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Pharmaceutical and personal care products-induced stress symptoms and detoxification mechanisms in cucumber plants☆

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ABSTRACT

Contamination of agricultural soils by pharmaceutical and personal care products (PPCPs) resulting from the application of treated wastewater, biosolids and animal wastes constitutes a potential environmental risk in many countries. To date a handful of studies have considered the phytotoxicity of individual PPCPs in crop plants, however, little is known about the effect of PPCPs as mixtures at environmentally relevant levels. This study investigated the uptake and transport, physiological responses and detoxification of a mixture of 17 PPCPs in cucumber seedlings. All PPCPs were detected at higher concentrations in roots compared to leaves, with root activity inhibited in a dose-dependent manner. At 5–50 μg/L, the mature leaves exhibited burnt edges as well as a reduction in photosynthesis pigments. Reactive oxygen species (ROS) production and lipid peroxidation increased with increasing PPCP concentrations; and their contents were greater in roots than in leaves for all PPCP treatments. Enzymes involved in various functions, including oxidative stress (superoxide dismutase and ascorbate peroxidase) and xenobiotic metabolism (peroxidase and glutathione S-transferase), were elevated to different levels depending on the PPCP concentration. Glutathione content gradually increased in leaves, while a maxima occurred at 0.5 μg L^{-1} PPCPs in roots, followed by a decrease thereafter. This study illustrated the complexity of phytotoxicity after exposure to PPCP mixtures, and provided insights into the molecular mechanisms likely responsible for the detoxification of PPCPs in higher plants.

1. Introduction

The use of reclaimed wastewater for irrigation and biosolids or animal wastes as fertilizers in agriculture is on the rise worldwide (Kinney et al., 2006; Malchi et al., 2014). There are many benefits from reuse of these waste materials, such as augmenting water supply, increasing soil nutrient content and improving crop yields (Carter et al., 2015). However, concerns remain about the safety of such practices (Boxall et al., 2012), as they introduce a multitude of trace contaminants, including pharmaceuticals and personal care products (PPCPs), into the agroecosystems (Bartrons and Peñuelas, 2017; Wu et al., 2013). In recent years, the fate of PPCPs in the soil-plant continuum has been extensively studied (Dalkmann et al., 2014; Grossberger et al., 2014; Xu et al., 2009). Furthermore, several studies have considered uptake and accumulation of subsets of PPCPs by different crop species (Bartrons and Peñuelas, 2017; Wu et al., 2015). Although many PPCPs are inherently bioactive substances, their toxicity to plants is comparatively less understood.

A few studies showed that exposure to PPCPs affected plant development and physiological functions (Bartrons and Peñuelas, 2017; Carter et al., 2015; Christou et al., 2016). For example, root growth and development were markedly reduced when pinto beans were exposed to chlortetracycline antibiotics (Batchelder, 1981). Tetracyclines and sulfonamides were shown to negatively affect seed germination (Liu et al., 2009a), and the influence varied among plant species and the different PPCPs considered in the study (Carvalho et al., 2014). Although such studies have shown various phytotoxic effects from PPCPs, the corresponding physiological and molecular mechanisms contributing to the toxicity were not adequately explored.

Once taken up by the plant root, PPCPs may be metabolized, leading to their detoxification, inactivation and sequestration (He et al., 2017; Wu et al., 2016). Another nodal point in the response of plant cells to xenobiotics is reactive oxygen species (ROS)

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generation, ultimately imposing oxidative stress to fundamental plant biomolecules (Ahammed et al., 2012; Liu et al., 2009b). Recent studies suggested that ROS overproduction-triggered oxidative damage may be the cause of the longer-term visual phytotoxic responses (Mittler, 2002), such as root growth inhibition and seed germination reduction. For instance, phenanthrene-induced oxidative stress in *Arabidopsis* was responsible for the observed reductions in germination and root growth and the damage to organelle structures (Liu et al., 2009b). On the other hand, plants have developed sophisticated antioxidant mechanisms to protect their cellular components from oxidative damage (Mittler, 2002). However, to our knowledge, so far little information is available on the potential impacts of PPCPs on ROS metabolism in higher plants, such as ROS production, oxidative damage and antioxidant system responses.

When exposed to single compounds of PPCPs, the observed toxic effects to plants were generally low (Migliore et al., 2003). However, PPCPs always enter agroecosystems as mixtures of many compounds. Comparison between individual and mixtures of PPCPs in aquatic organisms suggested that exposure to single PPCPs underestimated the actual environmental effect, and did not allow prediction of the risk of mixtures at environmentally relevant doses (Fernández et al., 2013).

This study was designed to evaluate PPCP accumulation and potential effects on ROS production and oxidative damage. Cucumber seedlings were exposed hydroponically to a mixture of 17 PPCPs at incremental levels (0, 0.5, 5, 50 μg L^{-1}) covering environmentally relevant occurrence (Christou et al., 2016; Meffe and de Bustamante, 2014; Wu et al., 2013). The molecular mechanisms involved in the detoxification of PPCPs were also explored.

2. Materials and methods

2.1. Chemicals and plant treatments

A total of 17 PPCPs were selected based on their occurrence in treated wastewater and biosolids. These compounds included 15 pharmaceutical compounds, i.e., acetaminophen, caffeine, meprobamate, atenolol, trimethoprim, carbamazepine, diazepam, gemfibrozil, primidone, sulfamethoxazole, dilantin, diclofenac, naproxen, ibuprofen and atorvastatin; and 2 personal care products, i.e., triclosan, triclocarban. The standards of these PPCPs were purchased from Alfa Aesar (Ward Hill, MA) and TCI America (Portland, OR), and their chemical purity was >98%. The corresponding deuterated standards, including their sources, are given in Text S1 in Supporting Information (SI). Stock solutions of each PPCP at 5, 50, 500 mg/L was prepared in methanol and stored at −20 °C before use. Solvents, including acetonitrile, methanol, and formic acid were Ultima grade (Fisher Scientific, Fair Lawn, NJ). All other chemicals and reagents used in enzyme activity measurement were of analytical grade or better. Purified water was obtained from a Milli-Q system (Millipore, Carrigtwohill, Cork, Ireland).

Cucumber (*Cucumis sativus* L.) seeds were obtained from Fisher Scientific (Fair Lawn, NJ). Seeds were germinated in a growth medium filled with a mixture of vermiculite and perlite (3/1, v/v) in a 72-hole plate. When the cotyledons were fully expanded, seedlings were transplanted to 500-mL glass jars filled with aerated, full-strength Hoagland nutrient solution and cultivated under controlled conditions (12 h/25 °C day and 12 h/25 °C night cycle; relative humidity of 75–80%). The nutrient solutions were renewed every 2 d. After 7 d, uniform seedlings were exposed to 0, 0.5, 5, or 50 μg L^{-1} PPCP mixture by spiking 50 μL of the stock solutions to the aerated, fresh hydroponic solution. In order to monitor the effect of different PPCP concentrations on the plant response, the solution was not changed during the short exposure experiment. Blank controls (with plant but no PPCPs) were set up simultaneously. The jars were wrapped with aluminum foil to prevent direct sunlight on the nutrient solution. Cucumber seedlings were sampled 7 d after the treatment, and the roots were gently dried with a paper towel, the cucumber plants were separated into roots and shoots (including stems and leaves). The content of reactive oxygen species (ROS), level of lipid peroxidation and activities of antioxidant enzymes in both roots and shoots were immediately analyzed after sampling. The remaining plant samples were frozen in liquid nitrogen and stored at −80 °C until processing. Each experiment was repeated at least three times.

2.2. Determination of chlorophyll content

The chlorophyll content in leaves was determined spectrophotometrically after chlorophyll extraction with 80% acetone (Liu et al., 2009b). Briefly, fresh leaves (0.2 g) were thoroughly homogenized with 15 mL of 80% acetone containing 0.2 g calcium carbonate and quartz sand until the tissue turned white. The homogenate was kept undisturbed for 5 min and was then filtered through a filter paper into a flask. The absorbance at 663, 646 and 470 nm was measured on a Cary 50 UV-Visible spectrophotometer (Varian, Palo Alto, CA). Chlorophyll *a* and *b* contents were calculated according to the following equations:

$$\text{Chlorophyll } a \text{ (mg g}^{-1}\text{ FW)} = (12.21A_{663}\text{-}2.81A_{645})/\text{FW} \quad (1)$$

$$\text{Chlorophyll } b \text{ (mg g}^{-1}\text{ FW)} = 20.13A_{645}\text{-}5.03A_{645}/\text{FW} \quad (2)$$

where A_{663} and A_{645} are absorbance at 663 nm and 645 nm for chlorophyll *a* and *b*, respectively, A_{470} is the absorbance at 470 nm for carotene, and FW is the fresh weight of leaves.

2.3. Determination of root activity

Root activity was evaluated according to the triphenyltetrazoliumchloride (TTC) method (Wang et al., 2010b). Roots were excised and incubated in freshly prepared 5 mL of 0.4% TTC and 5 mL potassium phosphate buffer (PBS; 60 mM; pH 7.0) at 37 °C for 2 h. The reaction was stopped by the addition of 2 mL of 1 M sulfuric acid. Blank controls were included using the same procedure, but sulfuric acid was added at the beginning of incubation. After incubation, the roots were extracted with 10 mL ethyl acetate and absorbance was measured at 485 nm on a Cary 50 UV-Visible spectrophotometer (Varian, Palo Alto, CA).

2.4. Determination of PPCPs in cucumber tissues

The freeze-dried plant tissue samples were extracted and analyzed following the same method as in Wu et al. (2012). Briefly, plant materials were ground to a fine powder and placed in 50 mL centrifuge tubes, spiked with deuterated PPCPs as recovery surrogates and mixed with 20 mL methyl *tert*-butyl ether (MTBE). The mixture was then extracted in an ultrasonic water bath (50/60 Hz, Fisher) for 20 min prior to centrifugation at 8000*g* for 20 min. The supernatant was collected in 40 mL glass vials. Pellets were subsequently extracted with 20 mL acetonitrile. The pooled supernatant from MTBE and acetonitrile extractions was concentrated to near dryness under nitrogen. The residue was then re-dissolved in 1 mL methanol, followed by addition of 20 mL deionized water. The extract was loaded onto an Oasis™ HLB cartridge (150 mg, Waters, Milford, MA) that was preconditioned with 6 mL methanol and 12 mL deionized water. The cartridge was eluted with 15 mL methanol under gravity and the eluate was evaporated to dryness under nitrogen. The residue was recovered in 1.5 mL

methanol:water mixture (v:v; 1:1) and filtered (PTFE, 0.2 μm, Millipore, Carrigtwohill, Cork, Ireland) before analysis.

The PPCPs were quantified on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) in combination with a Waters Micromass Triple Quadrupole mass spectrometer (QqQ) equipped with an electrospray ionization (ESI) 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). The mobile phase consisted ofwater/methanol/formic acid (v/v/v; 95/5/0.001) as mobile phase A and methanol as mobile phase B. The details of instrument analysis can be found in Wu et al. (2012).

2.5. Determination of ROS content

The O_2^- content was determined as in Sun et al. (2014). Plant tissue was homogenized in 2 mL of 65 mM phosphate buffer (pH 7.8) and then centrifuged at 5000 g for 10 min at 4 °C. An aliquot of 1.0 mL supernatant was mixed with 0.9 mL of 65 mM PBS (pH 7.8) and 0.1 mL of 10 mM hydroxylamine hydrochloride. The mixture was incubated at 25 °C for 20 min. After that, a subsample (1.0 mL) of the mixture was incubated with 1.0 mL of sulfanilamide (1% w/v in 1.5 M HCl) and 1.0 mL N-(1-naphthyl)-ethylenediamine dihydrochloride (0.02% w/v in 0.2 M HCl) at 25 °C for 30 min. The product of NO_2^- was measured using the UV-Vis spectrophotometer at 540 nm.

The content of H_2O_2 was determined after extraction by homogenizing plant tissues with 2 mL cold acetone (Wang et al., 2010a). After centrifugation at 5000g at 4 °C for 10 min, a 1.0 mL aliquot of the supernatant was mixed with 0.1 mL of 5% $TiSO_4$ and 0.1 mL ammonia. After centrifugation, the titanium-peroxide complex pellet was resuspended in 3.0 mL of 2 M H_2SO_4, and absorbance was determined at 415 nm with a standard curve generated with known concentrations of H_2O_2.

2.6. Determination of oxidative damage

Plasma membrane integrity was evaluated by staining roots with Evans blue solution (0.25%, w/v) as in Yamamoto et al. (2001) with minor modifications. After the PPCP treatment, roots were stained with Evans blue solution (0.25%, w/v) for 15 min, and the stained roots were washed thoroughly with deionized water. The trapped Evans blue was released by extracting the roots in 5.0 mL of *N*,*N*-dimethylformamide. Absorbance of the supernatant was determined spectrophotometrically at 600 nm.

The level of lipid peroxidation in roots and shoots of cucumber was measured in terms of malondialdehyde, which was determined according to the reaction with thiobarbituric acid as described in Yamamoto et al. (2001). Historical staining for lipid peroxidation was conducted with Schiff's reagent (Sigma-Aldrich, St. Louis, MO) (Yamamoto et al., 2001).

2.7. Determination of glutathione

For the measurement of reduced glutathione (GSH) and oxidized glutathione (GSSG), plant tissues were homogenized in 5 mL of cold 5% *meta*-phosphoric acid on ice. The homogenate was centrifuged at 12,000 g for 15 min at 4 °C, and the supernatant was used for analysis of GSH and GSSG (Jiang et al., 2012). A 0.5-mL aliquot of the supernatant was added to a reaction mixture containing 100 mM PBS (pH 7.0), 0.2 mM NADPH and 1 mM 5′5′-dithiobis-2-nitrobenzonic acid (DTNB, dissolved in PBS). The reaction was started by the addition of 3 U of glutathione reductase, and absorbance at 412 nm was measured after 5 min. For GSSG, 2-vinylpyridine was added to the neutralized supernatant to mask GSH. Simultaneously, the same volume of water was added for the total glutathione assay. The GSH concentration was obtained by subtracting the GSSG from the total GSH.

2.8. Assay of enzyme activities

Fresh plant tissue samples were frozen in liquid nitrogen, and homogenized in 50 mM PBS (pH 7.0) containing 1 mM EDTA and 1% PVP, with the addition of 1 mM ascorbate for ascorbate peroxidase. The homogenate was centrifuged at 12,000 g for 20 min at 4 °C, and the supernatant was used for the following enzyme assays (Sun et al., 2014, 2015). Protein content in enzyme extracts was determined by Coomassie brilliant blue G-250 with a standard curve using bovine serum albumin as the standard.

Measurement of superoxide dismutase (SOD; EC 1.15.1.1) activity was carried out by inhibiting photochemical reduction of nitro blue tetrazolium (NBT). The assay mixture contained 50 mM PBS (pH 7.8), 13 mM methionine, 75 μM NBT and 2 μM riboflavin. After addition of 100 μL of enzyme extract, the glass tubes were placed under light for 15 min, and then read at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT.

To determine ascorbate peroxidase (APX; EC 1.11.1.11) activity, a 200 μL aliquot of enzyme extract was added to the reaction mixture of 1 mM ascorbate and 0.3 mM H_2O_2 in 50 mM PBS. The absorbance changes were monitored at 290 nm for 3 min as ascorbate was oxidized, the enzyme activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbate.

Peroxidase (POD; EC 1.11.1.7) activity was monitored by oxidation of 0.2% guaiacol using 0.3% H_2O_2 after addition of 50 μL enzyme extract. The enzyme activity was calculated using an extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Glutathione S-transferase (GST; EC 2.5.1.18) activity was determined in 2 mL of a reaction mixture containing 50 mM PBS (pH 7.0), 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), 5 mM GSH and 100 μL enzyme extract. The GST activity was measured spectrophotometrically at 340 nm based on GSH-CDNB adduct synthesis using extinction coefficient 9.6 mM^{-1} cm^{-1} for GSH-CDNB.

2.9. Statistical analysis

Data are statistically analyzed using the SPSS package (version 11.0; SPSS, Chicago, IL). ANOVA was performed on the data sets, and the mean and standard deviation (SD) of each treatment as well as the least significant difference (LSD; $P < 0.05$ and $P < 0.01$) for each set of corresponding data were calculated.

3. Results

3.1. Changes in leaf chlorophyll content and root activity

To evaluate the sensitivity of cucumber plants to PPCPs, an initial dose-response experiment was carried out. Statistical analysis showed no significant difference in the biomass of plants grown in 0.5, 5 ng L^{-1} PPCP-spiked and control solutions (Table S1). However, treatment with PPCPs at 50 μg L^{-1} progressively caused an increase of leaf necrosis (Fig. 1a). The level of chlorophyll *a* and chlorophyll *b* decreased with increasing PPCP treatment rates (Fig. 1b). Meanwhile, root activity decreased by 15.4% and 28.2% after exposure to 5 and 50 μg L^{-1} PPCPs, respectively, as compared with the control (Fig. 1c).

3.2. Uptake and translocation of PPCPs in plants

To understand the pattern of PPCP uptake and translocation in cucumber seedlings, cucumber seedlings grown in solution with

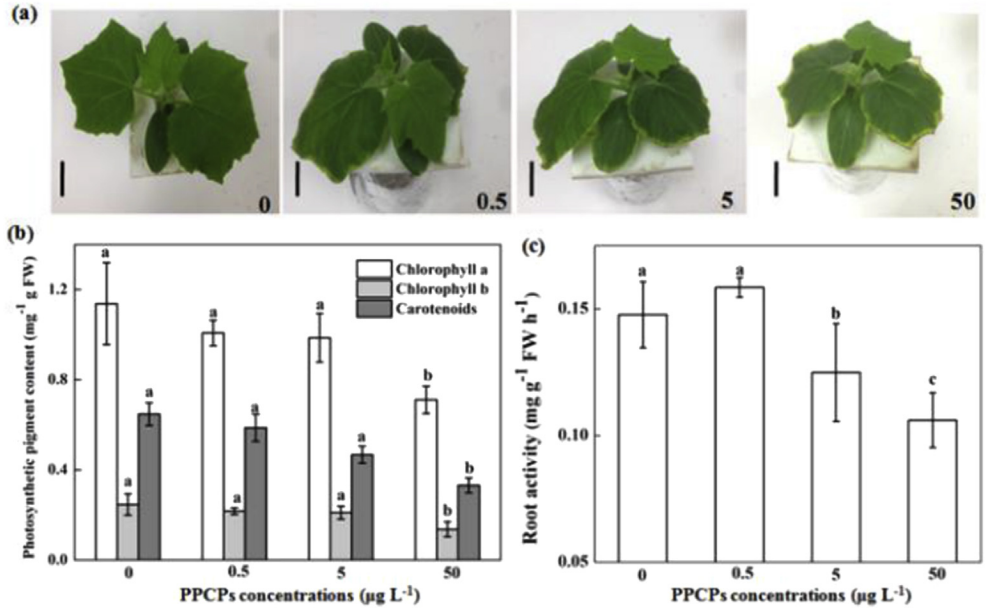

Fig. 1. Changes in (a) phenotype, (b) leaf photosynthetic pigments content and (c) root activity in 2-week old cucumber plants exposed to mixed PPCPs for 7 d before assayed. Bars, 1.5 cm. The values shown are mean ± SD (n = 3) and different letters indicate significant differences ($P < 0.05$) among treatments.

5 μg $L^{-1}$of PPCPs were used to analyze PPCP accumulation in shoots and roots (Fig. 2). All PPCPs were detected in the roots, with triclocarban (2.0×10^3 ng g^{-1}) found at the highest level, followed by naproxen (6.9×10^2 ng g^{-1}), triclosan (4.9×10^2 ng g^{-1}), sulfamethoxazole (4.6×10^2 ng g^{-1}), caffeine (4.5×10^2 ng g^{-1}), and gemfibrozil (4.2×10^2 ng g^{-1}). In shoots, meprobamate (1.9×10^2 ng g^{-1}), and trimethoprim (1.9×10^2 ng g^{-1}) were detected at the highest level, while acetaminophen was not detected.

3.3. PPCP-induced reactive oxygen species accumulation

Previous studies showed that reactive oxygen species (ROS) were produced when plants were exposed to xenobiotics (Liu et al., 2009a,b). However, little is known if exposure to PPCPs would induce intracellular ROS production in higher plants. It was found that even at 0.5 μg L^{-1}, PPCPs induced H_2O_2 accumulation in both shoots and roots, and the maximum elevation occurred at the highest concentration (50 μg L^{-1}), where H_2O_2 levels were about 3.0- and 3.2-fold higher in shoots and roots, respectively, than those from the control treatment (Fig. 3b). Additionally, $O_2^{\cdot-}$ did not increase significantly after exposure to PPCPs at 0.5 μg L^{-1} (Fig. 3a), which could be due to the fast conversion of $O_2^{\cdot-}$ to H_2O_2.

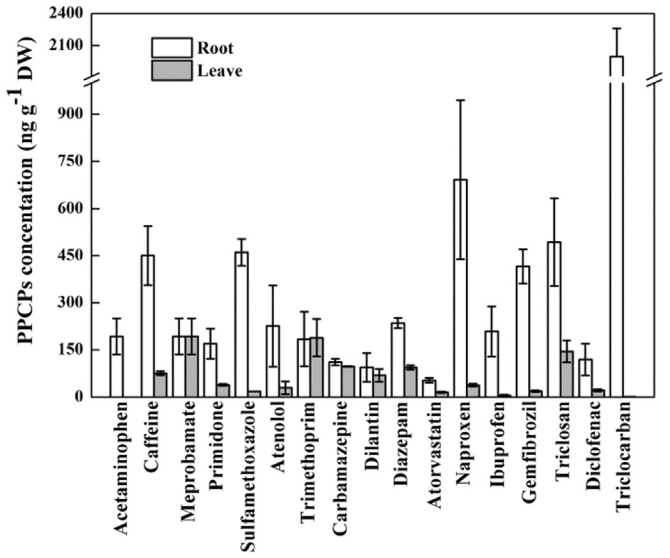

Fig. 2. Uptake and translocation of PPCPs in 2-week-old cucumber plants exposed to mixed PPCPs at 5 μg L^{-1} for 7 d. The values shown are mean ± SD (n = 3).

3.4. PPCP-induced oxidative damage to cucumber plants

Analysis of Evans blue uptake (Fig. 4) and malondialdehyde (MDA, Fig. 5) content showed that PPCPs caused oxidative damage to the plasma membrane and lipid fraction in plant seedlings; however the damage was less pronounced in the leaves. A significant increase in Evans blue uptake was found at 50 μg L^{-1} PPCPs in the root (1.93-fold over control) (Fig. 4a), and histochemical staining clearly indicated that cell death occurred in the root tip (Fig. 4b) that is the most active and sensitive region of the root. Lipid peroxidation measured as MDA increased in all of the stressed plants, and its content was higher in roots than in shoots in all of the PPCP treatments (Fig. 5a and b). These results were further confirmed by histochemical analysis using Schiff's reagent to detect lipid peroxidation in plants (Fig. 5c and d).

3.5. Effects on activities of antioxidant enzymes and glutathione content

Activities of several main antioxidant enzymes in cucumber plants were determined after exposure to PPCPs. After 7 d of cultivation, SOD showed the maximal activity in roots after exposure to PPCPs at 5 μg L^{-1} and decreased thereafter (Fig. 6a). Ascorbate peroxidase showed a dose-dependent response, increasing about 2.0- and 1.1-fold in roots and shoots, respectively, after exposure to PPCPs at 50 μg L^{-1} (Fig. 6b). Peroxidase is among the enzymes with a potential role in the detoxification of a variety of xenobiotics, and GSTs often detoxify exogenous compounds by

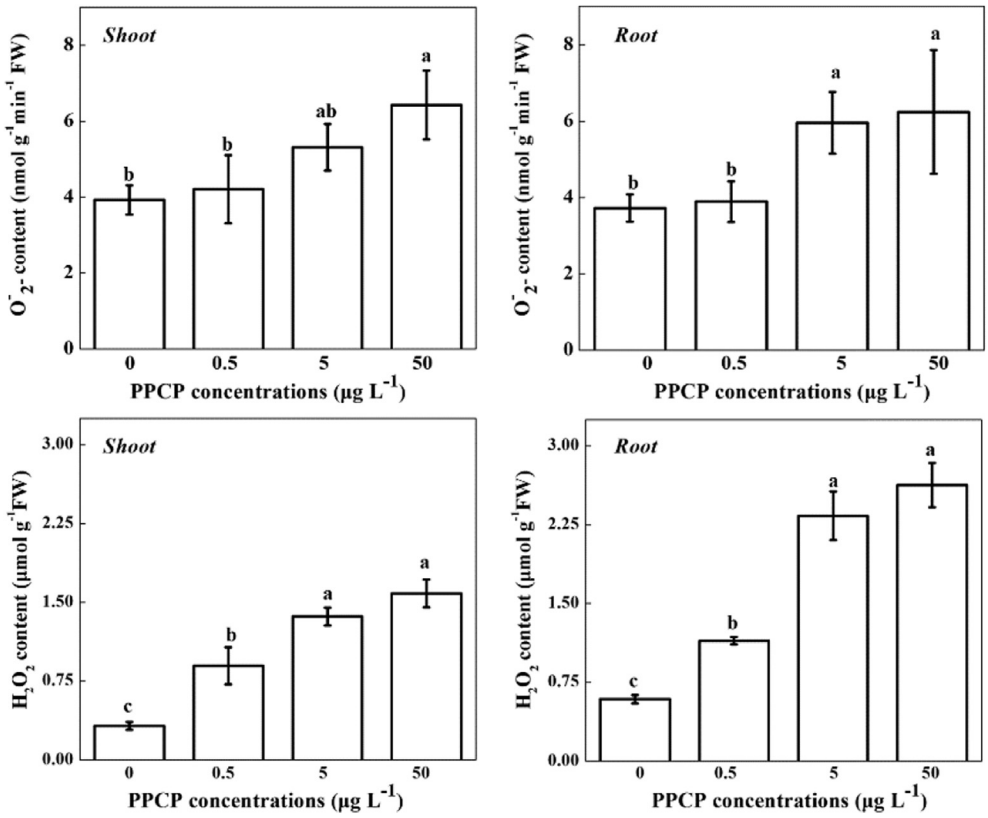

Fig. 3. PPCPs induced reactive oxygen species (ROS) accumulation in 2-week-old cucumber plants exposed to mixed PPCPs for 7 d. (a) O_2^- in shoot; (b) O_2^- in root; (c) H_2O_2 in shoot; and (d) H_2O_2 in root. The values shown are mean ± SD (n = 3) and different letters indicate significant differences (P < 0.05) among treatments.

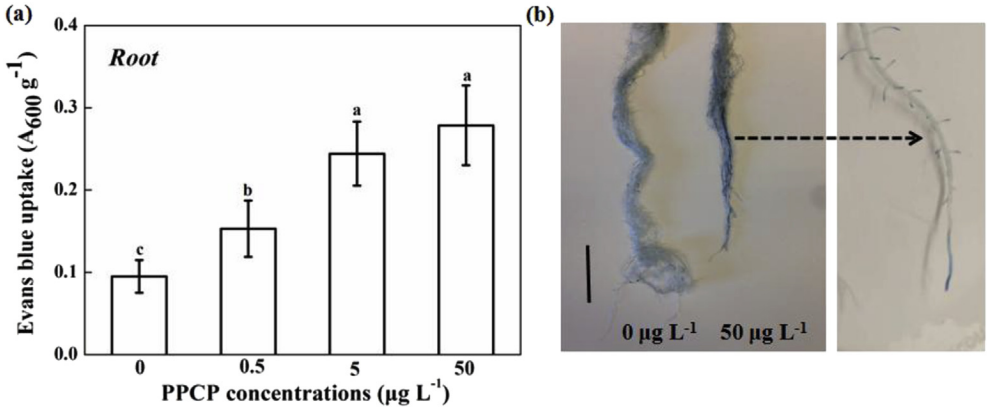

Fig. 4. PPCPs induced oxidative damage in 2-week-old cucumber plants exposed to mixed PPCPs for 7 d. (a) Evans blue uptake by roots; (b) Histochemical visualization of Evans blue staining. Bars, 1.5 cm. The values shown are mean ± SD (n = 3) and different letters indicate significant differences (P < 0.05) among treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conjugation with GSH. The total POD (Fig. 6c) and GST (Fig. 6d) activities both increased appreciably after exposure to PPCPs.

The GSH content increased in leaves after exposure to PPCPs, while the root showed a maximal GSH content at 0.5 μg L^{-1} PPCPs, followed by decreases thereafter (Fig. 7a). The decreases of GSH in roots may be due to GSH serving as an antioxidant for preventing oxidative damage, and also acting to detoxify PPCPs by conjugation. The GSSG content displayed little change when PPCP concentrations were low (<5 μg L^{-1}). However, when the PPCP concentration was increased to 50 μg L^{-1}, there was a significant increase in GSSG content (Fig. 7b).

4. Discussion

Results from the present study illustrated the physiological, biochemical and molecular mechanisms involved in the detoxification of PPCPs in plants by considering especially homeostasis of ROS and anti-oxidant metabolism. The results clearly showed that PPCP-induced morphological indicators changed at elevated PPCP concentrations (Fig. 1), and the impact was more pronounced in roots than shoots. The enhanced sensitivity of roots to PPCP toxicity may be due to the greater accumulation of PPCPs in the root (Fig. 2). Similar observations were previously reported in alfalfa, lettuce,

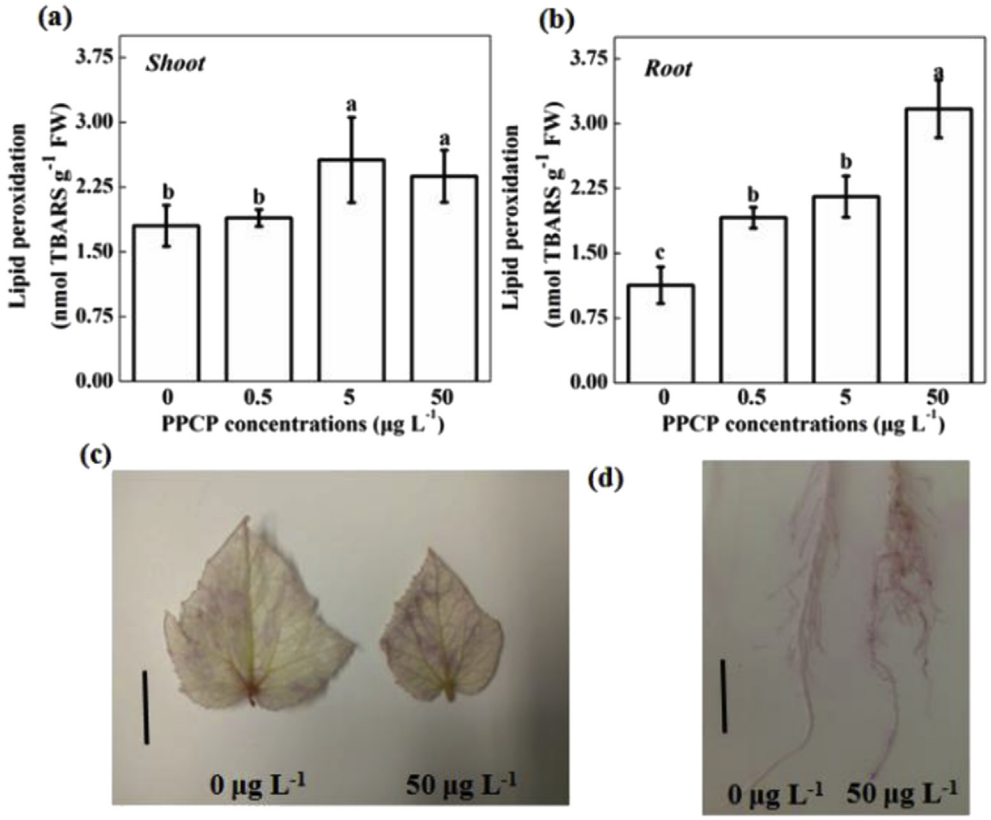

Fig. 5. PPCPs induced oxidative damage in 2-week old cucumber plants exposed to mixed PPCPs in nutrient solution for 7 d. (a) malondialdehyde (MDA) content in leaf; (b) MDA content in root; (c) Leaf after staining with Schiff's reagent; (d) Root after staining with Schiff's reagent. Bars, 1.5 cm. The values shown are mean ± SD (n = 3) and different letters indicate significant differences ($P < 0.05$) among treatments.

and pepper after PPCP exposure (Christou et al., 2016; Wu et al., 2013). In addition, Christou et al. (2016) suggested that PPCPs in a mixture displayed a different uptake pattern compared with that when exposed individually. The present study also showed that roots consistently accumulated PPCPs to a higher level than shoots when exposed to mixed PPCPs (Fig. 2). In shoots, the relative accumulation of individual PPCPs differed somewhat from that in Wu et al. (2013), where carbamazepine and diazepam were found at the highest levels, followed by meprobamate and trimethoprim. The discrepancy between the studies may be attributed to the different growth conditions, plant cultivars, and sampling intervals. Acetaminophen, however, was not detected in the plant tissues (Fig. 2), likely owing to its rapid metabolism after uptake (Huber et al., 2009).

Recent studies showed that contact with PPCPs was capable of inducing a complex set of physiological responses in higher plants (Bartrons and Peñuelas, 2017; Carvalho et al., 2014). Generally, contents of leaf pigments, including chlorophyll and carotenoids, provide valuable information about the physiological status of a plant. Here, a clear leaf necrosis and reduction in contents of chlorophyll and carotenoids were observed at higher PPCP concentrations (Fig. 1b). Findings from this and previous studies together suggest that PPCPs may significantly affect plant growth.

Prior to the induction of whole plant morphological effects, stress may also lead to physiological, biochemical and molecular changes within the plant. However, little information is available about the toxic effects in plants from a mechanistic perspective. A direct result of stress-induced cellular changes is the enhanced ROS accumulation, consequently imposing oxidative stress to biomolecules (Gill and Tuteja, 2010). Although additional research is needed to establish oxidative stress as the primary mechanism of PPCP toxicity to higher plants, it is clear that oxidative stress is involved in the development of PPCP-induced toxic symptoms. In this study, changes in ROS levels were observed in comparison to the control after exposure to PPCPs (Fig. 3), and the response occurred at much lower concentrations than that for morphological effects. Overproduction of ROS can cause cell damage and is the final consequence of oxidative stress. In the current study, the increase in ROS production (Fig. 3) coincided with the increase in membrane damage (Fig. 4) and lipid peroxidation (Fig. 5) in the cucumber plants, indicating the presence of oxidative stress. In a previous study, exposure to 10 μg L^{-1} of diclofenac, sulfamethoxazole, trimethoprim or 17α-ethinylestradiol did not induce significant lipid peroxidation in alfalfa leaves (Christou et al., 2016). Exposure to mixed PPCPs was found to exacerbate cytotoxicity to a rainbow trout gonadal cell line as compared to exposure to individual compounds (Fernández et al., 2013). These results indicated that studies using individual PPCPs might underestimate the actual environmental impacts of trace organic contaminants that usually occur as mixtures.

During the period of time in which cucumber plants were exposed to the PPCP mixture, an overall induction of enzymatic and non-enzymatic antioxidant systems was observed. Superoxide dismutase (SOD) constitutes the first line of defense against ROS, which can dismutate $O_2^{\cdot-}$ into the more stable H_2O_2 (Mittler, 2002). In this study, the root experienced more significant oxidative damage as a result of greater ROS accumulation than shoots, and elevated activities of SOD were observed in treatments below 5 μg L^{-1} of PPCPs (Fig. 6a). Above the 5 μg L^{-1} treatment rate, SOD activity in roots was significantly inhibited. In a previous study,

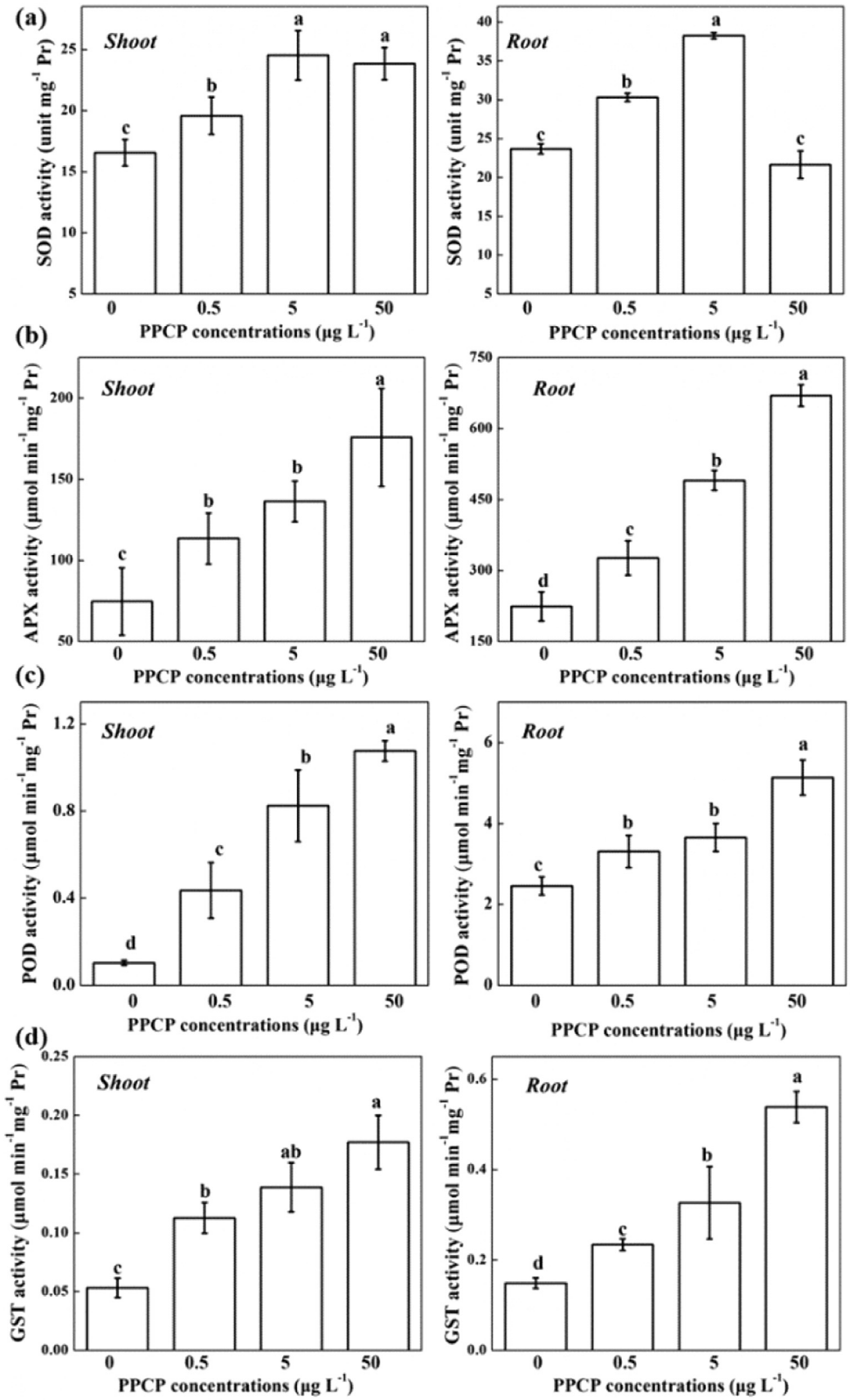

Fig. 6. Activities of antioxidant enzymes in 2-week-old cucumber seedlings exposed to mixed PPCPs for 7 d. (a) Superoxide dismutase (SOD); (b) Ascorbate peroxidase (APX); (c) Peroxidase (POD); and (d) Glutathione S-transferase (GST). The values shown are mean ± SD (n = 3) and different letters indicate significant differences ($P < 0.05$) among treatments.

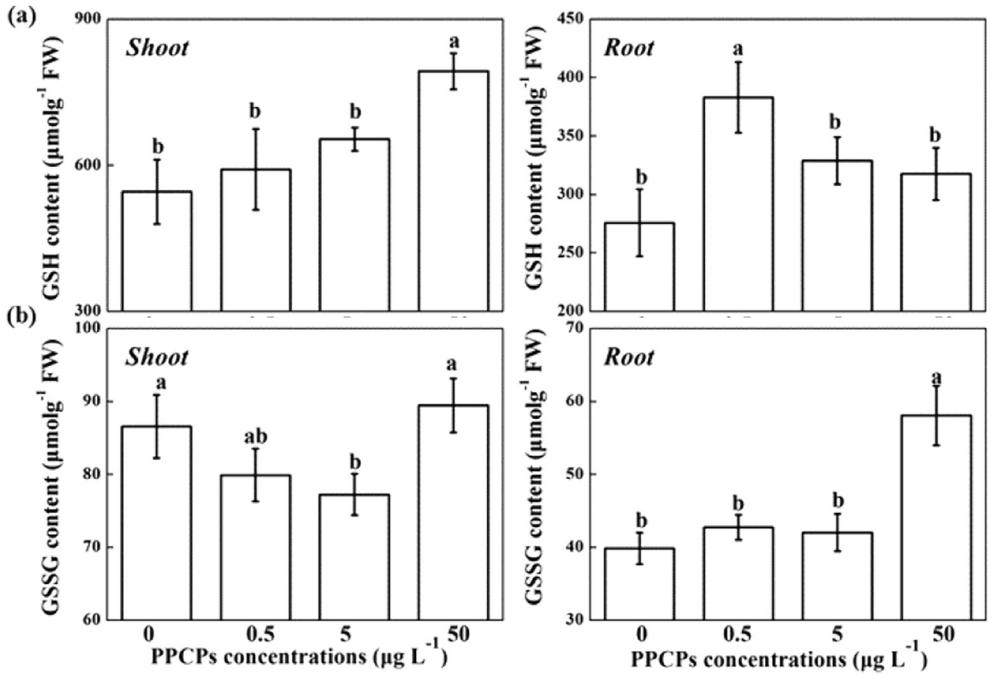

Fig. 7. Content of (a) reduced glutathione (GSH) and (b) oxidized content (GSSG) in 2-week-old cucumber seedlings exposed to mixed PPCPs for 7 d. The values shown are mean ± SD (n = 3) and dfferent letters indicate significant differences (P < 0.05) among treatments.

increased SOD activity was detected after exposure to paracetamol (An et al., 2009a), while decreased SOD activity was found under triclosan and galaxolide stress (An et al., 2009b) in wheat seedlings. A possible explanation for the decreased SOD activity may be that the oxidative stress exceeded the capability of the enzymatic machinery. It must be noted that there exist three forms of SOD isoenzymes: copper/zinc containing SOD, manganese containing SOD and iron containing SOD, which are all localized in different cellular compartments. Further research should take into account the specific roles of different SOD isoenzymes from different cellular compartments after exposure to trace xenobiotics. Using ascorbate as a reductant, H_2O_2 was reduced to water by ascorbate peroxidase (APX), the major component of the ascorbate-glutathione cycle (Noctor and Foyer, 1998). Thus the increase in APX activity (Fig. 6b) in the PPCP treated cucumber plants may be related to the functioning of the ascorbate-glutathione cycle that detoxifies H_2O_2 thereby preventing further damage. This was consistent with previous observations in *Brassica juncea* with acetaminophen treatment (Bartha et al., 2010). It has been shown that APX may be responsible for the fine modulation of ROS for signaling, whereas CAT might be responsible for the removal of excess ROS during stress. The significantly increased catalase (CAT) activity with increasing PPCP concentrations (Fig. S1) reaffirmed that the cucumber plants experienced serious oxidative stress when in contact with the PPCP mixture.

Beside anti-oxidation, another key role of POD and GSTs is their ability to inactivate toxic compounds (Bártíková et al., 2015; Coleman et al., 1997; Neuefeind et al., 1996). For example, Agostini et al. (2003) demonstrated the capacity of peroxidases to degrade the pesticide 2,4-dichlorophenol in a cell culture of *Brassica napus*. Xia et al. (2009) suggested that the induced activity of GST by chlorpyrifos indicated formation of glutathione S-conjugates to detoxify the insecticide in plants. In comparison with pesticides, only sporadic research has shown detoxification of PPCPs by POD and GST in plants. In the present study, activities of POD and GST increased in a dose-dependent manner in both roots and leaves after exposure to PPCPs (Fig. 6b). This observation was consistent with Bartha et al. (2014) and Huber et al. (2016), who observed oxidation of diclofenac by plant peroxidases, and also glutathione conjugation in *Typha latifolia*. These mechanistic studies, together with our results, clearly show that the POD and GST enzyme families may play an important role in transformation and conjugation of PPCPs in plants.

Glutathione is one of the major soluble low molecular weight antioxidants, and also the major non-protein thiol in plant cells (Noctor and Foyer, 1998), contributing to maintain the cellular redox homeostasis and signaling. Moreover, conjugation with xenobiotics by GSH may be a common pathway for plant metabolism of various man-made chemicals (Neuefeind et al., 1996). Glutathione conjugation with diclofenac (Bartha et al., 2014), 8:2 fluorotelomer alcohol (Zhang et al., 2016) and chlortetracycline (Farkas et al., 2007) have been previously observed in plants. It is well known that these processes require extensive utilization of reduced GSH as an electron donor and subsequently produce oxidized glutathione (GSSG). The changes in the cellular glutathione pool, specially the associated ratio of reduced to oxidized glutathione, play a central role in plant defense responses (Mittler, 2002). Glutathione homeostasis after exposure to trace levels of PPCPs, however, has not been well documented so far. In this study, the glutathione content increased at low PPCP doses, while decreased to normal levels at the highest PPCP treatment level (Fig. 7a). The GSSG content was unchanged when the PPCP concentrations were low (<5 μg L^{-1}), but showed a significant increase when the PPCP concentration was increased to 50 μg L^{-1} (Fig. 7b). The depletion observed in cellular GSH could contribute to the PPCP-induced oxidative stress and detoxification of xenobiotics. Meanwhile, the different responses of GSH in root and leaves indicated that roots may be the main site to express PPCP toxicity and induce PPCP detoxification. Given the dominance of GSH conjugates as observed for pesticides, it is possible that trace contaminants such as PPCPs may be removed similarly by GSH conjugation. It is therefore imperative to conduct further research to explore the mechanisms and pathways of PPCP phytotransformation by GSTs after uptake by plants.

On the whole, the present study provided evidence that some PPCPs may be translocated systemically, and ultimately posed toxicity effects in higher plants. Oxidative stress response may reflect the intensity of PPCP treatment and sensitivity of plant species to PPCPs, and may be used as indicators for early plant response to trace organics introduced into agroecosystems. Furthermore, plants may detoxify PPCPs through different mechanisms, including enhanced antioxidant defense systems to prevent oxidative damage and increased activities of xenobiotic-metabolizing enzymes. These mechanisms help maintain plant physiological, biochemical and molecular functional integrity, offering the possibility to use some of these endpoints as biomarkers for predicting phytotoxicity induced by PPCPs and likely other man-made chemicals. Further research is needed to evaluate the physiological and biological responses of plants in realistic field practices, such as irrigation with treated wastewater or fertilization with biosolids and animal wastes in agriculture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.11.041.

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