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Dissemination of antibiotics through the wastewater–soil–plant–earthworm continuum

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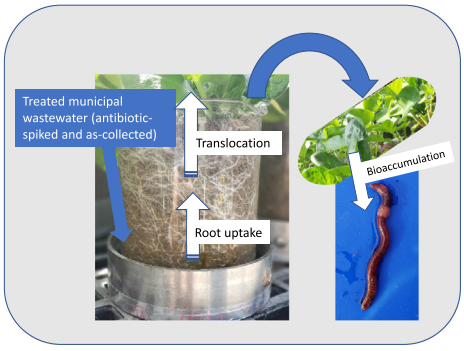
HIGHLIGHTS

- Transfers of trimethoprim, sulfamethoxazole, and sulfapyridine studied
- With as-collected TMW, no uptake by spinach and radish
- With spiked TMW, uptake by radish and particularly spinach
- Slow soil degradation, speciation, and root distribution important uptake factors
- Uptake by earthworms when fed spiked spinach and radish materials

GRAPHICAL ABSTRACT



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ABSTRACT

Under the ongoing climate change scenario, treated municipal wastewater (TMW) is a potential candidate for irrigated agriculture but may result in the exposure of agricultural environments to antibiotics. We studied the transfers of trimethoprim, sulfamethoxazole, and sulfapyridine in the TMW–soil–plant–earthworm continuum under greenhouse/laboratory conditions. Irrigation of potted spinach and radish with as-collected TMW resulted in no transfers of antibiotics into soil or plants owing to their low concentrations in the tertiary-treated TMW. However, TMW spiked with higher antibiotic concentrations led to transfers through this continuum. High initial inputs, slow soil degradation, and chemical speciation of the antibiotics, coupled with an extensive plant-root distribution, were important factors enhancing the plant uptake of antibiotics. In microcosm studies, transfers from vegetable materials into earthworms were low but showed potential for bioaccumulation. Such food chain transfers of antibiotics may be a driver for antibiotic resistance in agricultural systems, which is an area worthy of future study. These issues can perhaps be mitigated through high levels of TMW purification to effectively remove antibiotic compounds.

1. Introduction

Antibiotics are introduced into municipal sewage systems via various human activities, including (i) neglectful disposal of unused or expired medication and (ii) releases from pharmaceutical manufacturing plants and hospitals (Grossberger et al., 2014). Traditional wastewater treatment processes are relatively poor at removing trace contaminants of emerging concern (CECs) such as antibiotic compounds (Michael et al., 2012; Petrie et al., 2015). As such, antibiotics are routinely detected in TMW, in TMW-irrigated agricultural soils, in the runoff from such sites, and in surface and groundwater systems and sediments receiving TMW (Kolpin et al., 2002; Kinney et al., 2006; Fatta-Kassinos et al., 2011; Gottschall et al., 2012). This issue may be particularly acute in arid regions where water reuse is an expanding approach to addressing water shortages caused by

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climate change, urbanization, regional drought, and pollution (IPCC, 2009; WWAP, 2012).

As noted previously (Ben Mordechay et al., 2021, 2022; Pan and Chu, 2017), antibiotics found in the soil pore water of agricultural soils (e.g., as a result of TMW irrigation) may be taken up by crop plants and bioaccumulate within plant tissues. Relevant to the present work, Wu et al. (2014) reported trimethoprim (TMP) concentrations for a range of agricultural crops grown in contaminated soil under greenhouse conditions, with values ranging from 1.10 (spinach leaves) to 270 (pepper roots) ng g^{-1}. The plant concentrations of sulfonamides previously reported are typically lower, with values for sulfamethoxazole (SMZ) ranging from non-detectable levels (lettuce leaves) to 6.80 ng g^{-1} (cucumber roots) (Wu et al., 2013). However, little is presently known about whether antibiotics accumulated in plant materials can be further disseminated along a food chain, i.e., across trophic levels. To study the potential for such transfers experimentally, we herein focused on the wastewater–soil–plant–earthworm continuum. Earthworms can be considered a terrestrial organism of choice for better understanding the inputs of contaminants into food chains (Carter et al., 2021). Indeed, several studies have demonstrated that antibiotics and other pharmaceuticals can be taken up from soils and accumulate in earthworms (Berge and Vulliet, 2015; Carter et al., 2014; Carter et al., 2016; Kinney et al., 2008). Earthworms are at the base of many food chains; therefore, if chemicals are taken up into earthworms via soil and/or plant ingestion, the movement of these chemicals into the food web via bioaccumulation and biomagnification processes may be facilitated (Shore et al., 2014). Moreover, the selection pressure exerted by antibiotic compounds in the environment may facilitate the development and dissemination of antibiotic resistance in environmental bacteria (Christou et al., 2017; Su et al., 2017), e.g., in soils, plants, and animal guts. Given the global significance of the spread of antibiotic resistance (HM Government and Wellcome Trust, 2014; WHO, 2017; WHO, 2018; Murray et al., 2022), it is crucial to quantify antibiotic transfers through agricultural systems, where the processes driving the dissemination of antibiotic resistance are poorly understood. We are not aware of any previous work in which the transfers of antibiotics across the wastewater–soil–plant–earthworm continuum have been studied. Understanding antibiotic transfers to the animal gut in a relatively simplistic food chain may be a useful first step toward elucidating more complex and harder to study food chains, e.g., those including humans.

In the present work, pot experiments in which spinach and radish were irrigated with TMW (both as-collected (unspiked) and spiked with antibiotics) were conducted under greenhouse conditions to determine plant uptake. The vegetable materials from these experiments were then used as a food source for earthworms in microcosm studies to determine uptake into earthworm tissues. Based on a preliminary analysis of the TMW used in this study, we focused on the antibiotics TMP, SMZ, and sulfapyridine (SPD). TMP and SMZ are commonly found together in TMW as they are co-prescribed for the treatment of urinary tract infections, middle ear infections, bronchitis, diarrhea, shigellosis, and certain types of pneumonia. SPD is used to treat dermatitis herpetiformis, benign mucous membrane pemphigoid, and pyoderma gangrenosum. Sulfonamides are known to have relatively high levels of environmental (e.g., soil and water) mobility owing to their relatively low sorption potential (Thiele-Bruhn et al., 2004). TMP has also been shown to exhibit low levels of adsorption to environmental media (Lin and Gan, 2011). Owing to their potentially high level of environmental mobility, these compounds are ideal candidates for studying antibiotic transfers through agricultural systems.

2. Methods

2.1. Soil, wastewater, chemicals, earthworms

TMW was collected from a local wastewater treatment plant. This is a state-of-the-art plant employing membrane bioreactor tertiary treatment. The TMW was collected in 4-L amber, glass bottles, which were immediately capped, returned to the laboratory, and stored at 4 °C. This TMW has a pH of 7.37, electrical conductivity of 0.97 dS m^{-1}, and ionic composition (mg L^{-1}) of Ca = 66.0; K = 22.3; Mg = 11.0; Na = 91.0; B = 0.04; Fe = 0.05; Zn = 0.04; PO_4^{3-} = 8.3; SO_4^{2-} = 80.3; Cl = 106.9; $CaCO_3$; NO_3-N = 4.3; and Cu, Mn, Mo = non detectable. Preliminary qualitative analysis of this TMW (see Section 2.8.1) revealed trace levels of the antibiotics TMP, SMX, and SPD; these compounds therefore became the focus of this study. Analytical standards of the antibiotic compounds were purchased from Tokyo Chemical Industry, Japan (SMZ and SPD) or MP Biomedicals, Solon, OH (TMP). The internal standards TMP-$^{13}C_3$ and SMX-$^{13}C_6$ were purchased from Toronto Research Chemicals (Toronto, Canada). Oasis hydrophilic/lipophilic balance (HLB) cartridges (6 mL) were obtained from Waters Corporation, Milford, MA. The soil used in these studies was a sandy loam (75 % sand, 18 % silt, 8 % clay; 0.92 % organic matter; pH 7.1; Arlington series) collected from the upper 30 cm of field 2B of the University of California, Riverside, agricultural station. The earthworms *Eisenia fetida* (Lumbricidae) were purchased from The Worm Farm (www.thewormfarm.net; Durham, CA). Milli-Q water (18.2 μΩ) treated by UV light was used throughout.

2.2. Plant uptake experiments

To mitigate sorptive losses of antibiotics, non-sorbing apparatus (i.e., glass and stainless steel) were used in the pot experiments. As such, glass beakers (1 L) with a 1 cm-diameter hole in their base were used as experimental pots. The holes were covered by a stainless-steel mesh before the pots were filled with 1.527 kg of sandy loam soil pre-adjusted to 10 % gravimetric moisture content. This moisture content was attained by adding either the experimental TMW or Milli-Q (18.2 μΩ) water (depending on the treatment to be imposed; see Table 1) before mixing and passing the soil through a 5-mm sieve. The pots were then transferred to a greenhouse and each was placed in a 500-mL stainless steel dish. The pots were arranged according to a randomized block design with five replicates (A–E) per treatment. The eight treatments are shown in Table 1. The pots were covered with plastic bags and allowed to stand for 48 h before 160 mL of irrigation solution was added to each dish. As shown in Table 1, the irrigation solution was either Milli-Q water (control, CT); as-collected (unspiked) TMW (TMW); TMW spiked at 10 μg L^{-1} with TMP, SMX, and SPD (TMW10); or TMW spiked at 100 μg L^{-1} TMP, SMZ, and SPD (TMW100). The spiked treatments were considered to reflect the antibiotic concentrations of a less-processed TMW, i.e., these concentrations were also considered environmentally relevant. After 24 h, five spinach (*Spinacia oleracea*; cv. Gazelle) or radish (*Raphanus sativus*; cv. Cherry Belle) seeds were placed onto the soil surface and lightly covered with soil. The pots were then irrigated every 2 or 3 days. After germination, the three weakest plants were removed, leaving two plants per pot. All pots were irrigated with the same volume of solution on each occasion (typically 80–100 mL), with a total addition of 1770 mL over the 8 weeks of the experiment. The air temperature within the greenhouse was measured using a fine-wire thermocouple connected to a Campbell Scientific 21 ×

Table 1
Irrigation treatments used in the greenhouse plant uptake experiments.

Abbreviation	Crop	Irrigation*
CT_{Sp}	Spinach	Milli-Q water
TMW_{Sp}	Spinach	TMW
$TMW10_{Sp}$	Spinach	TMW + 10 μg L^{-1} antibiotics
$TMW100_{Sp}$	Spinach	TMW + 100 μg L^{-1} antibiotics
CT_{Rd}	Radish	Milli-Q water
TMW_{Rd}	Radish	TMW
$TMW10_{Rd}$	Radish	TMW + 10 μg L^{-1} antibiotics
$TMW100_{Rd}$	Radish	TMW + 100 μg L^{-1} antibiotics

Note: CT = Non-spiked control; TMW = treated municipal wastewater.

* Two weeks after seed planting, all irrigation solutions were amended with macro- and micro-nutrients through a half-strength Hoagland's solution, ensuring vigorous crop growth.

datalogger, with measurements made every 5 min and averaged over each hour of the experiment.

At the end of the pot experiment, the plants were harvested as their above- and below-ground portions and the fresh weights immediately determined, before being placed into plastic bags and stored at −60 °C. Within 48 h, the plant materials from replicates A–C were triple-rinsed in Milli-Q water, freeze dried, and ground in a mortar and pestle before being placed in glass vials and stored at −60 °C. The bulk soil was homogenized before sub-samples were freeze-dried, ground, and stored in glass vials at −60 °C prior to analysis.

2.3. Earthworm uptake experiments

Following the pot experiment described above, an earthworm (*E. fetida*) microcosm experiment was conducted using the spinach (leaves) and radish (tubers) materials harvested from the earlier pot experiment (replicates D and E combined) as food sources. The microcosm experiments broadly followed OECD Guideline 317 (OECD, 2010). To 150-mL glass beakers, 130 g of clean sandy loam soil (< 2 mm; 12 % moisture content, adjusted using Milli-Q water) was added to achieve approximately 5-cm soil depth. For each of the treatments listed in Tables 1, 0.8 g of finely chopped and homogenized plant material was thoroughly mixed into the soil on Day 0. Additional control (non-spiked) spinach and radish material was also surface-spiked with each of the three antibiotic compounds. This was done by adding dropwise a methanol solution containing the three compounds onto the surface of 0.8 g sub-samples of plant material and allowing the solvent to evaporate for 1 h in a fume hood. On a fresh weight basis, final spiked concentrations of 1 and 10 μg g^{-1} (denoted as SP1 and SP10, respectively) were thus achieved, giving a total of six treatments for each vegetable. Each beaker was then covered with a breathable fabric and placed in a controlled environment room held at 20 °C and 60 % relative humidity, and with a 16/8 h day/night light cycle. The beakers were arranged in a randomized block design with six replicates per treatment. After 1 week, an additional 0.8 g of plant material was added to each beaker together with one earthworm. Adult worms, i.e., with a visible clitellum, were used. The average mass of the added worms was 389 mg (standard deviation = 69 mg; n = 72). The relevant plant material (0.8 g) for each treatment was then added on a weekly basis over a 3-week exposure period. This feeding rate was consistent with the OECD Guideline 317 value of 7 mg food g^{-1} dry soil $week^{-1}$. The moisture content of the soil was maintained by the addition of Milli-Q water every two days. The food material and moisture were added by carefully pouring the soil from the beaker into a clean tray, setting the worm aside, thoroughly mixing in the appropriate amounts of food and water, and then replacing the soil and earthworm back into the original beaker. At these times, visual assessment confirmed that the earthworms consumed all the plant materials added the previous week. Three weeks after earthworm addition, the worms were removed from the soil, washed three times in Milli-Q water, dried by gently patting with an absorbent wipe, weighed, and placed into a petri dish for 24 h to purge their guts (Hartenstein et al., 1981; Arnold and Hodson, 2007). After this time, the worms were removed, re-weighed, and placed in a freezer at −20 °C to induce death. Next, the worms were freeze-dried, weighed again, and ground to a fine powder using a glass rod.

2.4. Plant material extraction

The plant material was extracted based on the method of Zheng et al. (2016). For this, 0.2 g of freeze-dried and ground plant material was weighed into a glass vial and 10 mL of methanol was added. The vials were then sonicated for 20 min (60 Hz) before being centrifuged at 2000 rpm for 20 min. The supernatant was removed into a glass tube before the extraction was repeated twice more to give a final extract volume of 30 mL. The extract was evaporated to dryness (Labconco RapidVap®) and reconstituted in 50 mL of pH-2 Milli-Q water by vortexing for 1 min. The solution was transferred to a polyethylene centrifuge tube and centrifuged at 8000 rpm for 20 min, after which the supernatant was passed through a pre-conditioned (10 mL methanol, 10 mL Milli-Q water, and 10 mL pH-2 Milli-Q water, sequentially) Oasis HLB solid-phase extraction cartridge for sample concentration and clean-up. Milli-Q water (10 mL) was then passed through the cartridge before drying under vacuum for 30 min. The antibiotic compounds were eluted from the cartridge using 10 mL methanol followed by 6 mL of methanol/acetone (1:1 v/v). The eluted solvent was then taken to dryness before reconstitution in 1 mL 10 % methanol and filtration through a 0.2-μm PTFE filter. Then, a 0.5-mL aliquot was transferred to an amber LC vial spiked with 50 μL of 1-μg mL^{-1} internal standard (TMP-$^{13}C_3$ and SMX-$^{13}C_6$). Samples were analyzed by LC–MS/MS (Section 2.8.2). For spinach, the extraction efficiencies of this procedure were determined as 71.4 % (±2.2 %), 59.9 % (±3.6 %), and 55.4 % (±4.4 %) (n = 8) for TMP, SMX, and SPD, respectively; for radish, the values were 81.2 % (±3.8 %), 45.0 % (±4.0 %), and 55.8 % (±2.7 %) (n = 8), respectively. The reported concentrations account for these recoveries.

2.5. Soil extraction

Soils from the pot experiment were extracted by adding 5 mL of methanol to 0.2 g of freeze-dried, ground soil in a glass vial. The vials were sonicated for 20 min (60 Hz) before being centrifuged for 20 min at 2000 rpm. The supernatant was then poured into a glass tube before the process was repeated twice more. The combined supernatant was then taken to dryness before being reconstituted in 1 mL of 10 % methanol and filtered through a 0.2 μm PTFE filter. A 0.5-mL aliquot of the sample was then transferred to an amber LC vial and spiked with 50 μL of a 1-μg mL^{-1} internal standard (TMP-$^{13}C_3$ and SMX-$^{13}C_6$). Samples were analyzed by LC–MS/MS (Section 2.8.2). The extraction efficiencies of this procedure were determined as 67.4 % (±4.7 %), 55.9 % (±2.7 %), and 55.3 % (±3.8 %) (n = 8) for TMP, SMX, and SPD, respectively. The reported concentrations account for these recoveries.

2.6. Earthworm extraction

To ensure sufficient earthworm mass for the extraction, worms were paired to yield three replicates for each treatment. Extraction was conducted by adding 2 mL of methanol to the ground material, sonicating for 20 min, centrifuging at 2500 rpm for 20 min, and removing the supernatant into a glass test tube. This was repeated twice more to give a final volume of 6 mL, which was then taken to dryness. Next, 10 mL of Milli-Q water at pH 2 was added to the dry tubes, which were then capped and vortexed for 1 min. The redissolved mixture was transferred to a polyethylene centrifuge tube and centrifuged at 8000 rpm for 20 min. The supernatant was then passed through a pre-conditioned solid phase extraction cartridge (Oasis HLB) for clean-up, followed by 10 mL of Milli-Q water and vacuum drying for 30 min. The antibiotic compounds were then eluted with 10 mL methanol. The methanol was taken to dryness before being reconstituted in 1 mL 10 % methanol. The 1-mL sample was then transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 20 min, followed by filtration through a 0.2-μm PTFE filter. An aliquot of 0.5 mL was then transferred to an amber LC vial spiked with 50 μL of a 1-μg mL^{-1} internal standard (TMP-$^{13}C_3$ and SMX-$^{13}C_6$). Samples were analyzed by LC–MS/MS (Section 2.8.2). The extraction efficiencies of this procedure were determined as 109.1 % (±3.5 %), 67.8 % (±4.5 %), and 65.0 % (±4.6 %) (n = 8) for TMP, SMX, and SPD, respectively. The reported concentrations account for these recoveries.

2.7. Determination of antibiotic degradation half-lives in the experimental soil

Antibiotic degradation experiments were conducted using the sandy loam soil. In 10-mL glass vials, an aqueous solution containing TMP, SMX, and SPD was added to 5 g of soil. The final soil concentration of each antibiotic was 100 ng g^{-1} and the final soil moisture content was 12 %. The vials were placed in an incubator at 25 °C, with triplicate vials moved to a −60 °C freezer (to prevent any further degradation of the compounds) at 0, 1, 2, 3, 5, 7, 10, and 14 days. The samples were then freeze

dried, ground, extracted using 10 mL of methanol as described above (Section 2.6), and analyzed by LC–MS/MS (Section 2.8.2). Losses of antibiotics over time were fitted with a first-order kinetic model to determine the half-life for each compound.

2.8. Antibiotic compounds analyses

2.8.1. Qualitative analysis by high resolution LC–MS/MS

For qualitative antibiotic identification, triplicate samples of the as-collected TMW (500 mL each) were passed through pre-conditioned HLB cartridges (~5 mL min^{-1}) for concentration and clean-up. The cartridges were then eluted with 10 mL methanol, which was dried under nitrogen and redissolved in 1 mL methanol prior to filtration (0.2 μm) and qualitative analysis using ultra high-performance liquid chromatography (UHPLC) coupled with high-resolution mass spectrometry (HRMS) (Q Exactive, Thermo Fisher Scientific). Briefly, 20 μL sample was loaded onto a C_{18} Atlantis-T3 column (particle size 3 μm, 3.0 × 150 mm) (Waters, Milford, CA) and eluted with nanopure water +0.1 % formic acid (A) and acetonitrile +0.1 % formic acid (B) at a gradient of 5 % B (0–1 min); 5–100 % B (1–8 min); 100 % B (8–20 min); 5 % B (21–26 min) at a flow rate of 0.35 mL min^{-1}. HRMS was equipped with electrospray ionization and acquired full-scan mass spectra at an *m/z* range of 100 to 1500 in positive and negative modes with a resolution of 70,000 at 200 *m/z*. Data-dependent tandem mass spectra were obtained at the exact masses of all investigated antibiotics with a normalized collision energy of 25. A previously compiled suspect list of contaminants of emerging concern, including antibiotics, was used to screen antibiotic residuals in the TMW (Xing et al., 2018). A previously reported suspect screening workflow was used to characterize the occurrence of antibiotic residuals (Xing et al., 2018). Briefly, suspect hits were acquired from full scan mass spectra using TraceFinder v4.1 EFS software (Thermo Fisher Scientific) against the self-compiled suspect database using exact masses of $[M + H]^+$ and $[M\text{-}H]^-$ in positive and negative modes, respectively. Suspect hits were identified using the following criteria: i) mass tolerance <5 ppm, ii) isotopic pattern score > 70 %, iii) signal intensities above 1×10^6, and iv) signal-to-noise ratio > 300. The structures of the suspect hits were further confirmed by comparing the MS2 profiles to those in the MassBank database (Xing et al., 2018).

2.8.2. Quantitative analysis by triple quadrupole LC–MS/MS

Quantitative analyses was conducted using an Agilent 1290 Infinity II HPLC coupled with an Agilent 6410B triple quadrupole MS/MS detector equipped with an electrospray ionization (ESI) interface. Waters Acquity UPLC C_{18} columns were used (guard column: 1.7 μm, 2.1 mm × 5 mm; analytical column: 1.7 μm, 2.1 mm × 50 mm) at a flow rate of 0.3 mL min^{-1}. The column temperature was set at 45 °C and the injection volume was 10 μL. Formic acid (0.1 %) was used as mobile phase A and acetonitrile as mobile phase B, with gradient conditions set as follows: 0–1 min 95 % A, 5 % B; 1–10 min ramp decrease 10 %/min A, ramp increase 10 %/min B, reaching 5 % A, 95 % B at 10 min; 10–15 min 5 % A, 95 % B; 15–20 min 95 % A, 5 % B. All analytes were analyzed in positive ion mode. Nitrogen from a nitrogen generator was used as the drying and nebulizing gas, and high purity nitrogen gas (99.999 % purity) was used as the collision gas. The conditions of the mass spectrometer were as follows: gas temperature, 325 °C; dry gas flow, 12 L min^{-1}; nebulizer, 50 psi; capillary voltage, 4000 V. Under these conditions, the retention times of TMP, SPD, and SMZ were 5.248, 5.288, and 6.694 min, respectively. Collection and treatment of data was performed using Mass Hunter software (Version B.01.04). Internal standards (TMP-$^{13}C_3$ and SMX-$^{13}C_6$) were added to the samples and calibration standards prior to analysis to account for matrix effects. Based on the compound retention times, TMP-$^{13}C_3$ was utilized as an internal standard for TMP and SPD analyses, whereas SMX-$^{13}C_6$ was used for SMX. Based on a serial dilution, the limit of quantitation (LOQ) was taken as 0.5 ng mL^{-1} for each compound. The limit of detection, determined as three times the standard deviation of 10 replicates at the LOQ concentration, was 0.1 ng mL^{-1} for TMP and SPD and 0.2 ng mL^{-1} for SMZ.

3. Results

3.1. Plant uptake experiments

During the greenhouse pot experiment, the hourly-averaged air temperature in the greenhouse ranged from 8.79 °C to 25.36 °C, with the lowest temperature typically observed at 7 am and the highest at 1 pm. The average temperature across the entire study period was 16.80 °C (standard deviation = 2.77 °C).

3.1.1. Wastewater and soil antibiotic concentrations

Prior to the experiment, the concentrations of TMP, SMZ, and SPD in the as-collected TMW were determined as 67 (±14), 285 (±11), and 77 (±3) ng L^{-1}, respectively. These concentrations were determined again at weeks 4 and 8 (i.e., the middle and end of the experiment) and were found not to have changed significantly over time relative to the initial concentrations (analysis of variance; $p > 0.05$ in each case). For the pot experiment, antibiotic transfers from the irrigation waters to the soil are shown as measured soil concentrations in Table 2. As expected, the compounds were not detected in the control treatments (irrigated with Milli-Q water). The compounds were also non-detectable in the TMW treatment, where antibiotic inputs to the soil from the wastewater were low (see "nominal" concentrations in Table 2, which were calculated based on the initial antibiotic

Table 2
Nominal (calculated) and measured concentrations (ng g^{-1}) of trimetropin (TMP), sufamethoxazole (SMZ), and sulfapyridine (SPD) in soil at the end of the spinach (Sp) and radish (Rd) pot experiments irrigated with unspiked control (CT) water, unspiked treated municipal wastewater (TMW), or TMW spiked with antibiotics at 10 μg L^{-1} (TMW10) or 100 μg L^{-1} (TMW100).

	TMP		SMZ		SPD	
	Theoretical*	Measured#	Theoretical*	Measured#	Theoretical*	Measured#
CT Sp	0.00	n.d.	0.00	n.d.	0.00	n.d.
CT Rd	0.00	n.d.	0.00	n.d.	0.00	n.d.
TMW Sp	0.06	n.d.	0.05	n.d.	0.01	n.d.
TMW Rd	0.06	n.d.	0.05	n.d.	0.01	n.d.
TMW10 Sp	8.72	3.70 (1.50)	1.77	0.08 (0.01)	1.74	0.06 (0.03)
TMW10 Rd	8.72	5.59 (0.53)	1.77	0.06 (0.04)	1.74	0.04 (0.02)
TMW100 Sp	87.2	24.11 (5.87)	17.7	2.35 (1.15)	17.4	0.37 (0.11)
TMW100 Rd	87.2	68.66 (2.68)	17.7	1.31 (0.29)	17.4	1.33 (0.52)

Note: Sp = spinach-planted treatment, Rd = radish-planted treatment, n.d. = non-detectable concentration. Standard deviations of measured values (n = 3) shown in parentheses.

Measured concentrations are for methanol extraction (total).

* Nominal values were calculated from antibiotic concentration in the irrigation water and added volume of irrigation water, followed by degradation correction to the end of the experiment (using $C_t = C_0 \times e^{-\lambda t}$, where C_0 is the original concentration at time of addition, C_t is the concentration at time t, λ is the first-order degradation rate constant for the compound [$\lambda = \ln(2) / t½$], and t is the elapsed time between the addition and the end of the experiment).

concentrations in the wastewater and the volumes of wastewater added, followed by degradation correction to the end of the experiment based on the measured half-life of each compound). In the TMW10 and TMW100 treatments, each compound was readily detected; however, the concentrations (particularly of SMZ and SPD) were found to be markedly lower than the nominal values. Indeed, the average measured concentrations were 2×, 18×, and 33× lower than the calculated nominal concentrations for TMP, SMZ, and SPD, respectively. The TMW100 treatment led to 6–33× increases in soil antibiotic concentrations when compared with the TMW10 treatment. Typically, the order of decreasing soil concentrations was TMP > SMZ > SPD. Differences in the soil concentrations of each compound were also observed between the radish and spinach treatments, although no consistent trend was noted.

3.1.2. Plant uptake of antibiotics

The measured plant (edible and non-edible portions) antibiotic concentrations at harvest are given in Table 3. Unsurprisingly, given the non-detectable soil concentrations in the unspiked CT and TMW treatments (Table 2), no antibiotics were detected in any plant organs (edible or non-edible portions) in these treatments. Nevertheless, the potential for antibiotics uptake from the soil by the plants is evidenced by the measured plant material concentrations in the antibiotic-spiked treatments TMW10 and TMW100. As would be expected, the TMW100 treatment led to greater plant concentrations (5–48× greater) across all compounds when compared with TMW10. In most cases, the order of decreasing plant concentrations was TMP > SMX > SPD, although exceptions were found for the spinach roots. In all but one case, in both the edible and non-edible portions, spinach exhibited much greater concentrations of each compound than did radish. The exception was TMP concentration in the non-edible portions for the TMW100 treatment (radish > spinach). Comparing those treatments where plant uptake was observed (TMW10 and TMW100), two-factor analysis of variance indicated significant differences ($p < 0.05$) in plant uptake between the spinach and radish treatments ($p = 0.006$ for TWM10 and $p = 2.8 \times 10^{-9}$ for TMW100) and between the three antibiotic compounds ($p = 0.02$ for TMW10 and $p = 8.4 \times 10^{-9}$ for TMW100).

Soil–plant bioaccumulation factors (BAFs) and root–shoot translocation factors (TFs) are shown in Table 4 for TMW10 and TMW100. Both sets of values, but particularly the BAFs, were greater for spinach than for radish across all three compounds. Indeed, for radish, the BAFs are considered very low across all compounds. The BAFs for SMZ and SPD were similar in magnitude for an individual crop, ranging from 46 to 162 for spinach and 1.2 to 3.0 for radish. Despite the much greater TMP concentrations measured in the plant material as compared with SMZ and SPD (Table 3), the BAFs for TMP were much lower than those of the other two compounds within a treatment for radish and, particularly, spinach. In contrast, the root–shoot TF value was much greater for TMP than for either SMZ or SPD within a treatment, with the overall trend following the order TMP > SMZ > SPD in each case. The SMZ and SPD TF values were generally very low (i.e., <1) across both vegetables, with the only exception being SMZ in the TMW10 spinach treatment (TF = 3.4). The TF values were consistently greater for spinach than for radish.

Table 3
Measured antibiotic concentrations (ng g^{-1}, dry weight basis) in spinach and radish organs from the pot experiment irrigated with unspiked control (CT) water, unspiked treated municipal wastewater (TMW), or TMW spiked with trimethoprim (TMP), sulfamethoxazole (SMZ), and sulfapyridine (SPD) at 10 μg L^{-1} (TMW10) or 100 μg L^{-1} (TMW100).

			TMP	SMZ	SPD
Edible portions	Spinach leaves	CT	n.d.	n.d.	n.d.
		TMW	n.d.	n.d.	n.d.
		TMW10	40.95 (0.32)	20.22 (0.88)	2.04 (0.83)
		TMW100	429.73 (45.41)	108.54 (35.12)	37.84 (5.87)
	Radish tubers	CT	n.d.	n.d.	n.d.
		TMW	n.d.	n.d.	n.d.
		TMW10	0.69 (0.09)	0.17 (0.11)	0.11 (0.06)
		TMW100	33.54 (17.64)	2.61 (0.40)	1.53 (0.05)
Non-edible portions	Spinach roots	CT	n.d.	n.d.	n.d.
		TMW	n.d.	n.d.	n.d.
		TMW10	3.88 (1.05)	3.98 (0.54)	3.54 (2.09)
		TMW100	28.44 (3.44)	163.39 (52.06)	83.68 (18.06)
	Radish leaves	CT	n.d.	n.d.	n.d.
		TMW	n.d.	n.d.	n.d.
		TMW10	2.82 (1.11)	0.13 (0.11)	0.03 (0.01)
		TMW100	83.34 (29.31)	0.95 (0.10)	0.51 (0.07)

Note: n.d. = non-detectable concentration. Standard deviation (n = 3) shown in parentheses.

Table 4
Antibiotic bioaccumulation factors (BAFs) and translocation factors (TFs) in the greenhouse pot experiment.

		Soil–edible portion BAF			Root–shoot TF		
		TMP	SMZ	SPD	TMP	SMZ	SPD
Spinach	CT	–	–	–	–	–	–
	TMW	–	–	–	–	–	–
	TMW10	17.2	162.4	62.2	16.4	3.4	1.1
	TMW100	17.8	46.2	103.1	15.1	0.7	0.5
Radish	CT	–	–	–	–	–	–
	TMW	–	–	–	–	–	–
	TMW10	0.1	3.0	2.4	4.1	0.7	0.3
	TMW100	0.5	2.0	1.2	2.5	0.4	0.3

3.2. Earthworm uptake experiments

The antibiotic concentrations in earthworm tissues after the 3-week exposure period are shown in Fig. 1. When radish and spinach from the CT, TMW, and TMW10 pot experiment treatments were used as food, no antibiotics were detected in earthworms. However, in the TMW100, SP1, and SP10 treatments, antibiotic accumulation was observed to varying degrees in earthworm tissues, with the three compounds having differing behaviors. For TMP, the uptake concentrations of spinach-fed worms across the treatments followed the order SP10 > TMW100 > SP1, whereas for radish-fed worms it was SP10 > SP1 > TMW100. In the TMW100 treatment, the TMP concentration in spinach-fed worms was greater than that of the radish-fed worms; however, the opposite was true in the SP1 and SP10 treatments. For SMZ, all treatments resulted in greater earthworm concentrations for spinach-fed worms than for radish-fed worms. For spinach-fed worms, the order of decreasing SMZ concentrations followed the order SP10 > TMW100 > SP1, whereas for radish-fed worms, the order was SP10 > SP1 > TMW100 (i.e., the same trends shown for TMP). For SPD, no antibiotic accumulation was observed in the TMW100 treatment for either the spinach- or radish-fed worms. The SPD concentrations followed the order SP10 > SP1 > TMW100 for both spinach- and radish-fed worms. The maximum average concentrations of TMP, SMZ, and SPD were 43 ng g^{-1} (radish-fed), 53 ng g^{-1} (spinach-fed), and 66 ng g^{-1} (spinach-fed), respectively (each observed in the SP10 treatment). The increase in vegetable antibiotic concentrations from SP1 to SP10 resulted in 2.0–14.7× (average 6.2×) increases in earthworm concentrations across all compounds.

The relation between the matrix (i.e., soil/plant material mixture) concentrations and the earthworm concentrations was considered using bioconcentration factors (BCFs). Overall, these values were low (Fig. 1), especially in spiked treatments (SP1 and SP10) where they ranged from 0.12 to 1.20 (average 0.39); in the TMW100 treatment, they ranged from 0 to 6.57 (average 1.82). The largest BCF values were observed for the TMW100 treatment: BCF = 6.57 for SMZ in spinach-fed worms, BCF = 3.15 for TMP in radish-fed worms, and BCF = 2.11 for TMP in spinach-fed worms. All other values were either very close to, or below, unity.

Mass balances for the experiments are shown in Table 5, indicating the distribution of each antibiotic among the various compartments as a percentage of the mass added. Any unaccounted-for mass was assumed to have degraded. Since no antibiotics were detected in the soil, plant, and earthworm compartments in the TMW treatment, the entire (albeit low)

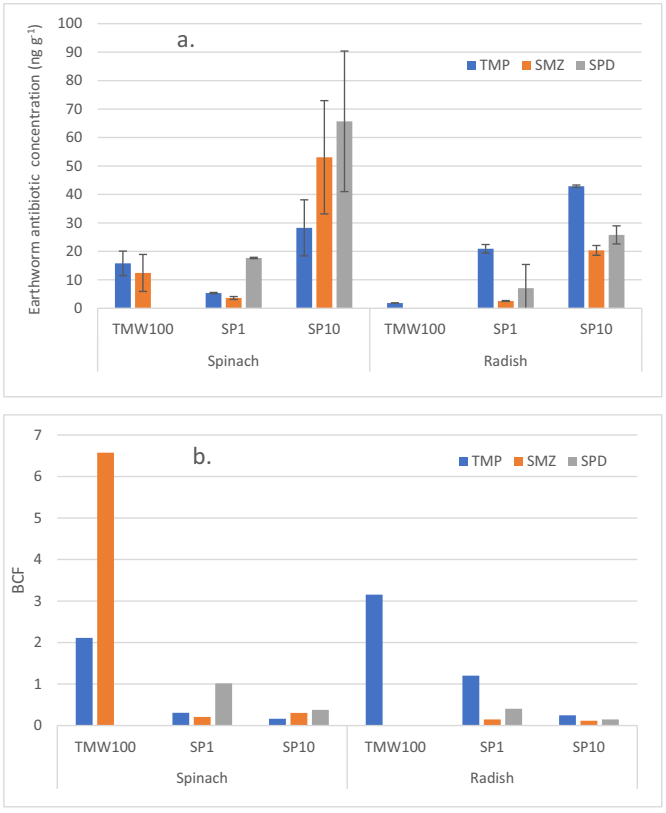

Fig. 1. (a) Earthworm tissue concentrations and (b) soil/vegetable mixture–earthworm bioconcentration factors (BCFs).

added mass of each compound was assumed to have degraded. In TMW10 and TMW100, SMZ and SPD were found to have largely degraded (97.4 to 99.6 %), given the relatively small percentages observed in the soil, plant, and earthworm compartments. In contrast, TMP was found in relatively large quantities in the soil compartment (24.6 to 70.1 %), although the percentages in the plant and earthworm compartments were again relatively low.

4. Discussion

In concurrence with the results of Al-Farsi et al. (2018), our results established that use of the TMW did not lead to detectable levels of antibiotics in the irrigated soils, i.e., no difference from the control. The reason for this is that the TMW was collected from a modern state-of-the-art facility that utilizes a membrane bioreactor for tertiary water treatment. Such treatment technologies are known to produce high-quality effluent with very low contaminant levels (Phoon et al., 2020). Thus, the trace amounts of antibiotics added to the pot soils irrigated with the TMW were likely diluted by the soil mass, as well as potentially degraded by chemical and biological processes in the soil, yielding non-detectable final soil concentrations. In contrast, when the irrigation wastewater was amended with 10 and 100 μg L^{-1} antibiotics (treatments TMW10 and TMW100), marked increases in soil antibiotic concentrations were found. Differences in soil concentrations among the compounds can be accounted for by their degradation half-lives, as determined in the batch degradation studies, as TMP = 115.5 d, SMZ = 5.26 d, and SPD = 5.16 d. As such, the much slower degradation of TMP led to much greater final soil concentrations than those for the faster degrading SMZ and SPD. However, even when taking into account the expected degradation losses of the compounds, it is evident that the measured soil concentrations in the TMW10 and TMW100 treatments were far lower than the theoretical values (Table 2). Since the additional losses cannot be entirely accounted for by plant uptake (Table 5 and below), we hypothesize that the compounds were actually degraded at a faster rate than that indicated by the preliminary batch studies. For example, photodegradation of the compounds in the soil was much more likely in the pot experiment (conducted in a greenhouse) than it was in the preliminary batch degradation study (conducted under dark conditions). Moreover, in the pot experiment, the presence of roots within the soil may have increased the rate of degradation of the compounds, perhaps owing to rhizosphere microbial activity and/or chemical interactions with root exudates. This process may also help explain the differences in soil concentrations between the radish and spinach treatments as the two crops have markedly different root structures and densities. Based on the relative differences between theoretical and measured concentrations, these processes seemingly impacted the compounds to differing extents, i.e., SPD was most affected, followed by SMZ and then TMP.

The measured final soil concentrations offer a highly plausible general explanation for the observed plant uptake patterns of the three compounds in the TMW10 and TMW100 treatments. As noted by Carter et al. (2021), the lack of uptake of some pharmaceuticals can be well explained by their degradation in soils: in cases where the dissipation of a compound is relatively rapid, its diminishing concentration in the soil matrix results in a smaller fraction being available for plant uptake (Boxall et al., 2006; Carter et al., 2014). As such, where degradation was slow and the soil concentrations remained relatively high (i.e., TMP), plant uptake and accumulation were consequently also relatively high; whereas SMZ and SPD uptake had relatively low uptake and accumulation owing to their faster dissipation and lower concentrations within the soil. This is also supported by the trend in decreasing plant concentrations matching that observed for soils, i.e., TMP > SMZ > SPD.

The relationship between soil and plant concentrations is more meaningfully expressed as the BAF, in which a value >1 is indicative of bioaccumulation. As such, in all cases except TMP in radish, antibiotic bioaccumulation was observed in the edible plant portions. The BAF values ranged from 0.1 to 3.0 in radish and 17.2 to 162.4 in spinach. For TMP and SMZ, Christou et al. (2017) reported BAF values of 0.18 to 6.44 and 0.47 to 5.41, respectively, for tomato fruits, which are comparable to the values found herein for radish. However, Wu et al. (2014) found that these compounds were not transferred (i.e., BAF = 0) from soil to various vegetables, e.g., celery, lettuce, cabbage, spinach, carrot, cucumber, bell pepper, and tomato. Similarly, Goldstein et al. (2014) found that SMZ and SPD were not

Table 5
Mass balances for the experimental systems expressed as a percentage of the mass of each antibiotic added.

		TMW			TMW10			TMW100		
		TMP	SMZ	SPD	TMP	SMZ	SPD	TMP	SMZ	SPD
Spinach	Soil	0.00 %	0.00 %	0.00 %	37.56 %	0.83 %	0.62 %	24.60 %	2.39 %	0.37 %
	Plant	0.000 %	0.000 %	0.000 %	0.771 %	0.199 %	0.059 %	0.541 %	0.165 %	0.056 %
	Earthworm	0.000 %	0.000 %	0.000 %	0.000 %	0.000 %	0.000 %	0.007 %	0.006 %	0.000 %
	Degraded*	100.00 %	100.00 %	100.00 %	61.67 %	98.97 %	99.32 %	74.85 %	97.44 %	99.57 %
Radish	Soil	0.00 %	0.00 %	0.00 %	56.70 %	0.56 %	0.46 %	70.06 %	1.34 %	1.36 %
	Plant	0.000 %	0.000 %	0.000 %	0.033 %	0.005 %	0.003 %	0.119 %	0.006 %	0.004 %
	Earthworm	0.000 %	0.000 %	0.000 %	0.000 %	0.000 %	0.000 %	0.001 %	0.000 %	0.000 %
	Degraded*	100.00 %	100.00 %	100.00 %	43.26 %	99.43 %	99.54 %	29.82 %	98.66 %	98.64 %

* The degraded mass was assumed based on the difference between the mass added and the amount found in each compartment.

transferred from soil to cucumber and tomato. Therefore, compared with other studies, our data indicate very high levels of TMP, SMZ, and SPD uptake by spinach in our study. We believe this may have been in part due to the optimal nutrient amendment of the TMW and the consequential rigor of plant growth, both above and below ground, which would have stimulated large transpiration fluxes and hence root uptake. The low concentration of organic matter content of our soil may also have increased compound availability for plant absorption.

The consistently higher level of uptake by spinach as compared with radish is considered a result of the extent of overlap between the plant roots and antibiotic soil distribution. Based on visual assessment, the spinach roots explored the entire soil mass, whereas a single tap root with only a small number (<10) of short (<20 mm) lateral roots extended from the radish tuber; therefore, the spinach roots were likely subject to much greater exposure to the antibiotic compounds. Overall, the BAFs showed the opposite pattern to uptake concentrations, i.e., sulfa-compounds > TMP. Therefore, although the TMP plant concentrations were higher, the sulfa-compounds were taken up more efficiently by the plants despite exhibiting lower soil concentrations. This was likely due to differences in the partitioning of the compounds into the solution phase. For example, the solid–liquid partitioning coefficients (K_d values; determined in preliminary experiments) for SMZ and SPD (1.32 ± 0.55 mL g^{-1} and 1.03 ± 0.45 mL g^{-1}, respectively) were much lower than that of TMP (28.92 ± 4.01 mL g^{-1}), which would be expected to facilitate root uptake of the sulfonamides relative to TMP. Being ionic compounds, these antibiotics may exist as various species with different charges depending on soil pH. At the prevalent pH of the experimental soil (pH = 7.2), TMP would be expected to exist as approximately equal fractions of cationic and neutral species, whereas SMX and SPD would be expected to exist mostly in anionic forms (Kocarek et al., 2016). On this basis, SMZ and SPD would partition much more readily into the liquid phase (owing to repulsion from the negatively charged soil surfaces) where they would be available for root uptake. In contrast, cationic TMP species would be expected to adsorb to the soil surfaces and possibly exterior root surfaces (Keerthanan et al., 2020; Zheng and Guo, 2021) via ion exchange processes and thus be relatively less available to the plant. Collectively, these sorption and speciation characteristics likely facilitated the higher BAFs found for the sulfonamides; however, their rapid degradation in the soil clearly limited their overall uptake (i.e., low plant tissue concentrations).

Transfers of contaminants from the roots of a plant to the above-ground portion can be characterized by TFs, with values of <1 indicating a relative accumulation of the antibiotics in the roots, i.e., the compounds are not effectively translocated to the above-ground biomass. In general, limited translocation was observed for SMZ and SPD in the current study. Low or absent root-to-shoot translocation has been observed previously for several pharmaceutical compounds (Herklotz et al., 2010; Eggen et al., 2011) including sulfonamide antibiotics (Migliore et al., 1995; Pan et al., 2014; Kurwadkar et al., 2017). The translocation of antibiotic compounds within a plant can be explained by movement of the chemicals in the plant xylem—from the roots upwards toward aerial tissues—which is driven by the transpiration stream (Li et al., 2018). Differences in translocation between compounds within the same system (i.e., the same transpiration stream) may therefore be a result of the chemical properties of the compounds and their interactions with plant cells (Pan et al., 2014; Carter et al., 2021). For example, Michelini et al. (2014) reported that the localization of sulfonamide compounds in roots could be due to possible metabolic transformation of the compounds or to protein binding in the roots. In contrast, TMP appeared to be readily translocated to the shoots, especially in spinach, suggesting that the chemical processes limiting the translocation of SMZ and SPD may not have impacted TMP in the same way, i.e., TMP was readily transported with the xylem flux. Consistently, the TF values were greater for spinach than radish (especially for TMP), which is likely a result of differences in the above/below ground biomass ratios of the two vegetables, which were 11.06 (±2.90) for spinach and just 0.26 (±0.08) for radish; therefore, a greater upward flux to the above ground biomass in spinach likely facilitated root-to-shoot translocation of the antibiotics.

No antibiotics were detected in the earthworms in the control and TMW10 treatments. In the TMW100 treatment, the tissue concentrations of the earthworms were relatively low (non-detectable to 15.8 ng g^{-1}), whereas in the spiked vegetable treatments (SP1 and SP10) the earthworm tissue concentrations ranged from 2.6 to 65.7 ng g^{-1}. In general, a low vegetable antibiotic concentration led to non-detectable levels in earthworm tissues. Our data indicate that antibiotic compounds can be transferred from vegetable material to animal tissues, provided that their concentrations in the food source are relatively high. There is a paucity of information on the uptake of antibiotics (and other organic compounds) from vegetable material into earthworms. Typically, earthworm uptake experiments have focused on transfers from contaminated soils, and although such research has been conducted for a range of pharmaceutical compounds, there is again a lack of information for antibiotics. For earthworm uptake of pharmaceuticals from soils, Carter et al. (2016) reported BCFs of 7.02–69.57 for diclofenac, 30.50–115.88 for orlistat, 14.09–20.42 for fluoxetine, and 1.05–1.61 for carbamazepine, which are typically greater than the BCF values found herein (0.12–6.57) for earthworm uptake from the soil/vegetable mixtures. As such, it is evident that the antibiotics overall showed a relatively low propensity for bioaccumulation in earthworm tissues. Interestingly, the BCF values for the spiked treatments (SP1 and SP10) were much lower than those for the TMW100 treatment, suggesting that when the compounds were taken up into the plant tissues (rather than being spiked onto the exterior surfaces), they were more bioavailable to the worms. Indeed, this treatment resulted in the largest BCF values: BCF = 6.57 for SMZ and BCF = 2.11 for TMP (both in spinach-fed worms) and BCF = 3.15 for TMP in radish-fed worms. Although these values are relatively low compared to those for many other organic compounds (Carter et al., 2016), they demonstrate a potential for these antibiotic compounds in vegetable materials to be transferred to, and in certain cases bioaccumulate in, animal tissues, i.e., to pass between trophic levels. To our knowledge, this is the first study to provide experimental data on this pathway for antibiotic compounds.

The presence of antibiotic-resistance determinants in food chains is currently a major safety concern; therefore, understanding how antibiotic compounds are transferred through such systems is important as this may be a key driver of resistance development within each compartment. Importantly, our data suggest that the use of a highly processed (e.g., membrane bioreactor-treated) wastewater for crop irrigation likely limits the dissemination of antibiotics through agricultural systems by reducing the initial input in the irrigation water. However, it was also apparent that the use of wastewater with higher (spiked) antibiotic concentrations led to significant antibiotic inputs to soil, which, in some cases, resulted in transfers through the wastewater–soil–plant–earthworm continuum. Therefore, less processed TMWs, with relatively high antibiotic concentrations (e.g., >10 μg L^{-1}), may represent a risk to agricultural systems. High initial inputs, slow soil degradation, and chemical speciation of the antibiotics, coupled with an extensive plant root distribution, were seemingly important factors enhancing the uptake of antibiotics into crops. Transfers from vegetable materials into earthworms were low but showed a potential for bioaccumulation depending on the concentration of the antibiotics in the TMW. Although the masses of antibiotics transferred through this continuum were very small relative to the amounts added via the (spiked) TMWs (Table 5), it is noted that small and consistent inputs of antibiotics to a biological compartment may be sufficient to induce the development of antibiotic resistance. While far beyond the scope of the current study, the potential for antibiotic transfers across trophic levels may have important implications for human health risks when associated with high concentrations of antibiotics in irrigation water and the development of antibiotic resistance.

CRediT authorship contribution statement

Daniel J. Ashworth: Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – original draft. **Abasiofiok M. Ibekwe:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Yujie Men:** Conceptualization, Funding acquisition,

Resources, Writing – review & editing. **Jorge F.S. Ferreira:** Conceptualization, Funding acquisition, Resources, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

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

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