.3, respectively. Translocation from roots to above-ground tissues was not detected for naproxen and ibuprofen, likely due to poor translocation or active plant metabolism (Figure 4). The active translocation of some CECs (e.g., sulfamethoxazole, carbamazepine, tetracycline, azithromycin, and trimethoprim) to flowers may present an interesting route of exposure of these contaminants to other non-target organisms such as pollinators.

Previous studies have used the pH-adjusted octanol-water partition coefficient, log $D_{ow,}$ to address the influence of molecular dissociation on plant uptake (Tanoue *et al*.,

2012; Wu *et al.*, 2013). Speciation of a compound was considered by calculating the fraction of neutral molecule (f_n) in the growth medium at pH 5.5, using the following equation (Trapp, 2009):

$$f_n = \frac{1}{1+10^{i(pH-pK_a)}} \qquad \text{(Eq. 3)}$$

where i is 1 for acids and -1 for bases. Log D_{ow} was then calculated using the following equation:

$$log\ D_{ow} = log\ K_{ow} + log\ f_n \qquad \text{(Eq. 4)}$$

Log D_{ow} was considered an indicator of potential root uptake, where chemicals with a higher log D_{ow} may easily sorb to roots (Tanoue *et al*., 2012; Wu *et al.*, 2013), therefore increasing the potential for uptake of CECs by the plant. We plotted the log BCF_{roots} against log D_{ow} and found a poor correlation between the two variables (r = 0.42), suggesting that other factors other than hydrophobicity also affected plant uptake of CECs (Figure 5). This was in contrast to Wu *et al*. (2013) who found an adequate correlation between log D_{ow} and BCF values of 20 CECs for four different vegetables. It is likely that CECs act on different biochemical processes. For example, carbamazepine regulates sodium ion flow across membranes, so it is possible that effects from one compound could affect the uptake of another (Carter *et al*., 2015). Christou *et al*. (2016) observed that a mixture of 4 CECs (diclofenac, trimethoprim, sulfamethoxazole, and 17α-ethinylestradiol) displayed different uptake and translocation patterns when exposed in a

mixture as compared to treatments with individual compounds, demonstrating the impact of mixture effects on plant uptake.

2.3.3. Effects of CEC mixtures on cucumber after long-term exposure

Above-ground and below-ground biomass were measured for cucumber plants at the end of a longer-term (30 d) exposure to the same CEC mixture in hydroponic solution at incremental levels. Biomass measurements are useful in measuring stress response, as deviations in growth from the control are indicative of the overall sum of response of the plant (Carter *et al.*, 2015). Although there were no significant differences in the biomass among the different CEC levels, there appears to be a dose dependent response when change in biomass, expressed as the percentage difference relative to the control, was considered (Figure 6). At the 20X treatment, the relative percentage differences in the average below ground, above ground, and total biomass from the control were -51.2 ± 20.9, -26.3 ± 34.1, and -33.2 ± 41.7%, respectively (Figure 6d). The greatest reduction in plant biomass occurred in the roots, and this finding was similar to Carter *et al*. (2015) who also observed a ~30% reduction in the below ground plant tissues of zucchini from the control when the plant was exposed to 10 mg kg^{-1} carbamazepine in soil. The observed reduction in above-ground biomass and total biomass along the dose-response curve (no change in root to shoot ratio) suggested that there was not simply further investment in photosynthetic or aerial tissues due to interferences in photosynthetic mechanisms, but rather that multiple aspects of the plant were affected without ways to mitigate the stress (Schmidt and Redshaw, 2015). It was also possible that the roots could not support an increase in aerial tissues, the common stress mitigation mechanism,

because the roots were also under stress and were unable to take up the necessary nutrients to promote growth.

2.3.4. Alterations in plant hormone homeostasis after CEC exposure

A hormone profile was analyzed to further understand the dose-response effect of chronic exposure to a mixture of CECs on cucumber plants (Figure 7). In this study, we focused on three phytohormones; auxin (indole-3-acetic acid, IAA), jasmonic acid (JA), and abscisic acid (ABA) because of their critical roles in regulation of a plant's development and stress-response.

The auxin profile was characterized by a hormesis effect along the dose-response curve when the leaves and stems were considered (Figure 7a). The solvent control did appear to have some stimulatory effect on auxin concentrations in the stems (71.72 ng g^{-1}, f.w.), but the change was not statistically significant ($p > 0.05$). A 6-fold increase was observed in the stem auxin concentrations at the 1X CEC treatment level (132.58 ng g^{-1}, f.w.) as compared to the control (21.62 ng g^{-1}, f.w.). The trend, however, was followed by a gradual decrease to 2-fold the control at the 10X CEC treatment rate (44.55 ng g^{-1}, f.w.). The leaf auxin concentrations significantly increased at the 1X and 10X CEC treatment rates to 16 and 11-fold, respectively, which was followed by a decrease at the 20X CEC treatment rate. A similar pattern in leaf auxin content was also observed by Carter *et al*. (2015) along a dose-response treatment of carbamazepine for zucchinis grown in soil. A similar pattern, however, was not visible for the auxin content in the roots or fruits at the end of 30 d cultivation in this study. Auxin is known to be involved in cell elongation and division of meristematic tissues. The observed increase of auxin in

the stems and leaves at CEC levels as low as the 1X treatment rate suggested that the stems and leaves were being signaled to grow in order to gain increased light exposure because of interferences with photosynthetic mechanisms, and/or decrease heat stress by allowing for more air flow.

Jasmonates are phytohormones that are involved in flower development, fruiting, reproduction, and plant defense. No clear trends or significant differences were observed in JA levels in any of the plant tissues along the dose-response curve (Figure 7b). This could be due to the time of sampling, as the plant was still in an early stage of development (approximately 50 d from seed), when flowering and fruiting was not the primary focus of the plant. Instead, at this point in development, increasing photosynthetic tissues was likely of the upmost importance.

ABA is a signaling hormone that communicates water stress to the plant. ABA levels were significantly elevated in the leaves with exposure to increasing levels of CECs and significantly decreased in the roots at environmentally relevant concentrations of the CEC mixture (Figure 7c, $p < 0.05$). ABA in the roots dropped from 56.5 ± 17.3 ng g^{-1} in the controls to 8.23= ± 9.5, 5.6 ± 2.2, and 11.8 ± 13.7 ng g^{-1} at the 1X, 10X, and 20X CEC treatment rates. Low ABA levels in the roots could indicate over-saturation by water at the root tips. In this study, we observed an approximate 20% decrease in root ABA levels when the plant was exposed to the CEC mixture at the 1X level as compared to the control, demonstrating that even exposure to CECs at low levels could significantly affect the homeostasis of this hormone. The decrease in the ABA levels coincided with visual symptoms of the roots, where the roots appeared to be over-saturated and less rigid

structurally, which also resulted in a 'shedding' of some small roots into the hydroponic medium. In the leaves, ABA was found to increase significantly at the 1X, 10X, and 20X CEC treatment levels (78.3 ± 11.6, 138.3 ± 37.6, and 165.39 ± 96.45 ng g^{-1}, respectively, ($p < 0.05$) from the control 42.6 ± 12.8 ng g^{-1} (Figure 7c). Elevated ABA levels can cause stomatal closure, thereby reducing transpiration in a plant's efforts to conserve water (Ahammed *et al.*, 2016). The increase of ABA in the leaves and its resulting effect on anti-transpiration and therefore decreased pulling force of nutrients to aerial tissues could be the reason for the reduction in above-ground biomass observed in this study. Anti-transpiration activity may also impose an impediment on plant growth by limiting gas exchange and impairing the plant's ability to adapt to additional stressors such as extreme temperatures (Munemasa *et al*., 2015). With stomatal closure, the plant's ability to mitigate heat stress by transpiring is also impacted, threatening its survival.

2.3.5. Phytohormone response to multiple stressors (CEC and heat stress)

Following cultivation in CEC-containing nutrient solution, a subset of cucumber plants was exposed for 4 d to heat stress at temperatures up to 41 °C in a greenhouse, and the plants were then sampled for hormone analysis. JA content was not significantly altered with the additional heat stress in the roots and stems (Figure 8a). Although, a significant effect on the JA content in the leaves was not observed following any of the lower level CEC treatments, JA content in the 20X treatment (417.0 ± 94.2 ng g^{-1}, f.w.) was significantly increased from JA content in plants exposed to heat without CEC exposure (166.9 ± 27.2 ng g^{-1}, f.w.) (Figure 8a, $p < 0.05$). A consistent trend across CEC treatments was a decrease in the JA content in the leaves in response to heat stress.

Jasmonates are important signaling molecules in plant defense, and therefore a decrease in JA content in leaves in response to heat stress across all CEC treatments has implications for plant survival when exposed to disease, wounding, or pathogens (Wani *et al.*, 2016). Although heat-stress exposed plants did not have statistically significant changes in JA content, this decreased trend in the JA content in leaves exposed to excessive heat conflicts with a study where heat shock (50 °C for 30 min) was found to result in an upregulation of JA pathway genes and its consequentially enhanced production in agarwood cells (Xu *et al.*, 2016). This could be due to the type of heat exposure (chronic vs. acute), the range of temperature exposure, or the difference at the cellular level (cell culture vs whole plant).

Auxin concentrations (59.8 ± 16.9 ng g^{-1}, f.w.) in the roots of the control treatments with heat stress were significantly elevated ($p < 0.05$) as compared to the 1X and 20X CEC treatments (21.6 ± 17.2 ng g^{-1}, f.w.) (Figure 8b). This finding showed how different types of stress may have opposing effects on plant hormone levels. In the stems, heat stress resulted in a significant decrease in auxin concentrations in plants in the 1X CEC treatment (65.3 ± 14.0 ng g^{-1}, f.w.) as compared to the 1X CEC treatment without heat stress (132.6 ± 54.3 ng g^{-1}, f.w.), however the 1X CEC treatment with the added heat stress was not statistically different from the control or the 20X CEC treatment. The role of auxins in cell division and elongation has recently been associated with being an adaptive growth response to high temperature tolerance as seedlings elongate to elevate photosynthetic and meristematic tissues away from the heat-absorbing soil, thereby allowing increased air circulation and cooling effects (Gray *et al.*, 1998). Leaf auxin

concentrations were not affected by heat stress (Figure 8b). Between the control and 20X CEC treatments, heat stressed plants displayed only slightly elevated auxin contents from their respective CEC treatments without the additional heat exposure.

ABA is integral to how plants mitigate heat stress. Elevated temperatures cause an increase in transpiration as the plant's mechanism to decrease leaf temperature (Ahammed *et al*., 2016). However, prolonged transpiration and dry soil conditions may alter a plant's strategy to mitigate heat stress due to the need to conserve water. Considering that we saw an increase in ABA content with increased CEC concentrations, we speculated that the cucumber plant's ability to adapt and mitigate heat stress would be further impaired due to exposure to CECs. The ABA content in cucumber tissues followed the same general trend under heat stress conditions as it did under non-heat stressed conditions (Figure 8c). No differences in root ABA content due to heat stress were observed within the treatments, however the 1X (20.0 ± 2.2 ng g^{-1}, f.w.) and 20X (17.8 ± 13.7 ng g^{-1}, f.w.) CEC treatment with heat stress were significantly decreased from the control with heat stress ($p < 0.05$) (64.1 ± 13.3 ng g^{-1}, f.w.) (Figure 8c). Similarly, significant differences in leaf ABA content due to heat stress were not observed within the same CEC treatment, but did show a consistent elevated trend across the range of CEC treatment levels when the plant was also subjected to heat stress. It must be noted that only a 4 d heat stress with somewhat moderate, but realistic, temperature regimes was examined in this study. If this observed trend were to continue, it could have a significant effect over the long-term health and development of the plant. It is possible that prolonged exposure to heat stress and the resulting increase in abscisic

acid content and anti-transpiration activity could ultimately affect biomass production and water use efficiency.

3. CONCLUSIONS AND FUTURE RESEARCH NEEDS

A challenge of promoting the use of recycled wastewater for agricultural irrigation and biosolids/animal wastes as soil amendments involves ensuring the productivity of crops grown using these practices. As treated wastewater and biosolids/animal wastes contain numerous CECs such as pharmaceuticals, it is important to understand whether or not plants' functions, including their tolerance to additional environmental stressors such as heat, pathogens, and salinity are compromised due to exposure to CECs. In this study, we utilized several endpoints, including germination, root length, biomass, and hormone homeostasis, to assess the response of vegetable plants to repeated exposure to CEC mixtures under hydroponic conditions. Significant changes in hormone homeostasis resulted from repeated exposure to the CEC mixture, often at environmentally relevant CEC concentrations. Because phytohormones such as abscisic acid, jasmonic acid, and auxin have effects on transpiration, stress-response, and cell division and elongation of meristematic tissue, changes in in their levels due to exposure to the CECs likely underlined the observed reductions in below and above-ground biomass, especially at the higher CEC levels. The analysis of phytohormones provided insights into long-term physiological consequences due to stress of CECs, such as biomass, flowering, and fruiting. While all treated wastewater and biosolids/animal wastes have their own unique mixtures of CECs with variable ranges of concentrations, the analysis of phytohormones

may be a useful screening tool as they are sensitive indicators of potential physiological response that is crucial for plant development, growth, and productivity.

While our study revealed potential perturbations to plant hormone homeostasis and physiological performance by low levels of CECs in mixture, our findings are limited in the sense that a hydroponic system was used, and that the response was not followed through the plant's entire life cycle. Future research should use soil as the growth media, as processes in the rhizosphere such as sorption and microbial degradation affect the availability of plant uptake of CECs. Additionally, 10 compounds likely represent only a very small fraction of chemical constituents in treated wastewater and biosolids/animal wastes, and future studies under field conditions using actual treated wastewater for irrigation or biosolids/animal wastes for soil amendment should be carried out to derive more pertinent information. Such knowledge is useful for promoting safe reuse of the valuable and yet under-utilized resources such as treated wastewater and biosolids/animal wastes, contributing to agricultural and environmental sustainability.

4. REFERENCES

1. Ahammed, G. J., Li, X., Zhou, J., Zhou, Y. H., & Yu, J. Q. (2016). Role of hormones in plant adaptation to heat stress. In *Plant Hormones under Challenging Environmental Factors,* 1-21.

2. Anderson, J. P., Badruzsaufari, E., Schenk, P. M., Manners, J. M., Desmond, O. J., Ehlert, C., et al. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. *The Plant Cell*, *16*(12), 3460-3479.

3. Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., et al. (2010). Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry*, *29*(3), 730-741.

4. Archer, E., Petrie, B., Kasprzyk-Hordern, B., & Wolfaardt, G. M. (2017). The fate of pharmaceuticals and personal care products (PPCPs), endocrine disrupting contaminants (EDCs), metabolites and illicit drugs in a WWTW and environmental waters. *Chemosphere*, *174*, 437-446.

5. Batchelder, A. R. (1982). Chlortetracycline and Oxytetracycline Effects on Plant Growth and Development in Soil Systems 1. *Journal of environmental quality*, *11*(4), 675-678.

6. Battisti, D. S., & Naylor, R. L. (2009). Historical warnings of future food insecurity with unprecedented seasonal heat. *Science*, *323*(5911), 240-244.

7. Boxall, A. B., Johnson, P., Smith, E. J., Sinclair, C. J., Stutt, E., & Levy, L. S. (2006). Uptake of veterinary medicines from soils into plants. *Journal of Agricultural and Food Chemistry*, *54*(6), 2288-2297.

8. Boyd, G. R., Reemtsma, H., Grimm, D. A., & Mitra, S. (2003). Pharmaceuticals and personal care products (PPCPs) in surface and treated waters of Louisiana, USA and Ontario, Canada. *Science of the total Environment*, *311*(1-3), 135-149.

9. Burns, E. E., Thomas-Oates, J., Kolpin, D. W., Furlong, E. T., & Boxall, A. B. (2017). Are exposure predictions, used for the prioritization of pharmaceuticals in the environment, fit for purpose? *Environmental toxicology and chemistry*, *36*(10), 2823-2832.

10. Carpenter, D. O., Arcaro, K., & Spink, D. C. (2002). Understanding the human health effects of chemical mixtures. *Environmental health perspectives*, *110*(Suppl 1), 25.

11. Cheng, Y., Han, Y., Wang, Q., & Wang, Z. (2006). Seasonal dynamics of fine root biomass, root length density, specific root length, and soil resource availability in a Larix gmelinii plantation. *Frontiers of Biology in China*, *1*(3), 310-317.

12. Christou, A., Antoniou, C., Christodoulou, C., Hapeshi, E., Stavrou, I., Michael, C., et al. (2016). Stress-related phenomena and detoxification mechanisms induced by common pharmaceuticals in alfalfa (Medicago sativa L.) plants. *Science of the Total Environment*, *557*, 652-664.

13. Christou, A., Michael, C., Fatta-Kassinos, D., & Fotopoulos, V. (2018). Can the pharmaceutically active compounds released in agroecosystems be considered as emerging plant stressors? *Environment international*, *114*, 360-364.

14. Clarke, S. M., Cristescu, S. M., Miersch, O., Harren, F. J., Wasternack, C., & Mur, L. A. (2009). Jasmonates act with salicylic acid to confer basal thermotolerance in Arabidopsis thaliana. *New Phytologist*, *182*(1), 175-187.

15. Creelman, R. A., & Mullet, J. E. (1995). Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. *Proceedings of the National Academy of Sciences*, *92*(10), 4114-4119.

16. D'Abrosca, B., Fiorentino, A., Izzo, A., Cefarelli, G., Pascarella, M. T., Uzzo, P., & Monaco, P. (2008). Phytotoxicity evaluation of five pharmaceutical pollutants detected in surface water on germination and growth of cultivated and spontaneous plants. *Journal of Environmental Science and Health, Part A*, *43*(3), 285-294.

17. Daughton, C. G., & Ternes, T. A. (1999). Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environmental health perspectives*, *107*(Suppl 6), 907.

18. Daughton, C. G., & Ternes, T. A. (1999). Pharmaceuticals and Personal Care Products in the Environment: Agents of Subtle Change? *Environ. Health Perspect*, *1*(6), 7907–7938.

19. de Wit, M., Lorrain, S., & Fankhauser, C. (2014). Auxin-mediated plant architectural changes in response to shade and high temperature. *Physiologia plantarum*, *151*(1), 13-24.

20. Dieter, C. A., Maupin, M. A., Caldwell, R. R., Harris, M. A., Ivahnenko, T. I., Lovelace, J. K., et al. (2018). *Estimated use of water in the United States in 2015* (No. 1441). US Geological Survey.

21. Dobrá, J., Černý, M., Štorchová, H., Dobrev, P., Skalák, J., Jedelský, P. L., ... & Vanek, T. (2015). The impact of heat stress targeting on the hormonal and transcriptomic response in Arabidopsis. *Plant science*, *231*, 52-61.

22. Du, L., & Liu, W. (2012). Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. *Agronomy for Sustainable Development*, *32*(2), 309-327.

23. Dudley, S., Sun, C., Jiang, J., & Gan, J. (2018). Metabolism of sulfamethoxazole in Arabidopsis thaliana cells and cucumber seedlings. *Environmental Pollution*.

24. Fahad, S., Nie, L., Chen, Y., Wu, C., Xiong, D., Saud, S., et al. (2015). Crop plant hormones and environmental stress. In *Sustainable Agriculture Reviews*, 371-400.

25. Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Current opinion in plant biology*, *9*(4), 436-442.

26. Furlong, E. T., Batt, A. L., Glassmeyer, S. T., Noriega, M. C., Kolpin, D. W., Mash, H., & Schenck, K. M. (2017). Nationwide reconnaissance of contaminants of emerging concern in source and treated drinking waters of the United States: Pharmaceuticals. *Science of the Total Environment*, *579*, 1629-1642

27. Glassmeyer, S. T., Furlong, E. T., Kolpin, D. W., Batt, A. L., Benson, R., Boone, J. S., et al. (2017). Nationwide reconnaissance of contaminants of emerging concern in source and treated drinking waters of the United States. *Science of the Total Environment*, *581*, 909-922.

28. Gray, W. M., Östin, A., Sandberg, G., Romano, C. P., & Estelle, M. (1998). High temperature promotes auxin-mediated hypocotyl elongation in Arabidopsis. *Proceedings of the National Academy of Sciences*, *95*(12), 7197-7202.

29. Herklotz, P. A., Gurung, P., Heuvel, B. V., & Kinney, C. A. (2010). Uptake of human pharmaceuticals by plants grown under hydroponic conditions. *Chemosphere*, *78*(11), 1416-1421.

30. Hillis, D. G., Fletcher, J., Solomon, K. R., & Sibley, P. K. (2011). Effects of ten antibiotics on seed germination and root elongation in three plant species. *Archives of environmental contamination and toxicology*, *60*(2), 220-232.

31. Holling, C. S., Bailey, J. L., Heuvel, B. V., & Kinney, C. A. (2012). Uptake of human pharmaceuticals and personal care products by cabbage (Brassica

campestris) from fortified and biosolids-amended soils. *Journal of Environmental Monitoring*, *14*(11), 3029-3036.

32. Huber, C., Bartha, B., & Schröder, P. (2012). Metabolism of diclofenac in plants–Hydroxylation is followed by glucose conjugation. *Journal of hazardous materials*, *243*, 250-256.

33. Jjemba, P. K. (2002). The effect of chloroquine, quinacrine, and metronidazole on both soybean plants and soil microbiota. *Chemosphere*, *46*(7), 1019-1025.

34. Jones-Lepp, T. L., & Stevens, R. (2007). Pharmaceuticals and personal care products in biosolids/sewage sludge: the interface between analytical chemistry and regulation. *Analytical and Bioanalytical Chemistry*, *387*(4), 1173-1183.

35. Kidd, K. A., Blanchfield, P. J., Mills, K. H., Palace, V. P., Evans, R. E., Lazorchak, J. M., & Flick, R. W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences*, *104*(21), 8897-8901.

36. Kienzler, A., Bopp, S. K., van der Linden, S., Berggren, E., & Worth, A. (2016). Regulatory assessment of chemical mixtures: Requirements, current approaches and future perspectives. *Regulatory Toxicology and Pharmacology*, *80*, 321-334.

37. Kinney, C.A., Furlong, E.T., Werner, S.L., Cahill, J.D. (2006). Presence and distribution of wastewater-derived pharmaceuticals in soil irrigated with reclaimed water. *Environ. Toxicol. Chem*. 25, 317-326.

38. Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., & Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999− 2000: A national reconnaissance. *Environmental science & technology*, *36*(6), 1202-1211.

39. Kostich, M. S., Batt, A. L., & Lazorchak, J. M. (2014). Concentrations of prioritized pharmaceuticals in effluents from 50 large wastewater treatment plants in the US and implications for risk estimation. *Environmental Pollution*, *184*, 354-359.

40. Kotak, S., Larkindale, J., Lee, U., von Koskull-Döring, P., Vierling, E., & Scharf, K. D. (2007). Complexity of the heat stress response in plants. *Current opinion in plant biology*, *10*(3), 310-316.

41. Kurelec, B. (1992). The multi-xenobiotic resistance mechanism in aquatic organisms. *Critical reviews in toxicology*, *22*(1), 23-43.

42. Larkindale, J., Hall, J. D., Knight, M. R., & Vierling, E. (2005). Heat stress phenotypes of Arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. *Plant physiology*, *138*(2), 882-897.

43. Li, Z. J., Xie, X. Y., Zhang, S. Q., & Liang, Y. C. (2011). Negative effects of oxytetracycline on wheat (Triticum aestivum L.) growth, root activity, photosynthesis, and chlorophyll contents. *Agricultural Sciences in China*, *10*(10), 1545-1553.

44. Lishman, L., Smyth, S. A., Sarafin, K., Kleywegt, S., Toito, J., Peart, T., ... & Seto, P. (2006). Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. *Science of the Total Environment*, *367*(2-3), 544-558.

45. Liu, F., Ying, G. G., Tao, R., Zhao, J. L., Yang, J. F., & Zhao, L. F. (2009). Effects of six selected antibiotics on plant growth and soil microbial and enzymatic activities. *Environmental Pollution*, *157*(5), 1636-1642.

46. Liu, H., Weisman, D., Ye, Y. B., Cui, B., Huang, Y. H., Colón-Carmona, A., & Wang, Z. H. (2009). An oxidative stress response to polycyclic aromatic hydrocarbon exposure is rapid and complex in Arabidopsis thaliana. *Plant Science*, *176*(3), 375-382.

47. Macherius, A., Eggen, T., Lorenz, W., Moeder, M., Ondruschka, J., & Reemtsma, T. (2012). Metabolization of the bacteriostatic agent triclosan in edible plants and its consequences for plant uptake assessment. *Environmental science & technology*, *46*(19), 10797-10804.

48. Macherius, A., Seiwert, B., Schröder, P., Huber, C., Lorenz, W., & Reemtsma, T. (2014). Identification of plant metabolites of environmental contaminants by UPLC-QToF-MS: the in vitro metabolism of triclosan in horseradish. *Journal of agricultural and food chemistry*, *62*(5), 1001-1009.

49. Macková, H., Hronková, M., Dobrá, J., Turečková, V., Novák, O., Lubovská, Z., et al. (2013). Enhanced drought and heat stress tolerance of tobacco plants with ectopically enhanced cytokinin oxidase/dehydrogenase gene expression. *Journal of experimental botany*, *64*(10), 2805-2815.

50. *Managing water under uncertainty and risk*. (2012). Paris: UNESCO.

51. Melotto, M., Underwood, W., Koczan, J., Nomura, K., & He, S. Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell*, *126*(5), 969-980.

52. Miege, C., Choubert, J. M., Ribeiro, L., Eusèbe, M., & Coquery, M. (2009). Fate of pharmaceuticals and personal care products in wastewater treatment plants–conception of a database and first results. *Environmental Pollution*, *157*(5), 1721-1726.

53. Migliore, L., Cozzolino, S., & Fiori, M. (2003). Phytotoxicity to and uptake of enrofloxacin in crop plants. *Chemosphere*, *52*(7), 1233-1244.

54. Migliore, L., Godeas, F., De Filippis, S. P., Mantovi, P., Barchi, D., Testa, C., et al. (2010). Hormetic effect (s) of tetracyclines as environmental contaminant on Zea mays. *Environmental Pollution*, *158*(1), 129-134.

55. Mittler, R., Finka, A., & Goloubinoff, P. (2012). How do plants feel the heat?. *Trends in biochemical sciences*, *37*(3), 118-125.

56. Munemasa, S., Hauser, F., Park, J., Waadt, R., Brandt, B., & Schroeder, J. I. (2015). Mechanisms of abscisic acid-mediated control of stomatal aperture. *Current opinion in plant biology*, *28*, 154-162.

57. Nemhauser, J. L., Hong, F., & Chory, J. (2006). Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. *Cell*, *126*(3), 467-475.

58. Novak, P. J., Arnold, W. A., Blazer, V. S., Halden, R. U., Klaper, R. D., Kolpin, D. W., et al. (2011). On the need for a national (US) research program to elucidate the potential risks to human health and the environment posed by contaminants of emerging concern.

59. Ostonen, I., Püttsepp, Ü., Biel, C., Alberton, O., Bakker, M. R., Lõhmus, K., et al. (2007). Specific root length as an indicator of environmental change. *Plant Biosystems*, *141*(3), 426-442.

60. Pauwels, L., Inzé, D., & Goossens, A. (2009). Jasmonate-inducible gene: what does it mean? *Trends in plant science*, *14*(2), 87-91.

61. Prosser, R. S., & Sibley, P. K. (2015). Human health risk assessment of pharmaceuticals and personal care products in plant tissue due to biosolids and manure amendments, and wastewater irrigation. *Environment international*, *75*, 223-233.

62. Ramegowda, V., & Senthil-Kumar, M. (2015). The interactive effects of simultaneous biotic and abiotic stresses on plants: mechanistic understanding from drought and pathogen combination. *Journal of plant physiology*, *176*, 47-54.

63. Rogers, H. H., Prior, S. A., Runion, G. B., & Mitchell, R. J. (1995). Root to shoot ratio of crops as influenced by CO 2. *Plant and soil*, *187*(2), 229-248.

64. Seo, J. S., Joo, J., Kim, M. J., Kim, Y. K., Nahm, B. H., Song, S. I., et al. (2011). OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ proteins in a jasmonate signaling pathway leading to drought tolerance in rice. *The Plant Journal*, *65*(6), 907-921.

65. Schmidt, W., & Redshaw, C. H. (2015). Evaluation of biological endpoints in crop plants after exposure to non-steroidal anti-inflammatory drugs (NSAIDs): Implications for phytotoxicological assessment of novel contaminants. Ecotoxicology and environmental safety, 112, 212-222.

66. Speirs, J., Binney, A., Collins, M., Edwards, E., & Loveys, B. (2013). Expression of ABA synthesis and metabolism genes under different irrigation strategies and atmospheric VPDs is associated with stomatal conductance in grapevine (Vitis vinifera L. cv Cabernet Sauvignon). *Journal of experimental botany*, *64*(7), 1907-1916.

67. Stocker, T. (Ed.). (2014). *Climate change 2013: the physical science basis: Working Group I contribution to the Fifth assessment report of the Intergovernmental Panel on Climate Change*. Cambridge University Press.

68. Sun, C., Dudley, S., Trumble, J., & Gan, J. (2018a). Pharmaceutical and personal care products-induced stress symptoms and detoxification mechanisms in cucumber plants. *Environmental Pollution*, *234*, 39-47.

69. Sun, C., Dudley, S., McGinnis, M., & Gan, J. (2018b). Hydrogen peroxide mediates triclosan-induced inhibition of root growth in wheat seedlings. *Environmental Pollution*.

70. Tang, J. Y., Busetti, F., Charrois, J. W., & Escher, B. I. (2014). Which chemicals drive biological effects in wastewater and recycled water?. *Water research*, *60*, 289-299.

71. Trapp, S. (2009). Bioaccumulation of polar and ionizable compounds in plants. In *Ecotoxicology modeling,* 299-353.

72. United States, EPA, Office of Chemical Safety and Pollution Prevention. (2012, January). *Ecological Effects Test Guidelines OCSPP 850.4100: Seedling Emergence and Seedling Growth*. Retrieved from http://www.epa.gov/ocspp

73. United States, EPA, Office of Chemical Safety and Pollution Prevention. (2012, January). *Ecological Effects Test Guidelines OCSPP 850.4230: Early Seedling Growth Toxicity Test*. Retrieved from http://www.epa.gov/ocspp

74. United States, EPA, Office of Chemical Safety and Pollution Prevention. (2012, January). *Ecological Effects Test Guidelines OCSPP 850.4150: Vegetative Vigor*. Retrieved from http://www.epa.gov/ocspp

75. Van Noordwijk, M., & Brouwer, G. (1991). Review of quantitative root length data in agriculture. In *Developments in Agricultural and Managed Forest Ecology* (Vol. 24, pp. 515-525).

76. Wang, Y., Mopper, S., & Hasenstein, K. H. (2001). Effects of salinity on endogenous ABA, IAA, JA, and SA in Iris hexagona. *Journal of chemical ecology*, *27*(2), 327-342.

77. Wani, S. H., Kumar, V., Shriram, V., & Sah, S. K. (2016). Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. *The Crop Journal*, *4*(3), 162-176.

78. Wilkinson, S., Kudoyarova, G. R., Veselov, D. S., Arkhipova, T. N., & Davies, W. J. (2012). Plant hormone interactions: innovative targets for crop breeding and management. *Journal of experimental botany*, *63*(9), 3499-3509.

79. Wu, C., Witter, J. D., Spongberg, A. L., & Czajkowski, K. P. (2009). Occurrence of selected pharmaceuticals in an agricultural landscape, western Lake Erie basin. *Water Research*, *43*(14), 3407-3416.

80. Wu, X., Ernst, F., Conkle, J. L., & Gan, J. (2013). Comparative uptake and translocation of pharmaceutical and personal care products (PPCPs) by common vegetables. *Environment international*, *60*, 15-22.

81. Wu, X., Conkle, J. L., Ernst, F., & Gan, J. (2014). Treated wastewater irrigation: uptake of pharmaceutical and personal care products by common vegetables under field conditions. *Environmental science & technology*, *48*(19), 11286-11293.

82. Wu, X., Dodgen, L. K., Conkle, J. L., & Gan, J. (2015). Plant uptake of pharmaceutical and personal care products from recycled water and biosolids: a review. *Science of The Total Environment*, *536*, 655-666.

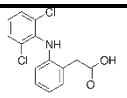
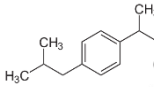
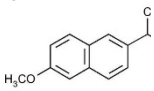
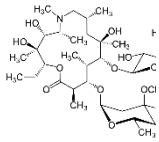
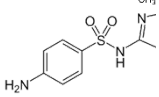
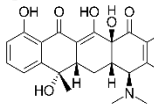
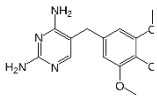
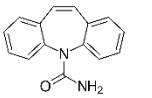
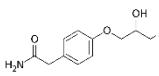
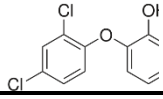
83. Wu, X., Fu, Q., & Gan, J. (2016). Metabolism of pharmaceutical and personal care products by carrot cell cultures. *Environmental Pollution*, *211*, 141-147.

84. Xie, X., Zhou, Q., He, Z., & Bao, Y. (2010). Physiological and potential genetic toxicity of chlortetracycline as an emerging pollutant in wheat (Triticum aestivum L.). *Environmental toxicology and chemistry*, *29*(4), 922-928.

85. Xu, Y. H., Liao, Y. C., Zhang, Z., Liu, J., Sun, P. W., Gao, Z. H., et al. (2016). Jasmonic acid is a crucial signal transducer in heat shock induced sesquiterpene formation in Aquilaria sinensis. *Scientific reports*, *6*, 21843.

86. Yoon, J. Y., Hamayun, M., Lee, S. K., & Lee, I. J. (2009). Methyl jasmonate alleviated salinity stress in soybean. *Journal of Crop Science and Biotechnology*, *12*(2), 63-68

Table 1. Physico-chemical properties and classification of CECs used in this study.

Therapeutic class	Compound	Structure	CAS Number	Formula	Molecular weight (g mol^{-1})	Log K_{OW}	pK_a	Source[a]
Analgesic anti-inflammatory	Diclofenac		15307-86-5	$C_{14}H_{11}Cl_2NO_2$	296.14	4.51	4.15	c
Analgesic anti-inflammatory	Ibuprofen		15687-27-1	$C_{13}H_{19}O_2$	206.29	3.97	4.91	c
Analgesic anti-inflammatory	Naproxen		22204-53-1	$C_{14}H_{14}O_3$	230.27	3.18	4.15	c
Antibiotic	Azithromycin		83905-01-5	$C_{38}H_{72}N_2O_{12}$	749.00	4.02	8.74, 9.45	f
Antibiotic	Sulfamethoxazole		723-46-6	$C_{10}H_{11}N_3O_3S$	253.28	0.89	1.8, 5.6	a
Antibiotic	Tetracycline		60-54-8	$C_{22}H_{24}N_2O_8$	444.44	-1.3	3.3, 7.8, 9.6	d, e
Antibiotic	Trimethoprim		738-70-5	$C_{14}H_{18}N_4O_3$	290.32	0.91	7.12	b, c
Anti-epileptic	Carbamazepine		298046-4	$C_{15}H_{12}N_2O$	236.27	2.45	2.3	a
Betablocker	Atenolol		29122-68-7	$C_{14}H_{22}N_2O_3$	266.34	0.16	9.6	c
Antimicrobial	Triclosan		3380-34-5	$C_{12}H_7Cl_3O_2$	288.37	4.76	7.9	c

[a] Malchi *et al.*, 2014. [b] Kinney *et al.*, 2008. [c] Wu *et al.*, 2012. [d] Leston *et al.*, 2016. [e] Pailler *et al.*, 2009. [f] McFarland *et al.*, 1997.

Table 2. Experimental concentrations of the CECs in the mixture. The 1X Median represents the median concentration of CEC present in treated wastewater effluents as determined from the literature.

Compound	Experimental concentrations in μg L^{-1} (ppb)				
	1X Median	2X Median	5X Median	10X Median	20X Median
Diclofenac	0.5	1	2.5	5	10
Ibuprofen	1	2	5	10	20
Naproxen	2	4	10	20	40
Azithromycin	0.1	0.2	0.5	1	2
Sulfamethoxazole	0.1	0.2	0.5	1	2
Tetracycline	0.2	0.4	1	2	4
Trimethoprim	0.1	0.2	0.5	1	2
Carbamazepine	0.5	1	2.5	5	10
Atenolol	0.15	0.3	0.75	1.5	3
Triclosan	0.1	0.2	0.5	1	2

Table 3. MS parameters for targeted compounds and surrogate standards.

Compound	MRM (m/z) Quantification	CV/CE	Qualification	CV/CE
ESI+				
Sulfamethoxazole	254 > 92	34/28	254 > 156	34/14
Sulfamethoxazole-d4	258 > 96	32/26	258 > 160	32/16
Atenolol	267 > 145	40/26	267 > 72	40/22
Atenolol-d7	274 > 79	42/20	274 > 145	42/24
Trimethoprim	291 > 230	50/22		
Trimethoprim-d9	300 > 234	50/22		
Carbamazepine	237 > 194	30/18	237 > 179	36/32
Carbamazepine-d10	247 > 204	38/30	247 > 187	40/32
Tetracycline	445 > 154	28/26	445 > 410	32/18
Azithromycin	750 > 83	50/56	750 > 592	38/20
Naproxen	231 > 170	24/24	231 > 115	24/52
Naproxen-d3	234 > 173	22/24	234 > 144	22/40
Ibuprofen	207 > 119	20/24	207 > 57	20/24
Indole-3-acetic acid	176 > 130	26/28	176 > 103	26/32
Indole-3-acetic acid-d7	183 > 109	26/32	183 > 137	26/28
Jasmonic acid	211 > 133	24/14	211 > 151	24/12
ESI-				
Ibuprofen	205 > 161	18/8		
Ibuprofen-d3	208 > 164	18/8		
Triclosan	287 > 35	22/8		
Triclosan-d3	290 > 35	25/10		
Diclofenac	294 > 250	22/20	294 > 214	22/20
Diclofenac-d4	298 > 254	25/10		
Abscisic acid	263 > 153	24/14	263 > 204	24/18
Abscisic acid-d6	269 > 159	26/12	269 > 210	26/18

Table 4. Deuterated surrogate standard recoveries.

Compound	Recovery % (mean ± SD)
Atenolol-d7	55.5 ± 26.8
Azithromycin-d5	48.3 ± 10.1
Carbamazepine-d10	82.4 ± 35.8
Sulfamethoxazole-d4	13.6 ± 4.8
Trimethoprim-d9	60.1 ± 39.2
Naproxen-d3	61.6 ± 17.1
Diclofenac-d4	75.5 ± 28.4
Ibuprofen-d3	85.2 ± 13.7
Triclosan-d3	78.7 ± 17.4
Abscisic acid-d6	84.2 ± 10.6
Indole-3-acetic acid-d7	90.8 ± 21.3

Table 5. Limits of detection (LOD) and limits of quantification (LOQ) of targeted compounds.

Compound	LOQ (S/N = 10, ppb)	LOD (S/N =3, ppb)
Sulfamethoxazole	1	0.1
Sulfamethoxazole-d4	1	0.1
Atenolol	1	0.5
Atenolol-d7	1	0.5
Trimethoprim	1	0.1
Trimethoprim-d9	1	0.1
Carbamazepine	1	0.1
Carbamazepine-d10	1	0.1
Tetracycline	5	1
Azithromycin	1	0.5
Naproxen	1	0.1
Naproxen-d3	1	0.1
Indole-3-acetic acid	1	0.5
Indole-3-acetic acid-d7	1	0.5
Ibuprofen	10	5
Ibuprofen-d3	10	5
Triclosan	20	10
Triclosan-d3	20	10
Diclofenac	1	0.5
Diclofenac-d4	1	0.5
Abscisic acid	5	2
Abscisic acid-d6	5	2
Jasmonic acid	10	5

Table 6. Measured concentrations of selected CECs in hydroponic solutions.

	1X			10X			20X		
Compound	Initial	3 d	3 d + plants	Initial	3 d	3 d + plants	Initial	3 d	3 d + plants
Atenolol	1.02 ± 0.02	0.43 ± 0.00	0.30 ± 0.01	2.34 ± 0.16	1.86 ± 0.01	2.30 ± 0.12	4.45 ± 0.05	3.68 ± 0.23	2.98 ± 1.63
Carbamazepine	0.52 ± 0.03	0.74 ± 0.03	0.37 ± 0.04	3.88 ± 0.05	3.49 ± 0.24	3.46 ± 0.08	7.35 ± 0.12	7.10 ± 0.21	5.06 ± 2.74
Ibuprofen	0.74 ± 1.02	0.22 ± 0.31	0.16 ± 0.21	11.52 ± 1.30	2.98 ± 0.09	3.34 ± 0.70	24.73 ± 2.50	8.01 ± 0.00	8.99 ± 1.22
Diclofenac	0.39 ± 0.51	0.43 ± 0.09	0.30 ± 0.06	5.97 ± 0.22	1.81 ± 0.43	1.26 ± 0.20	9.47 ± 4.38	3.79 ± 0.38	2.70 ± 0.62
Azithromycin	0.14 ± 0.02	0.16 ± 0.01	0.06 ± 0.01	0.67 ± 0.09	0.63 ± 0.37	0.87 ± 0.02	1.63 ± 1.29	1.49 ± 0.05	1.05 ± 1.11
Naproxen	2.37 ± 0.19	2.28 ± 0.01	0.95 ± 0.05	17.36 ± 0.31	13.03 ± 0.93	9.17 ± 0.46	36.71 ± 1.72	22.54 ± 0.59	29.95 ± 10.14
Sulfamethoxazole	0.45 ± 0.08	0.12 ± 0.00	0.07 ± 0.00	0.88 ± 0.02	0.93 ± 0.13	0.62 ± 0.00	1.73 ± 0.16	1.65 ± 0.04	1.10 ± 0.40
Trimethoprim	0.04 ± 0.03	ND	ND	0.83 ± 0.01	0.31 ± 0.08	0.36 ± 0.01	1.84 ± 0.03	0.64 ± 0.01	0.49 ± 0.34
Tetracycline	0.07 ± 0.00	0.07 ± 0.00	0.04 ± 0.00	0.32 ± 0.07	0.13 ± 0.01	0.10 ± 0.00	0.83 ± 0.27	0.51 ± 0.05	0.20 ± 0.06
Triclosan	0.09 ± 0.04	0.08 ± 0.02	0.04 ± 0.01	1.03 ± 0.12	0.91 ± 0.19	0.79 ± 0.09	2.14 ± 0.14	1.72 ± 0.21	1.26 ± 0.24

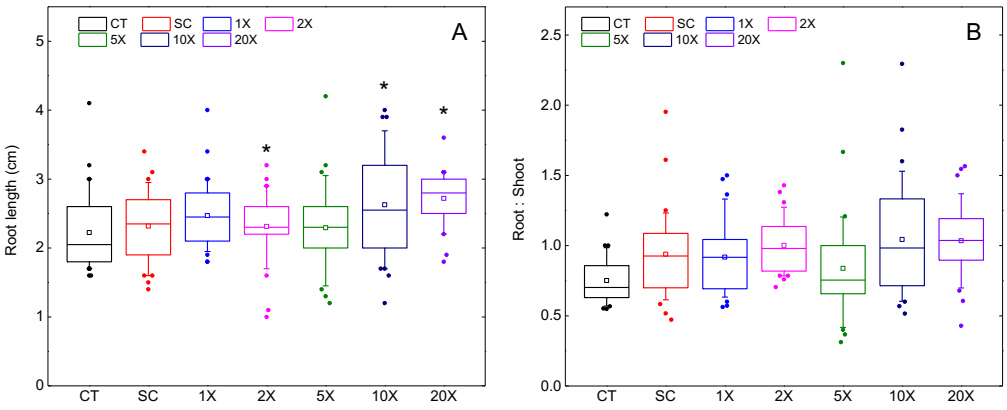


Figure 1. Root length (A) and root to shoot biomass ratio (B) of lettuce seedlings in a 7 d germination study. The boxplot represents the median (line), mean (x), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers), and outliers as dots. Treatments marked with an asterisk have means that are significantly different from the control ($p < 0.05$). CT control; SC solvent control (0.02% DMSO).

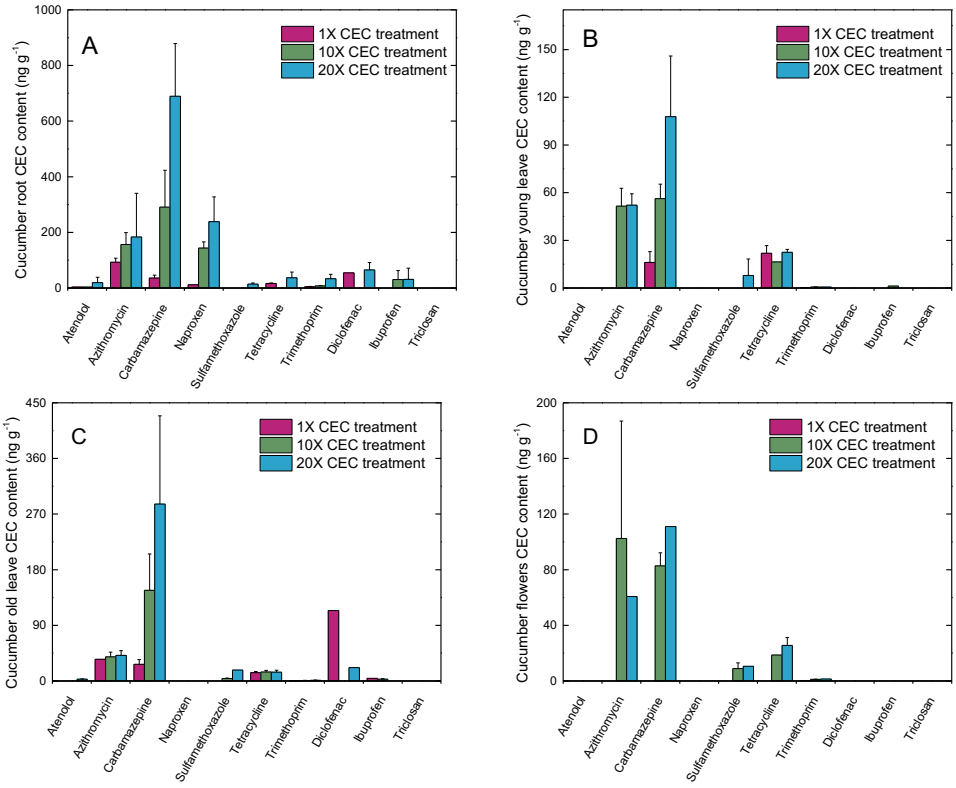


Figure 2. Concentrations of CECs at different treatment levels in (A) roots, (B) young leaves, (C) old leaves, and (D) flower tissues of cucumbers.

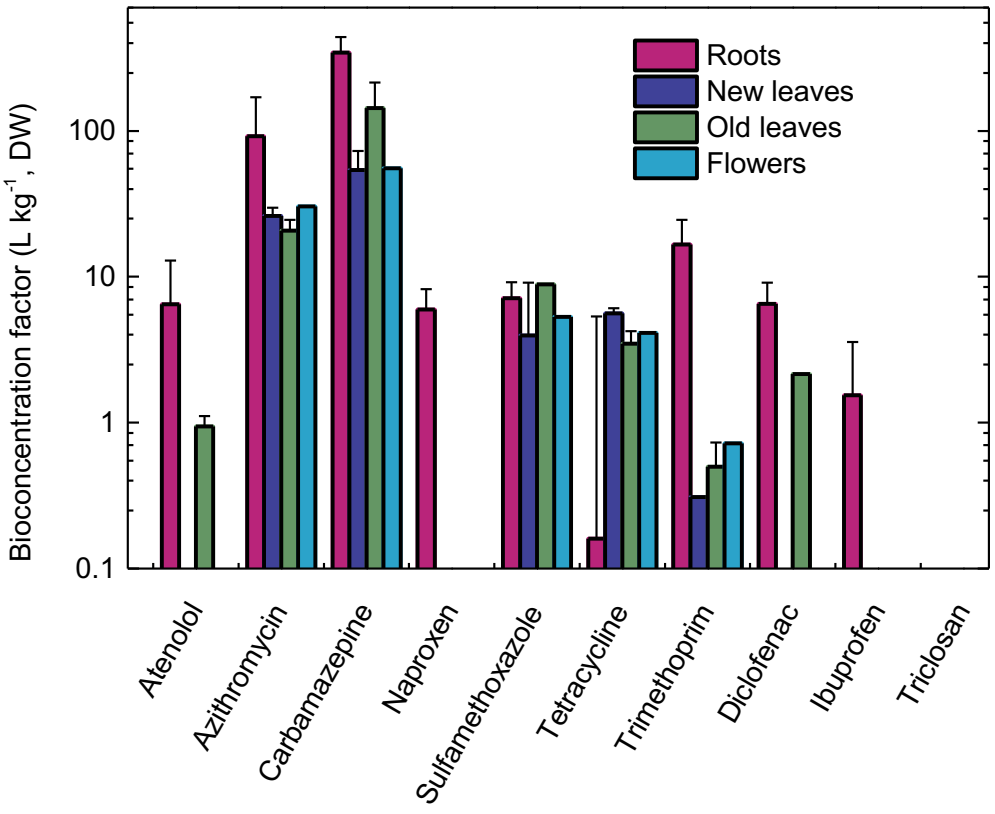


Figure 3. Bioconcentration factor (BCF) of targeted CECs in cucumber tissues. Data represents the mean at the 20X CEC amendment level of three samples for each tissue. Bars represent the mean ± standard deviation (n=3).

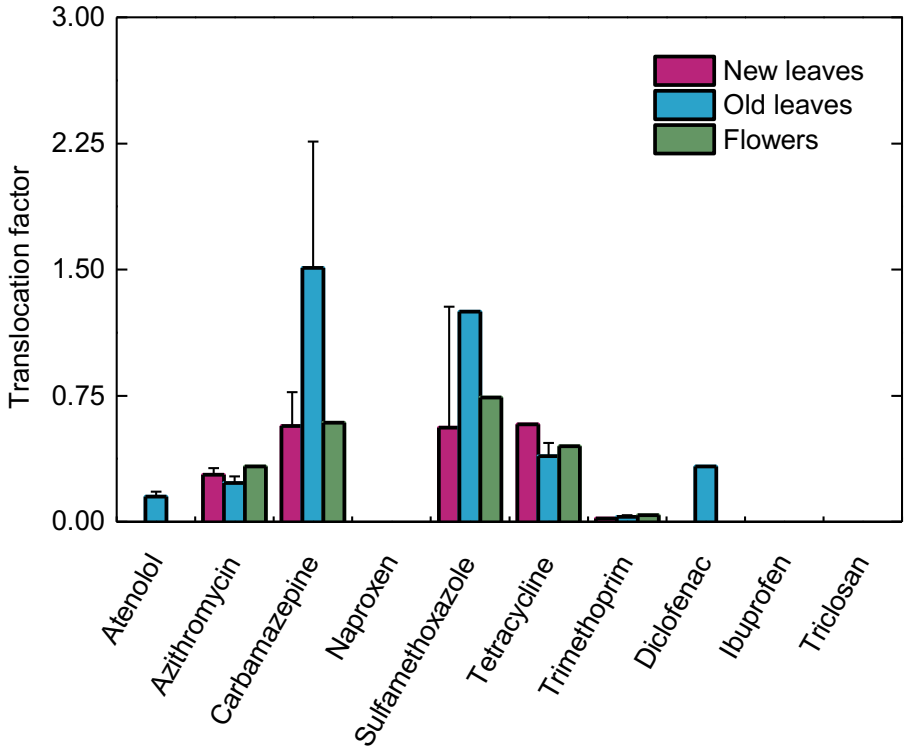


Figure 4. Translocation factor of targeted CECs in cucumber tissues at the 20X CEC amendment level. Bars represent the mean ± the standard deviation.

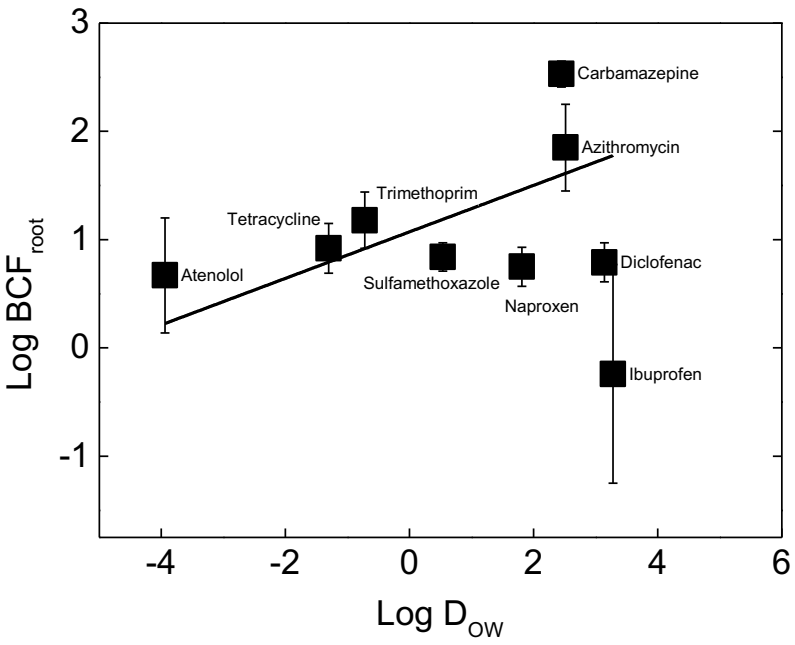


Figure 5. Correlation between log BCF_{root} and log K_{ow} for PPCPs measured in cucumber plant tissues at the 20X PPCP amendment level. Bars represent the mean ± the standard deviation.

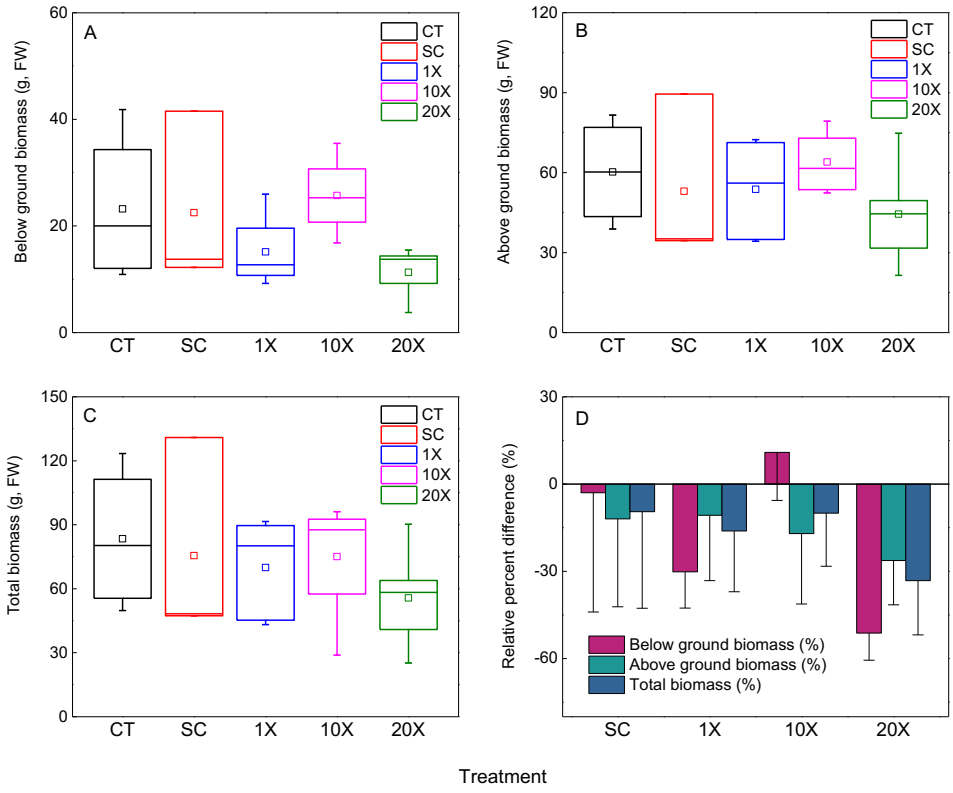


Figure 6. Biomass measurements after 30-d exposure to a CEC mixture at different rates. The boxplot represents the median (line), mean (square), 25th and 75th percentiles (box), and 10th and 90th percentiles (whiskers) for (a) above ground biomass, (b) below ground biomass, and (c) total biomass. Percentage differences in biomass between treatments and the control are shown in (d) where bars represent the mean ± standard error (n=3). CT, control; SC solvent control (0.02% DMSO).

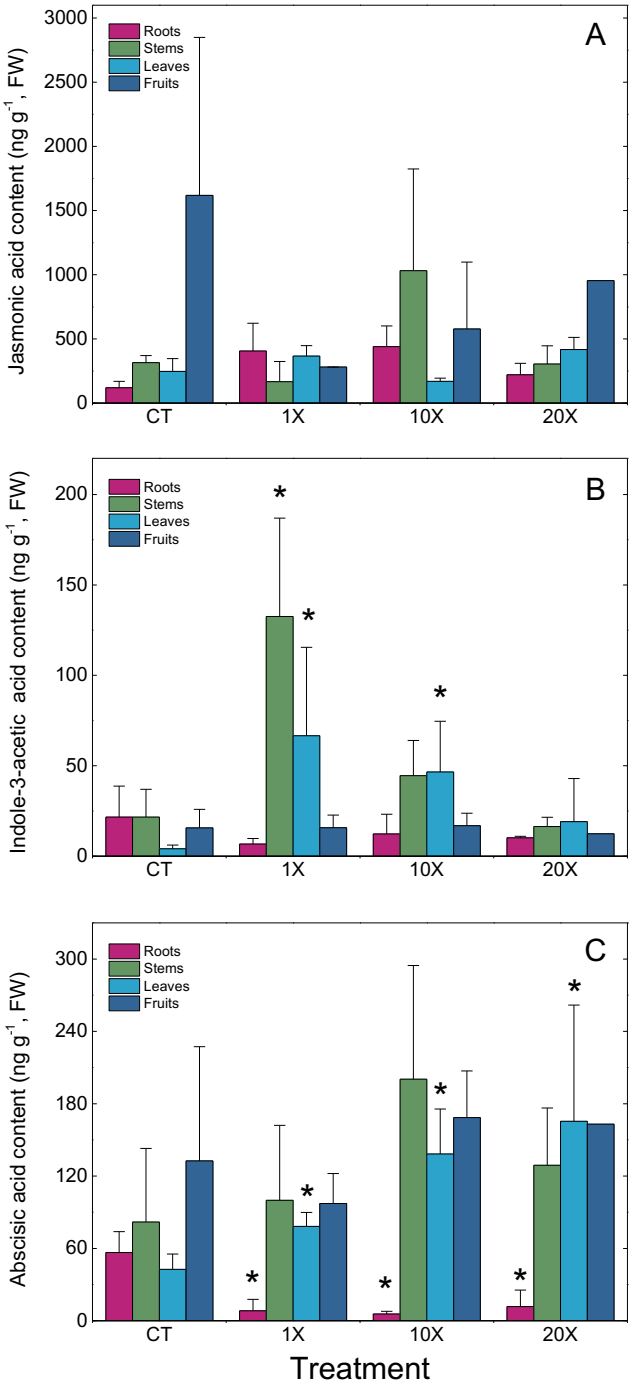


Figure 7. Levels of (A) jasmonic acid, (B) auxin, and (C) abscisic acid in cucumber tissues after a 30 d exposure to the CEC mixture at different treatment rates. Bars represent the mean ± standard deviation. Asterisks (*) indicate a significant difference ($p < 0.05$) from the control. CT, control treatment.

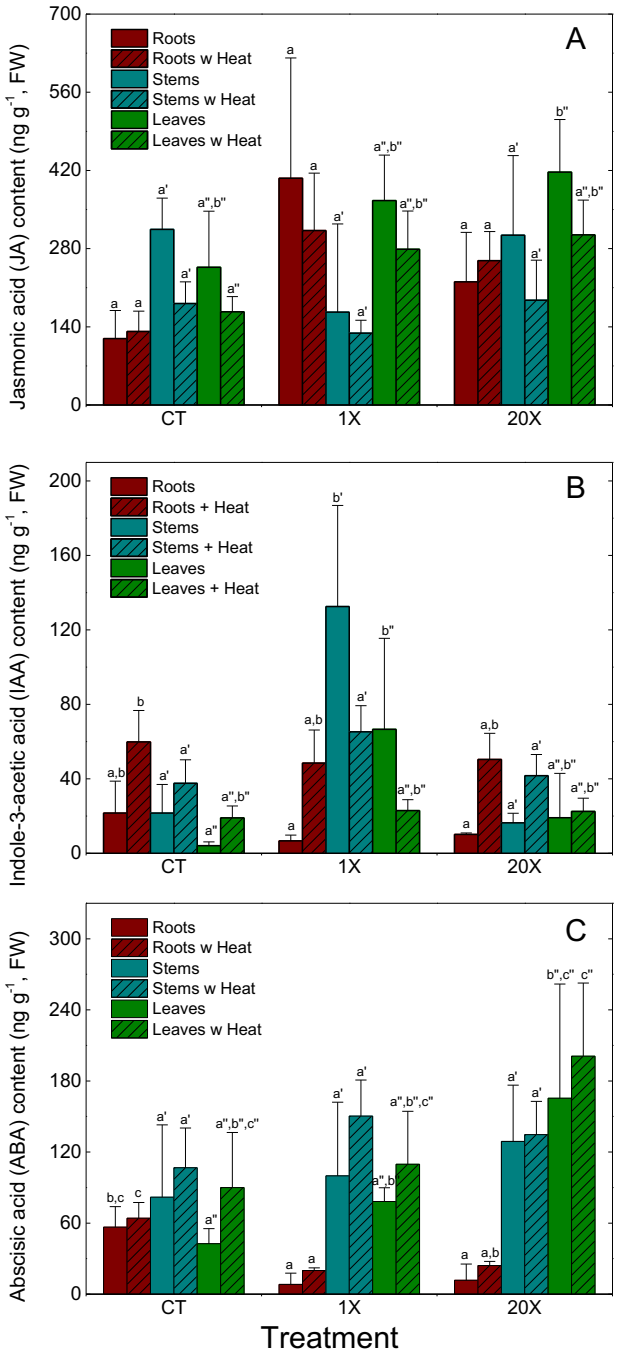


Figure 8. Levels of (A) jasmonic acid, (B) indole-3-acetic acid, and (C) abscisic acid in cucumber tissues after a 30-day exposure to CEC mixture at incremental CEC levels. Etched bars represent the treatment with additional heat exposure. Bars represent the mean ± standard deviation. Bars without a common letter indicate a statistically significant difference ($p < 0.05$) as determined by one-way ANOVA with Scheffe's post hoc test. CT, control treatment.