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1 **Uptake of antibiotics and their toxicity to lettuce following routine irrigation with**
2 **contaminated water in soils with increasing sand content**

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17 Antibiotic, uptake, toxicity, soil texture, water reuse

18 **Running Title**

19 Antibiotic uptake and toxicity to lettuce

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Abstract

To address the issue of global freshwater shortages, wastewater has become an increasingly valuable alternative for crop irrigation. As a result, trace levels of emerging contaminants, including antibiotics, may occur in water used for food production. The objective of this study was to investigate how soil texture affected the availability and uptake of three chemically diverse antibiotics (lincomycin, oxytetracycline, and sulfamethoxazole) by lettuce grown in soils comprised of a silt clay and increasing percentages of sand. Lettuce was irrigated routinely with antibiotic amended water (1 mg/L) from seed germination through the first harvest (40 days), switched to control water, and fate monitored at day 45 and 50. Sulfamethoxazole was the only compound where tissue concentrations increased with increasing sand concentrations to 24.7 ng/g fresh weight (FW). Lincomycin was most readily accumulated with increasing concentrations observed at the second harvest in both the loam (68.3 ng/g FW) and sandy soils (66.6 ng/g FW). Apparent toxicity of the antibiotic mixture resulted in decreasing plant mass (37-72 %) with increasing sand content. Results from this study show that soil texture impacts plant growth, contaminant transport, plant uptake, and toxic effects, which all contribute to, observed concentrations in edible plant portions.

1 Introduction

2 Wastewater is an increasingly valuable resource as a potential alternative to freshwater
3 owing to the fact that population growth and climate change have depleted water supplies
4 necessary for crop irrigation (Boxall, 2010; Monteiro and Boxall, 2010; Michael *et al.*, 2013).
5 The growing use of wastewater for crop irrigation coupled with increasing use of
6 pharmaceuticals, such as antibiotics, increases the potential for agroecosystem contamination by
7 these emerging contaminants (Toze, 2006; Du and Liu, 2012; Williams-Nguyen *et al.*, 2016).
8 While the practice of wastewater reuse for agriculture has long been implemented in Israel,
9 Jordan, Peru, and Saudi Arabia (WHO, 1989; Azov and Shelef, 1991), its increasing acceptance
10 is demonstrated by recent studies evaluating its viability in other regions including India (Salidas
11 *et al.*, 2015), Tanzania (Kahila *et al.*, 2014), and Vietnam (Trinh *et al.*, 2013). Typically, some
12 form of treatment is recommended prior to use of recycled wastewater; however, it has been
13 estimated that 20 million hectares of agricultural land is irrigated directly with untreated
14 wastewater (WHO, 2006). In some cities in developing countries, up to 60 % of vegetables
15 consumed locally have been grown with untreated wastewater which was valued significantly
16 higher than traditional sources of irrigation water by area farmers (Ensick and vander Hoek,
17 2007). Once introduced into the agroecosystem, pharmaceutical contaminants present in
18 wastewater are capable of transport and uptake into plants (Thiele-Bruhn, 2003; Fatta-Kassinos
19 *et al.*, 2011; Pan and Chu, 2017; Sallach *et al.*, 2015).

20 In the case of treated wastewater recycling, treatment technologies are not entirely
21 effective for removal of these chemicals. The efficacy in removal of antibiotics from wastewater
22 in the treatment process is dependent on physicochemical properties, which vary considerably
23 between antibiotic compounds. The result is a range of removal efficiencies from 4 % for

1 oxytetracycline to 100 % for sulfadimethazine (Verlicchi *et al.*, 2012). However, one of the
2 primary advantages in wastewater reuse is the recycling of nutrients, otherwise removed, with
3 great expense, in the treatment process (Duran-Alvarez and Jimenez-Cisneros, 2014). Life cycle
4 assessment studies have evaluated the use of wastewater management strategies that include the
5 separation and application of toilet fractions, a source of pharmaceutical contamination in raw
6 municipal wastewater, to agricultural applications with minimal treatment (Spangberg *et al.*,
7 2014). Furthermore, management practices associated with concentrated animal feeding
8 operations (CAFOs) often involve the application of highly contaminated wash and runoff water
9 to agricultural lands. While some regulation exists regarding treatment requirements necessary
10 for the reuse of wastewater, including recent EU regulations on the topic (European
11 Commission, 2016), they have traditionally focused on nutrient management rather than
12 contaminant control, with very limited consideration of emerging contaminants including
13 antibiotics and resulting antibiotic resistance (Paranychianakis *et al.*, 2015). As a result,
14 agricultural wastewater reuse provides an additional pathway for antibiotics and other
15 pharmaceutical contaminants to move within the agroecosystem (Bradford *et al.*, 2008).

16 The combination of direct irrigation with untreated wastewater, insufficient management
17 of agricultural wastewater, and the potential for nutrient reuse in municipal sourced wastewater
18 may lead to increased exposure of pharmaceutical contamination, greater than the levels
19 typically observed in wastewater treatment effluent. For example, antibiotics in raw agricultural
20 wastewater have been detected at mg/L levels (Zilles *et al.*, 2005, Bartelt-Hunt *et al.*, 2011), with
21 concentrations as high as 20 mg/L in wastewater lagoons (Peak *et al.*, 2007).

22 Hydroponic studies, where plants are exposed to antibiotics in a nutrient solution, have
23 been conducted to characterize the mechanisms of root uptake and translocation of compounds in

1 staple vegetables (Chuang *et al.*, 2015; Herklotz *et al.*, 2010; Liu *et al.*, 2013; Wu *et al.*, 2013).
2 Incorporating soil-compound interactions and bioavailability, uptake from spiked soil regimes
3 has also been investigated (Boxall *et al.*, 2006; Hawker *et al.*, 2013; Carter *et al.*, 2014, Chung *et*
4 *al.*, 2017). Uptake resulting from other known exposure routes including the land application of
5 manure (Kumar *et al.*, 2005; Dolliver *et al.*, 2007; Kang *et al.*, 2013) and municipal biosolids
6 (Wu *et al.*, 2010; Holling *et al.*, 2012; Sabourin *et al.*, 2012; Wu *et al.*, 2015), as well as
7 irrigation with contaminated water at concentrations representing various degrees of treatment
8 (Azanu *et al.*, 2016; Jones-Lepp *et al.*, 2010; Tanoue *et al.*, 2012; Wu *et al.*, 2013; Goldstein *et*
9 *al.*, 2014; Sallach *et al.*, 2015) have also been investigated for a number of pharmaceutical
10 contaminants and antibiotic compounds.

11 The degree of uptake is dependent upon environmental factors, properties of the
12 compounds, and the plants themselves (Briggs *et al.*, 1982; Wu *et al.*, 2013; Carter *et al.*, 2014;
13 Goldstein *et al.*, 2014). Of the studies that have investigated uptake via soil systems, most have
14 investigated only a single soil type, with a few exceptions (Kang *et al.*, 2013; Goldstein *et al.*,
15 2014; Zhang *et al.*, 2015). Of the few studies that have investigated the impact of soil properties
16 on plant uptake, conclusions have been inconsistent. For example, in two studies investigating
17 the uptake of sulfamethoxazole, increased (Kang *et al.*, 2013) and decreased (Goldstein *et al.*,
18 2014) uptake was attributed to higher clay contents of the respective soils in each study.

19 The aim of this study was to investigate the soil sorption behavior and corresponding
20 uptake of chemically diverse antibiotics by leaf lettuce, *Lactuca sativa* cv. Greenstar, to establish
21 relationships between soil texture and antibiotic uptake at concentrations of 1 mg/L representing
22 the reuse of untreated wastewater. The hypothesis is that an increasing proportion of sand
23 compared to clay in soil would increase the bioavailability and subsequent uptake of antibiotics

1 by lettuce. Batch sorption experiments with three antibiotics (lincomycin, oxytetracycline and
2 sulfamethoxazole) individually and as a mixture were conducted to determine soil-water
3 partitioning coefficients (K_d). Unlike a previous study, where contaminants were inoculated in a
4 single irrigation event (Zhang et al. 2015), in the current study lettuce grown in three soils of
5 varying textures were exposed to the antibiotics via irrigation water routinely throughout the 40
6 day growth period under greenhouse conditions. Analysis of lettuce shoots, and soil collected
7 from the top and bottom of the soil profile were used to ascertain relationships between sorption
8 and accumulation/translocation to the edible plant portions. In addition, after the first lettuce
9 harvest, irrigation with contaminated water was replaced with clean dechlorinated water and a
10 second and third harvest was conducted 5 and 10 days later to track the fate and mobility of each
11 compound in the soil-plant system.

12 **Materials and Methods**

13 *Chemicals and Reagents.*

14 Lincomycin, roxithromycin, doxycycline hyclate, and demeclocycline hydrochloride
15 were purchased from Sigma-Aldrich (St. Louis, MO). Sulfamethoxazole and oxytetracycline
16 were obtained from MP Biomedicals, LLC (Solon, OH). $^{13}\text{C}_6$ -Sulfamethazine was purchased
17 from Cambridge Isotope Laboratories (Andover, MA). Standard stock solutions were prepared
18 with HPLC grade methanol and stored dark at -20°C . Surrogate and internal standard spiking
19 solutions were prepared in methanol at the University of Nebraska-Lincoln (UNL) Water
20 Sciences Laboratory. Calibration standards (0.1 – 5 ng/ μL) were prepared prior to each analysis
21 in 3:1 (v:v) solution of Nanopure water (Barnstead, Dubuque, IA) and methanol.

22 *Batch Sorption Study.*

1 For each soil, duplicate batch sorption reactors were prepared for each compound
2 individually as well as together as a mixture. For lincomycin and sulfamethoxazole, 5 g of soil
3 was combined with 25 mL of water with antibiotic concentrations of 10, 50, 100, 500, and 1,000
4 µg/L in 50 mL polypropylene tubes. A soil to water ratio of 0.5 g in 40 mL water was used for
5 oxytetracycline at concentrations of 100, 500, 1000, 1500, 5000 µg/L. Reactors containing a
6 mixture of all three antibiotics were prepared with the same concentrations and soil to water ratio
7 as lincomycin and sulfamethoxazole. To provide the most accurate comparison of greenhouse
8 experimental conditions, de-chlorinated water was taken from the greenhouse and, along with
9 soil, was sterilized at 125°C and 15 psi. Soil and water were then mixed and allowed to
10 equilibrate for 24 hrs at 20°C prior to spiking with antibiotics. Concentrations in eluent solution
11 were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.
12 Additional details and validation are provided in Supplementary Material.

13 *Greenhouse Study.*

14 Three soils were prepared by mixing coarse sand and Sharpsburg silt clay at 75:25, 50:50 and
15 25:75 ratios by weight. The resulting soil properties were characterized at Midwest Laboratories
16 (Omaha, NE) and reported in Table 1. Soils were classified as sand, sandy loam, and loam.
17 Vegetable production flats, comprised of six 17-cm x 12-cm x 7-cm pots, were prepared in
18 triplicate for each soil type. Each flat represented a single treatment unit. An additional flat was
19 prepared for each soil type for control samples with no antibiotic exposure. Seeds of a leafy
20 lettuce, *Lactuca sativum* cv. Greenstar, were planted in each soil type at an initial density of 8
21 seeds per pot. Upon germination, lettuce was thinned to 4 plants per pot. A final thinning, to two
22 plants per pot, took place upon the emergence of the plumule and first true leaves. Plants were
23 grown in a greenhouse with temperature controlled at 15-18°C and 16 h of daily light.

1 Flats were sub-irrigated with 2 L of water to stimulate germination followed by 1 L
2 watering events at the first indication of leaf wilting throughout the growth cycle. Sub-irrigation
3 was conducted to simulate various types of furrow irrigation. Amended irrigation water was
4 prepared by spiking dechlorinated tap water with the lincomycin, oxytetracycline, and
5 sulfamethoxazole at a final concentration of 1 mg/L. Antibiotic spiking solutions were prepared
6 weekly and stored frozen at -20°C, and amended irrigation water was prepared fresh,
7 immediately before use for all irrigation events. Control flats were irrigated with the same
8 volume of dechlorinated water with no antibiotic amendment. Treatment and control flats were
9 ordered randomly on greenhouse benches and rotated at each watering to reduce biases related to
10 variations in greenhouse microclimates and samples of soil and plant tissues were taken in
11 triplicate at each harvest.

12 After 40 days, a single pot from each flat was randomly selected for harvest. Remaining
13 lettuce continued to grow, however, antibiotics were not added to the irrigation water after the
14 first harvest. A second and third harvest of lettuce and soil were collected at 45 and 50 days
15 respectively. At all harvests, lettuce plants were cut at the cotyledonary node, just above the soil
16 surface. Plant material was weighed, rinsed, and blotted dry prior to storage in plastic sample
17 bags. Soil was carefully removed from the pot and the top and bottom 1.5 cm of the soil profile
18 was collected separately in a sample storage bag for analysis. A subset of top and bottom soil
19 was used to determine moisture content. Collected samples were immediately taken to the UNL
20 Water Science Laboratory for further processing. A diagram detailing the subirrigation method
21 as well as soil and lettuce harvesting is provided in the supplementary material (Supplementary
22 Fig. S2).

1 *Lettuce and Soil Extraction.* Antibiotics were solvent extracted from lettuce and quantified by
2 LC-MS/MS following the method described in previous studies (Zhang *et al.*, 2015, Sallach *et*
3 *al.*, 2016), and soil extraction followed a two-step organic solvent – aqueous extraction from
4 methods that are also described in previous work (Sallach *et al.*, 2015). Additional details of the
5 extraction and analytical methods are provided in the Supplementary Material. Concentrations
6 are reported on a fresh weight (FW) basis, as moisture content in lettuce tissues were not
7 influenced by soil type or harvest date.

8 *Data Analysis.*

9 Statistical analysis was performed using Graphpad Prism V6 (Graphpad Software, Inc., La Jolla,
10 CA, USA) using 2-way ANOVA and Tukey's multiple comparisons test to determine
11 significance.

12 **Results and Discussion**

13 *Uptake as a function of soil texture.*

14 Measured concentrations in lettuce and soils were evaluated as a function of soil texture
15 and time. Sulfamethoxazole was the only compound that followed the hypothesis that increased
16 percentage of sand in the soil mixture would result in increased bioavailability and subsequent
17 uptake in the lettuce shoots when exposed to the antibiotic mixture in all irrigation events at the
18 time of the first harvest (Figure 1). The concentration of sulfamethoxazole in lettuce grown in
19 sand (25 ng/g FW) was greater than that grown in sandy loam (8.1 ng/g FW) or loam (3.3 ng/g
20 FW). Similar to our previous work in a different soil (Sallach *et al.*, 2015), lincomycin was
21 detected at the highest concentration of the three compounds in all three soils. The higher uptake
22 of lincomycin results from its ionic speciation at the soil pH range in the current study (7.1-7.4).

1 Lincomycin, with a pK_a of 7.6) existed in its cationic and neutral species. Sulfamethoxazole (1.6,
2 5.7) and oxytetracycline (3.57, 7.49, and 9.44) were dominated by their anionic and zwitterionic
3 forms. Uptake of positively charged ions has been demonstrated to be higher than other charged
4 organic ions (Goldstein *et al.*, 2014). Unlike sulfamethoxazole, plant concentrations of
5 lincomycin did not increase with increasing sand content in the soil. The highest concentration at
6 the first harvest (40 days) was in the lettuce grown in the sandy loam soil (58 ng/g FW).
7 Oxytetracycline concentrations were also highest in the lettuce grown in the sandy loam soil
8 (18.3 ng/g FW).

9 As shown in Table 2, leaf bioconcentration factors (BCFs) were determined by dividing
10 the concentration in the lettuce leaves by the average concentration measured in the soil (Figure
11 2). BCFs represent an uptake efficiency that incorporates translocation from roots to the edible
12 lettuce portion of the plant. BCFs for lincomycin and oxytetracycline are similar in both the loam
13 and sandy soils with values ranging from 0.023-0.028. Increased uptake efficiency was observed
14 for both compounds in lettuce grown in the sandy loam soil resulting in greater BCFs of 0.076
15 for lincomycin and 0.054 for oxytetracycline. Unlike the other two compounds,
16 sulfamethoxazole uptake efficiency increased with increasing sand soil content from a low of
17 0.010 in loam to a high of 0.111 in the sand soil. Sulfamethoxazole BCFs determined in this
18 study are consistent with BCF values reported in hydroponic systems where uptake and
19 translocation were also found to be low (Herklotz *et al.*, 2010; Wu *et al.*, 2013).

20 To add further insight into the influence of soil texture on the mobility of these
21 antibiotics, batch sorption experiments for each compound in each soil were performed. Batch
22 sorption experiments of the three compounds as a mixture also were conducted to replicate
23 conditions in the greenhouse trial. The resulting isotherms are provided in the supplementary

1 information (Supplementary Fig. S4) while a summary of soil partitioning coefficients (K_d) and
2 R^2 values of the linear regressions are shown in Table 2. As a single solute, oxytetracycline was
3 most influenced by soil texture with K_d values of 1107, 485, and 260 L/Kg in loam, sandy loam,
4 and sand respectively. Lincomycin sorption was highest in the loam soil with a K_d of 10 L/Kg,
5 but was slightly lower in sandy loam compared to the sand at 5.5 and 5.9 L/Kg respectively.
6 Sulfamethoxazole sorption to soils was not measureable at low concentrations (10-100 ng/mL) in
7 any of the soils (Supplementary Fig. S4), with all of the compound accounted for in solution,
8 which is supported by the findings of Huang and Weber (1998) who found that aqueous phase
9 concentrations within two orders of magnitude in difference could increase the time to reach
10 sorption equilibrium from a few hours for higher concentrations to several months at low
11 residual solution phase concentrations. Surprisingly, sulfamethoxazole at the higher solution
12 concentrations showed no difference in K_d value between the three soils. When all compounds
13 were present at the same concentrations in the multi-solute isotherms, the range of K_d values for
14 lincomycin increased to 3.9-15.3 L/Kg where the least amount of sorption occurred in sandy
15 loam and highest sorption in loam soils. An apparent decrease in sorption to both loam (1.9 L/Kg
16 to 0.4 L/Kg) and sand (1.9 L/Kg to 1.0 L/Kg) soils occurred for sulfamethoxazole when all
17 compounds were present in the mixture. However, reductions in R^2 values may indicate a
18 deviation from linear sorption for sulfamethoxazole when in a mixture. For this reason,
19 Freundlich isotherms were also modelled to the data for mixtures of antibiotics (Table 2).
20 Generally, the two parameter model (K_F and n) better represented the mixture data and yielded
21 R^2 values ≥ 0.98 in all instances except for sulfamethoxazole in loam ($R^2=0.49$). For
22 lincomycin, n values approaching one in all three soils, confirms linearity. Oxytetracycline was
23 not detected in solution, indicating that all of the compound present adsorbed to the soil. This

1 was not unexpected as the concentration range and soil-water ratio necessary to determine the
2 individual oxytetracycline sorption isotherms were far greater than the other two compounds due
3 to its highly sorptive behavior. These results show the influence of competitive sorption when
4 compounds are present as a mixture. In practical applications, multiple compounds are likely to
5 occur as mixtures and these results suggest that sorption behavior is likely to be impacted.

6 Overall, the confluence of data collected in this study shows that sorption is not the
7 driving factor behind the accumulation of antibiotics in lettuce shoots. Even in the case of
8 sulfamethoxazole, where increasing lettuce concentrations corresponded with increasing sand
9 content, this behavior was not supported by the batch sorption isotherms that showed that
10 changes in soil texture had no measurable effect on sulfamethoxazole sorption. However, the
11 results do show that growth in a soil system, in general, does have a large impact on uptake
12 trends compared to hydroponic systems. For example, Chuang *et al.*, (2015) showed that uptake
13 and translocation of oxytetracycline in lettuce grown hydroponically resulted in leaf
14 concentrations twice that of lincomycin which were, again, twice as high as sulfamethoxazole.
15 While similar, sulfamethoxazole in this study was found at low concentrations (3.3-24.7 ng/g
16 FW), oxytetracycline leaf concentrations (11.3-18.4 ng/g FW) were lower than lincomycin
17 concentrations (23.5-29.5 ng/g FW). This difference is partially explained by the high sorption
18 partitioning of oxytetracycline, resulting from the dominant cation exchange mechanism for
19 tetracycline compounds (Sassman and Lee, 2005), in all three soils which acts to reduce its
20 mobility and corresponding bioavailability to the plant, factors not accounted for in hydroponic
21 studies.

22 Partitioning coefficients did correlate strongly with the distribution of the antibiotics
23 throughout the soil profile. Comparing the concentrations in the top and bottom of the soil profile

1 (Figure 2) shows that oxytetracycline, with highest K_d values in all three soils, remained mostly
2 in the bottom layer. In comparison, the compound with the lowest sorption and lowest K_d values
3 for all three soils was sulfamethoxazole which was detected at higher concentrations in the top
4 soil layer.

5 *Fate in soil and lettuce.*

6 After the first harvest, all remaining lettuce pots were irrigated with the control dechlorinated tap
7 water and samples were collected 5 (harvest 2) and 10 (harvest 3) days later. The leaf
8 concentration of lincomycin grown in the sandy loam soil decreased at both subsequent harvests
9 (Figure 1).Lettuce grown in the sand and loam soils showed highest concentrations detected at
10 the second harvest. Even without additional amendment, the relatively high solubility of
11 lincomycin (13 g/L) likely allowed for desorption and resuspension into the uncontaminated pore
12 water, making it available for uptake in the irrigation events following the first harvest.

13 The lettuce concentrations of sulfamethoxazole in the sandy loam soil remained constant
14 at all three harvests at around 8 ng/g FW. In the loam soil, the concentration increased slightly at
15 each harvest while the opposite occurred in the sand soil where a decreasing concentration trend
16 was observed.

17 Oxytetracycline concentrations in lettuce were highest at the first harvest for all three
18 soils. However, a sharp decrease in concentration was observed at harvest 2 before a slight
19 increase in concentration at harvest 3. In fact, concentrations in lettuce harvested at 45 days were
20 below the detection limit for a number of the replicates in all three soils (Figure 2).

21 Oxytetracycline, even as a zwitterion, maintains a positively charged functional group and as a
22 result, cation exchange is more favorable than hydrophobic partitioning, which results in high

1 sorption affinity (Sassman and Lee, 2005). High sorption and low solubility (0.022 g/L) limit its
2 ability to desorb and reincorporate into the uncontaminated irrigation water that was used
3 following the first harvest.

4 While the concentration of toxicants in edible plant portions is an important measure for
5 the understanding of human exposure of emerging contaminants, it is not enough to reveal all of
6 the behaviors of the dynamic soil-plant system over time. This is because the measure of
7 concentration is dependent upon both the rate of uptake of the contaminant as well as the rate of
8 growth of the plant. Therefore, examining the total mass of accumulation, or net accumulation,
9 provides insight into the movement of the antibiotics with time. Net accumulation was calculated
10 by multiplying the contaminant concentrations in the lettuce plants by the average plant mass at
11 the time of harvest (Figure 3). For both sulfamethoxazole and lincomycin, even in instances
12 when the concentration decreased, antibiotic uptake continued in the five days between harvest 1
13 and 2. This result highlights how increasing plant mass effectively dilutes contaminant
14 concentrations, an observation noted in a previous study (Sallach *et al.*, 2015). Net lincomycin
15 uptake continued to increase from harvest 2 to harvest 3 in the sand soils. However, in the loam
16 and sandy loam soil the total accumulated mass of lincomycin decreased from harvest 2 to 3.
17 This indicates that degradation of lincomycin occurred within the lettuce plant at a rate that
18 exceeded uptake. Degradation of sulfamethoxazole is also apparent in lettuce grown in the sand
19 soil where net accumulation decreased between harvest 2 and 3. While pharmaceutical
20 degradation is known to occur in the environment, few studies have demonstrated its occurrence
21 in vegetable production (Goldstein *et al.*, 2014). Further, this highlights the importance of the
22 significant research gap where the fundamental understanding of the fate and biological impact of
23 antibiotic metabolites is not well known (Williams-Nguyen *et al.*, 2016).

1 In soil, antibiotic transport and degradation both factor into the soil concentrations over
2 the course of the three harvests. Generally, concentrations of each of the three compounds in
3 both the top and bottom soil profile were reduced over the course of the ten days during which
4 no additional antibiotics were added to the system. First order decay functions were generated
5 for the 10-day time period between harvest 1 and 3 and degradation rate constants, k , and
6 compound half-lives, $t_{1/2}$, were calculated. Values are summarized in Table 2 while isotherms
7 and calculations are provided in the supplemental information. Based upon the partition
8 coefficient K_d (Table 2), both sulfamethoxazole and oxytetracycline distributions in the soil
9 profile behaved as expected. Because subirrigation requires irrigation water to flow from the
10 bottom, up through the soil profile, we would expect the more sorptive compounds to be
11 concentrated in the bottom soil layer. Oxytetracycline concentrations in the top profile were far
12 lower than concentrations found in the bottom for all three soils. The least sorptive compound,
13 sulfamethoxazole ($K_d=0.4-1.9$) was found at higher concentrations in the top soil as compared
14 with the bottom in all soils and at all harvests. Both of these compound specific trends are
15 supported by transport studies that show tetracycline mobility to be limited while sulfonamides
16 may pose a risk to surface and groundwater contamination (Blackwell *et al.*, 2007; Watanabe *et*
17 *al.*, 2010; Kim *et al.*, 2012; Srinivasan and Sarmah *et al.*, 2014). With a half-life ranging 3.4-3.7
18 days, lincomycin demonstrated the most rapid and consistent decay in all three soils. Although
19 soils were exposed to the same concentrations of three antibiotics, higher initial concentrations of
20 lincomycin were detected at the first harvest. This high concentration of the most degradable
21 compound in our system may be a result of an initial lag phase in biodegradation, whereby the
22 compound was able to build up in the soil during the first 40 days where irrigation with
23 contaminated water retarded degradation via alteration in the microbial community. Irrigation

1 with uncontaminated water over the course of days 41-50 may have allowed the native bacteria
2 population to recover leading to the rapid degradation of the compound. This lag phase behavior
3 has been observed in other soil degradation studies and was attributed to the presence of a
4 sulfonamide, also included in our study, which has been shown to temporarily disrupt soil
5 bacteria populations (Monteiro and Boxall, 2009). Lincomycin and sulfamethoxazole
6 degradation rate decreased with increasing sand content from 8.3 days in loam to 14.6 days in
7 sand. This was expected as biological activity is known to decrease with increasing coarseness of
8 soil texture (Wardle, 1992). Oxytetracycline degradation was most rapid in the loam soil ($t_{1/2}$ =
9 6.6 days) but unlike sulfamethoxazole, was most persistent in sandy loam ($t_{1/2}$ =20.9 days).
10 Compared to other values reported, half-lives of oxytetracycline and sulfamethoxazole were on
11 the same order of magnitude, but higher, than the biodegradation rates of a sulfonamide
12 (sulfamethazine) and tetracycline (chlortetracycline) antibiotic in a silt loam soil (Topp *et al.*,
13 2013). In strong agreement with our work, half-lives have been reported for sulfamethoxazole
14 under aerobic and anaerobic conditions ranging from 9.0 to 18.3 days (Lin and Gan, 2011).

15 *Effects of routine irrigation with contaminated water*

16 In a previous study, which evaluated the uptake of these three compounds by lettuce in
17 the same soil mixtures, a single exposure event was conducted with water spiked 5x higher than
18 the antibiotic concentrations in the current study (Zhang *et al.*, 2015). Results from the prior
19 study showed that 48 hours after exposure, only sulfamethoxazole was detected in lettuce leaves
20 above detection limits (Zhang *et al.*, 2015). Consistent with results from the current study,
21 sulfamethoxazole concentrations in lettuce increased with increasing percentage of sand in the
22 soil mixture. However, when routine irrigation with contaminated water occurred throughout the
23 growth cycle of the lettuce, both lincomycin and oxytetracycline were detected in leaves, and

1 their concentrations in lettuce leaves exceeded sulfamethoxazole concentrations. The
2 significance of this, which was revealed by differences in exposure regimes between the two
3 studies, suggests that the processes by which oxytetracycline and lincomycin are internalized by
4 lettuce roots and translocated throughout the shoots are more time dependent than the kinetics
5 involved with sulfamethoxazole uptake. As discussed previously, this is supported by results in
6 the fate investigation for lincomycin where the total mass taken up by lettuce shoots increased
7 significantly in the five days following the final irrigation with spiked water (Figure 3).

8 *Toxicity.*

9 The growth of lettuce was affected by the soil texture, where the sand soil mixture
10 resulted in significantly ($P < 0.0001$) reduced mass of lettuce compared to both the loam and
11 sandy loam soil (Figure 4). The difference in lettuce plant mass between loam and sandy loam
12 was not statistically significant in the control group ($P = 0.146$). For all soil types, irrigation with
13 antibiotic amended water resulted in significantly decreased lettuce growth compared with its
14 respective control ($P < 0.0001$). The relative impact of the spiked water on the mass of lettuce
15 increased with increasing sand content in the soil. A decrease of 37 %, 55 %, and 72 % of plant
16 mass between controlled and treated plants was determined for lettuce grown in loam, sandy
17 loam, and sand soil respectively. High percentage decreases in plant material (up to 60%) have
18 also been associated with the pharmaceutical carbamazepine at similar soil concentrations
19 (Carter *et al.*, 2015). Furthermore, leaf discoloration and reduction in photosynthetic pigments
20 resulted from carbamazepine exposure, consistent with the discoloration, yellowing, of leaves
21 from lettuce grown in the sandy soil from the antibiotic spiked water. Lettuce was able to recover
22 as soil concentrations declined in the 10 days between harvest 1 and harvest 3 where leaves from
23 all three soils showed no signs of stress. These significant growth reductions suggest that

1 agricultural productivity may be negatively impacted by the use of recycled wastewater, a
2 significant research gap, recently identified, relating to antibiotics in the agroecosystem
3 (Williams-Nguyen *et al.*, 2016).

4 Sulfonamide antibiotics, including sulfamethoxazole, have been shown to inhibit the
5 growth of rice at a concentration of 0.1 mg/L and maize grown in soil at 10 mg/kg (Liu *et al.*,
6 2009; Michelini *et al.*, 2012). However, rice sensitivity to tetracyclines was less acute as
7 concentrations in soil as high as 300 mg/kg, tetracyclines did not affect plant growth but did
8 effect seed germination (Liu *et al.*, 2009). This likely is attributed to tetracycline's high
9 adsorption to soils (Table 2) and is supported by the findings of Norman where root growth was
10 inhibited by oxytetracycline in a hydroponic system, but had no effect in soils (Norman, 1955).
11 Oxytetracycline in hydroponic systems has also been shown to reduce plant growth in alfalfa;
12 however, at concentrations of 1 mg/L, the concentration of oxytetracycline in our irrigation
13 water, no effect was observed (Kong *et al.*, 2007). Lincomycin has been shown to be toxic to a
14 number of algae strains at the $\mu\text{g/L}$ level (Andreozzi *et al.*, 2006). Attributing toxicity to specific
15 antibiotics in a mixture is not possible, as mixture toxicities can have unpredictable and
16 concentration dependent synergistic or antagonistic effects (Liu *et al.*, 2008; Yang *et al.*, 2008;
17 Gonzalez-Pleiter *et al.*, 2013). Consistent with our study, antibiotic toxic effects have also been
18 shown to be dependent upon soil characteristics; where plants were more sensitive in sandy loam
19 than with a high clay soil (Batchelder, 1982). Not only was the sand soil, without antibiotic, the
20 least ideal for optimal plant growth, it also amplified the toxic effect of the antibiotics to lettuce.

21 **Conclusions**

1 This study confirmed that soil texture plays an important role in the uptake of antibiotics
2 by lettuce. However, correlation between increasing sand content and subsequent uptake and
3 translocation was only observed for sulfamethoxazole. This is because soil composition not only
4 affected the bioavailability of the contaminants but also the health of the plant. When irrigation
5 was switched to non-contaminated water, lettuce recovery was observed resulting in an increase
6 in growth rate. In addition, examination of the net accumulation of antibiotic compounds by
7 lettuce plants over time revealed that degradation of lincomycin and sulfamethoxazole within the
8 lettuce leaves occurred over the 10-day harvesting period. Results from this study should help in
9 the evaluation of best management practices for the use of recycled wastewater for irrigation.
10 Areas with sandy soil should pay particularly close attention to plant toxicity resulting in
11 decreased yield. Furthermore, due to the persistence and mobility of antibiotic compounds in the
12 soil-plant system, a “finishing” period, utilizing uncontaminated irrigation water, may be suitable
13 to reduce the concentrations of antibiotics in vegetables meant for consumption. The time needed
14 to realize this reduction is dependent upon both contaminant and soil characteristics.

15 **Supplementary Material.** Equilibration time study of antibiotic batch sorption reactors
16 (Supplementary Fig. S1). Method Validation for soil and lettuce samples (Supplementary Table
17 S1). Schematic of soil-plant system using subirrigation (Supplementary Fig. S2), average of top
18 and bottom soil concentrations (Supplementary Table S2), equations related to decay functions
19 (Equations 1-2), antibiotic decay in soils (Supplementary Fig. S3), and linear sorption isotherms
20 (Supplementary Fig. S4).

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7

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