Environmental Research 164 (2018) 197–203

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

# Impact of wastewater effluent containing aged nanoparticles and other components on biological activities of the soil microbiome, *Arabidopsis* plants, and earthworms

Jia Liu[a,f,*], Philip C. Williams[a], Jane Geisler-Lee[b], Boyd M. Goodson[c,f], Masoud Fakharifar[a], Meisam Peiravi[a], Da Chen[d], David A. Lightfoot[e], Max E. Gemeinhardt[c]

[a] Department of Civil and Environmental Engineering, Southern Illinois University, 1230 Lincoln Dr., Carbondale, IL 62901, USA
[b] Department of Plant Biology, Southern Illinois University, 1125 Lincoln Dr., Carbondale, IL 62901, USA
[c] Department of Chemistry and Biochemistry, Southern Illinois University, 1245 Lincoln Dr., Carbondale, IL 62901, USA
[d] School of Environment, Guangzhou Key Laboratory of Environmental Exposure and Health, and Guangdong Key Laboratory of Environmental Pollution and Health, Jinan University, Guangzhou, Guangdong, China
[e] Department of Plant, Soil and Agricultural Systems, Southern Illinois University, 1205 Lincoln Dr., Carbondale, IL 62901, USA
[f] Materials Technology Center, Southern Illinois University, 1230 Lincoln Dr., Carbondale, IL 62901, USA

ARTICLE INFO

*Keywords:*
Metallic nanoparticle
Wastewater effluent
*Arabidopsis thaliana*
*Eisenia fetida*
Soil microbiome
Cyanobacteria
*Trichodesmium*

ABSTRACT

The amount of engineered nanomaterials (ENMs) in the environment has been increasing due to their industrial and commercial applications. Different types of metallic nanoparticles (NPs) have been detected in effluents from wastewater treatment plants (WWTPs). The effluents have been reclaimed for crop irrigation in many arid and semi-arid areas. Here, a soil micro-ecosystem was established including a microbiome, 4 *Arabidopsis thaliana* plants, and 3 *Eisenia fetida* earthworms, for a duration of 95 days. The impact of wastewater effluent (WE) containing aged NPs was studied. WE was taken from a local WWTP and exhibited the presence of Ti, Ag, and Zn up to 97.0 ± 9.4, 27.4 ± 3.9, and 4.1 ± 3.6 μg/L, respectively, as well as the presence of nanoscale particles (1–100 nm in diameter). The plants were irrigated with WE or deionized water (DIW). After 95 days, significantly higher concentrations of extractable Ti and Zn (439.2 ± 24.4 and 9.0 ± 0.5 mg/kg, respectively) were found in WE-irrigated soil than those in DIW-irrigated soil (161.2 ± 2.1 and 4.0 ± 0.1 mg/kg). The extractable Ag concentrations did not differ significantly between the WE- and DIW-irrigated soil. Although microbial biomass carbon and nitrogen were not significantly reduced, the population distribution of the microbial communities was shifted in WE-irrigated soil compared to the control. The abundance of cyanobacteria (Cyanophyta) was increased by 12.5% in the WE-irrigated soil as manifested mainly by an increase of *Trichodesmium spp*., and the abundance of unknown archaea was enhanced from 26.7% in the control to 40.5% in the WE-irrigated soil. The biomasses of *A. thaliana* and *E. fetida* were not significantly changed by WE exposure. However, *A. thaliana* had a noticeable shortened life cycle, and corrected total cell fluorescence was much higher in the roots of WE-irrigated plants compared to the control. These impacts on the soil micro-ecosystem may have resulted from the aged NPs and/or the metal ions released from these NPs, as well as other components in the WE. Taken together, these results should help inform the reuse of WE containing aged NPs and other components in sustainable agriculture.

## 1. Introduction

In the United States (U.S.), approximately 12 billion gallons of municipal wastewaters generated are discharged into rivers, oceans, or estuaries on a daily basis (Council, 2012). This amount equals about 4% of the total fresh water use in the U.S. (Maupin et al., 2014). Even larger fractions of available fresh water are utilized for agricultural irrigation, corresponding to 32% and 70% of total freshwater withdrawals for the U.S. and for the world, respectively (Maupin et al., 2014). As freshwater has become a scarce commodity worldwide, all societies need to reclaim and reuse wastewater effluents (WEs) for sustainability. Effluents from wastewater treatment plants (WWTPs) are a cost-effective resource when reclaimed to irrigate agricultural land and scenery landscape in many dry areas. In Israel, a desert country, about 86% of its

* Corresponding author.
*E-mail address:* jialiu@siu.edu (J. Liu).

https://doi.org/10.1016/j.envres.2018.02.006
Received 10 November 2017; Received in revised form 4 January 2018; Accepted 5 February 2018
Available online 28 February 2018
0013-9351/ Published by Elsevier Inc.

treated sewage has been reclaimed for crops irrigation (Harris, 2015); in contrast in the U.S., that number is less than 1%. However according to the U.S. EPA, WEs have been reclaimed and reused in agricultural fields in at least eleven states such as California and Texas, for economically important crops such as citrus and wheat (EPA, 2012). However, the reuse of WEs cannot be performed haphazardly; incautious use of such WE could potentially result in surface and ground water contamination, long-term impacts on soil, plants, and local fauna, as well as health concerns for the local human population (Ahmadi and Merkley, 2009; Laposata and Dunson, 2000).

The physicochemical characteristics of WEs, such as total suspended solids (TSS), chemical oxygen demand (COD), biochemical oxygen demand (BOD), pH, and the concentrations of heavy metals, pharmaceuticals, personal care products, and nutrients (N, P, K) - as well as their possible effects on the plants, organisms, and soil - have been reviewed and examined (Aristi et al., 2015; Drury et al., 2013; Miller et al., 2016; Wakelin et al., 2008; Yadav et al., 2002). Previous studies have also found that reuse of WEs in arid and semi-arid countries result in a variety of technical, legal, institutional, and socio-economic issues (Mizyed, 2013). For example, irrigation with WEs could change soil chemical characteristics by causing a significant decrease of soil pH and infiltration rate, or an increase in organic matter and electrical conductivity (Bedbabis et al., 2014). Moreover, the practice may cause variable responses of soil microbiota, shift the population distributions of microbial communities (Becerra-Castro et al., 2015), or lead to the accumulation of trace metals in leafy vegetables (Qureshi et al., 2016).

Although some regulations exist to help ensure the safety of effluents for human health and the environment, most of them do not consider the probable existence of engineered nanomaterials (ENMs) in the WEs or their potential effects. Trace amounts of nanoparticles (NPs) exist naturally in the environment. However in recent years, the application of ENMs in various industries and domestic products has tremendously increased nanomaterial concentrations in the environment. The most common metallic NPs are those composed of $TiO_2$, ZnO, and Ag. Many of these nanomaterials have ended up in WWTPs (Madela et al., 2016). For the ENMs that cannot be removed in primary and secondary sludge, they could be discharged into rivers and lakes along with WEs. By reclaiming WEs in agricultural lands, crops are susceptible to be exposed to these ENMs. Numerous studies have focused on the effects of pristine NPs on a single species. For example, studies have investigated the toxicity of pristine Ag NPs to *Escherichia coli* (Pal et al., 2007) or to *Arabidopsis thaliana* (Geisler-Lee et al., 2013; Kaveh et al., 2013). Those metallic NPs may damage biological cells through physical attack and/or through reactive oxygen species (ROS) that may be generated. Ions released from the metallic NPs may also be transported into cells and affect their physiology. NPs were recently found to undergo transformations in WWTPs and aged NPs in biosolids, if any, were believed to have minimal impact upon soil microbial communities in one study (Durenkamp et al., 2016); however, reduced microbial biomass and shifted microbial community composition was found in another study (Judy et al., 2015). Similarly, exposure to aged NPs in biosolids posed low risks to crops in one study (Wang et al., 2016), while inhibited nodulation of *Medicago truncatula* plants in another study (Judy et al., 2015). Impacts of WEs containing aged NPs on the physical properties of constructed wetlands have been studied as well as those on aquatic microbial communities (Auvinen et al., 2017; Zuluaga, 2016), although impacts of WEs containing NPs have not been studied on specific organisms in soils. In addition, to date there remain no systematic study on the effects of WE-containing NPs on a given ecosystem (i.e., one including plants, microorganisms, and animals such as earthworms), as well as the effects (positive or negative) on organism-organism interactions in the ecosystems.

The objective of this study was to investigate the effects of reclaimed WEs on a soil micro-ecosystem, which consisted of soil microorganisms, *A. thaliana* plants, and *Eisenia fetida* earthworms. It was anticipated that the presence of trace amounts of NPs would be detected in the WE, and the impact on each type of organism would be demonstrated for WE irrigation compared to deionized water (DIW) irrigation for a duration of 95 days. First, the fraction of particulate matter with nanoscale dimensions would be quantified, and the concentrations of key metal ions would be detected in WE and WE-irrigated soil; second, the biomass of *A. thaliana*, fluorescence intensity of its roots, the biomass of *E. fetida* and soil microorganisms - as well as the composition of the microbial communities - would be measured with WE irrigation, and compared with DIW irrigation. To our knowledge, the present work is the first study on the influence of reclaimed WE containing aged NPs and a variety of other components on a soil micro-ecosystem.

## 2. Materials and methods

### 2.1. Ecosystem setup

Up to 280 g of potting soil (Propagation Mix, Sungro Horticulture) and 280 g of garden soil (Black Gold) were manually and thoroughly mixed for 10 min with 700 mL of deionized water. Seeds of *A. thaliana* were treated by 25% (v/v) Clorox (household bleach) for 10 min and rinsed in DIW thoroughly three times before planting the seeds in each of the four corners of the pot. The pot was kept in the dark at 4 °C for 4 days before they were transferred to humidity domes with 12 h light/12 h dark cycles for 95 days. WE from Carbondale Southeast WWTP (Carbondale, IL) was used to irrigate *A. thaliana*. The majority of residential wastewater in the city is currently treated by this plant. The effluent was shaken well each time before irrigation. The control pot was irrigated with DIW. Watering interval, dosage, and humidity were controlled and comparable to the control to keep the soil moisture to maintain the environment for germination and growth of the plants. *E. fetida* were hydrated in DIW and placed in a covered tray on wet paper towels in a growth chamber at 20 °C overnight to purge the gut of each earthworm of any soil material. The mass of each earthworm was recorded before they were added to the pots: a total of three earthworms per pot were added at 35 days after planting (DAP) when there were at least 14 leaves on all the plants. The endogenous microorganisms from the soils and the microorganisms introduced by the WE were studied.

### 2.2. Plant and earthworm analysis

At 95 DAP when *A. thaliana* turned yellowish, they were harvested. The fresh weights of shoots, roots, and seeds of *A. thaliana* were measured and recorded. About 1 cm of the root tips was cut for reactive oxygen species (ROS) measurement. Root tips were soaked in 0.25 μM of 2′,7′- dichlorofluorescin diacetate (H2DCF-DA) for 15 min. Then the roots were washed several times with doubly-distilled water and the fluorescence of ROS was observed using an excitation filter at 485 nm and an emission filter with a transmission cut-off at 535 nm and imaged by using a Leica DM 5000B compound microscope equipped with UV fluorescence and a Q-Imaging Retiga 2000R digital camera. The fluorescence intensity was measured using ImageJ software (http://rsb.info.nih.gov/ij/). *E. fetida* were extracted from the soil after the plants were harvested. The earthworms were hydrated and washed in DIW, and placed in the growth chamber overnight before they were weighed. The weight ratio of earthworms - calculated in terms of mass measured after exposure to soil irrigated by WE, divided by that measured prior to exposure - was compared to the control obtained with earthworms harvested from soil irrigated with DIW.

### 2.3. Soil pH

Soil pH was measured using a DIW suspension. Gravimetric soil moisture was first determined by drying the soil at 100 °C for 48 h. A dry soil equivalent of 1 g was mixed with DIW to reach a soil-to-liquid ratio of 1:5 (w/v). The suspension was stirred vigorously and the slurry

was allowed to settle for 1 h before pH measurement (HQD portable meter, Hach, Loveland, Colorado, US).

### 2.4. Extraction and analysis of nanoparticles from soil

Soil from the rhizosphere was collected. One part of the collected soil was saved for microorganism analysis (see below); the remaining soil was subject to the analysis of aged NPs. Aged NPs were extracted by a method modified from that of Schwertfeger et al. (Schwertfeger et al., 2016). A total of 3 g of dry-soil equivalent was mixed with 2.5 mM tetrasodiumpyrophosphate (TSPP) in DIW in a glass beaker to reach a soil-to-reagent ratio of 1:10 (w: v). Then another 50 mL of DIW was added to the sample. Particles were dispersed and released from the soil suspension by shaking at 200 rpm (radius 6.5 mm) for 1 h, followed by sonicating with 80% amplitude for 800 s (Sonics VC505, 500 W Ultrasonic Processor, Sonics & Materials, Inc., Newtown, CT, USA). During sonication, the probe tip was left 2 cm below the surface of the soil suspension and the glass beaker was placed in a cooler (< 20 °C or in ice) to prevent the influence of increased temperature on the stability of soil organic colloids throughout the process. After that, an additional 250 mL of DIW was added to the suspension to further dilute the sample to 330 mL in total, and the suspension was allowed to settle overnight. WE, collected for more than 95 days previously, was also allowed to settle overnight to remove any large particles. Up to 6 mL of the supernatant was collected.

Particle size distribution was analyzed by dynamic light scattering (DLS, DynaPro NanoStar, Wyatt Technology, Santa Barbara, CA, USA). For each sample, 10 scans were acquired and the data was manually averaged and graphed in terms of particle size as a function of the fractional mass of the particulate matter. For the WE, a single sample was studied; for the soil extracts, the graphs were calculated by averaging over 2 samples each (i.e., 20 scans total).

Metal concentrations of Ti, Zn, and Ag were analyzed by inductively coupled plasma mass spectrometry (Agilent ICP-MS 7500) with an ESI SC-4 high throughput auto-sampler. Ga was used as an internal standard for the ICP-MS analysis. These metals were selected as $TiO_2$, ZnO, and Ag NPs were considered to be the most likely substances comprising the ENMs in this study.

### 2.5. Microorganism analysis

After the plants were harvested, soil from the rhizosphere was collected to analyze microbial biomass and microbial community diversity. Chloroform fumigation extraction was conducted to determine the microbial biomass in the soil according to the protocol from Allison (Allison, 2008). Briefly, 2 g dry-soil and 30 mL of chloroform, along with several boiling chips, were placed in a vacuum desiccator. Vacuum was drawn and the chloroform was kept boiling for 2–3 min before venting. A total of three additional vacuum-venting cycles were repeated and the chloroform vapor was kept in the desiccator for the last cycle by not venting the desiccator. The desiccator was kept in the dark for three days. Then the chloroform vapor was removed by repeated venting. Indigenous microorganisms were killed during the fumigation process, and their carbon and nitrogen became extractable. Both the fumigated soil and the non-fumigated soil with equivalent weights were mixed separately with 0.5 M of $K_2SO_4$ to reach a soil-reagent ratio of 1:10 (w: v). The suspension was shaken for 25 min at 75 rpm (radius 6.5 mm) to facilitate extraction of the microbial biomass into the salt solution. The samples were then filtered through pre-leached (with 0.5 M $K_2SO_4$) Whatman No. 1 filter paper. The extract was stored at − 4 °C before testing.

Microbial biomass carbon was analyzed by a TOC analyzer (Shimadzu TOC-L CSN, Shimadzu Corporation, Kyoto, Japan). A combustion tube for high salt samples with B-type halogen scrubber and $SO_3$ mist catcher was installed in the analyzer. Microbial biomass nitrogen was determined using a UV–vis spectrophotometer according to an environmental protection standard from China (China, 2012). Briefly, a total of 15 g of NaOH was dissolved in 300 mL DIW. A total of 40 g of digesting and oxidizing agent $K_2S_2O_8$ was dissolved in 600 mL DIW under 50 °C. After both solutions cooled down to room temperature, they were mixed and transferred to a polyethylene bottle, and the final volume was adjusted to 1000 mL. $KNO_3$ was dried at 110 °C for 2 h and 0.7218 g of $KNO_3$ was dissolved in 1000 mL DIW. A standard solution of $KNO_3$ was prepared by adding 10 mL of the aforementioned solution to 90 mL of DIW for the standard nitrogen curve. An aliquot of 4 mL of the extracted sample was diluted to 10 mL, and 5 mL of the $K_2S_2O_8$ and NaOH solution was added. The serum vials were closed tightly and autoclaved at 120 °C for 30 min. After the tubes were cooled down, the solution was mixed thoroughly and 1.0 mL of HCl (0.119 g/mL) was added. DIW was added to dilute the solution to a final volume of 26 mL. Absorbance at 220 nm and 275 nm was measured by UV–vis spectroscopy. Duplicate samples were prepared and tested, and DIW was used as the blank. Microbial biomass nitrogen was calculated using the following three Eqs. (1)–(3):

$$A_b = A_{b220} - 2A_{b275} \quad (1)$$

$$A_s = A_{s220} - 2A_{s275} \quad (2)$$

$$A_r = A_s - A_b \quad (3)$$

Where $A_b$ is the absorbance at zero concentration, $A_s$ is the absorbance at the test concentration, and $A_r$ is the absorbance difference; the total nitrogen concentration ρ (mg/L) in sample was then calculated by Eq. (4):

$$\rho = \frac{(A_r - a) \times f}{bV} \quad (4)$$

where $a$ is the slope of the standard nitrogen curve, $b$ is the intercept of the standard nitrogen curve, $V$ is the sample volume (mL), and $f$ is the dilution factor.

A powerSoil® DNA isolation kit (MO BIO) was used for the DNA extraction. The 16S rRNA gene V4 variable-region PCR primers 515/806 were used and the PCR was performed under the following conditions: 94 °C for 3 min; followed by 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 min for 28 cycles; and a final elongation step at 72 °C for 5 min. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Ion Torrent Personal Genome Machine. Operational taxonomic units (OTUs) clustering at 3% divergence were generated and were taxonomically classified using BLASTn against a database derived from RDPII and NCBI.

## 3. Results

### 3.1. Properties of wastewater effluent and experimental soil

The WE was found to contain 97.0 ± 9.4, 27.4 ± 3.9, and 4.1 ± 3.6 μg/L of total Ti, Zn, and Ag, respectively (Fig. 1). NPs with size of ~2–35 nm in diameter were present in the WE (Fig. 2a). The

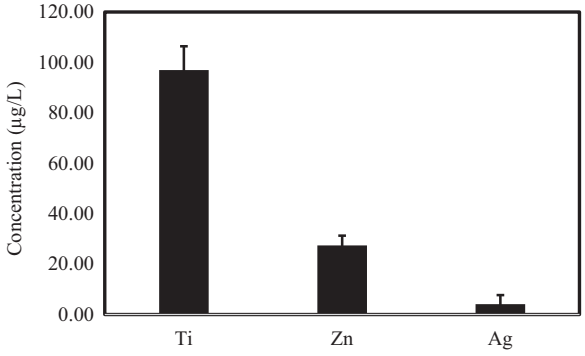


**Fig. 1.** Total metal concentrations in wastewater effluent.

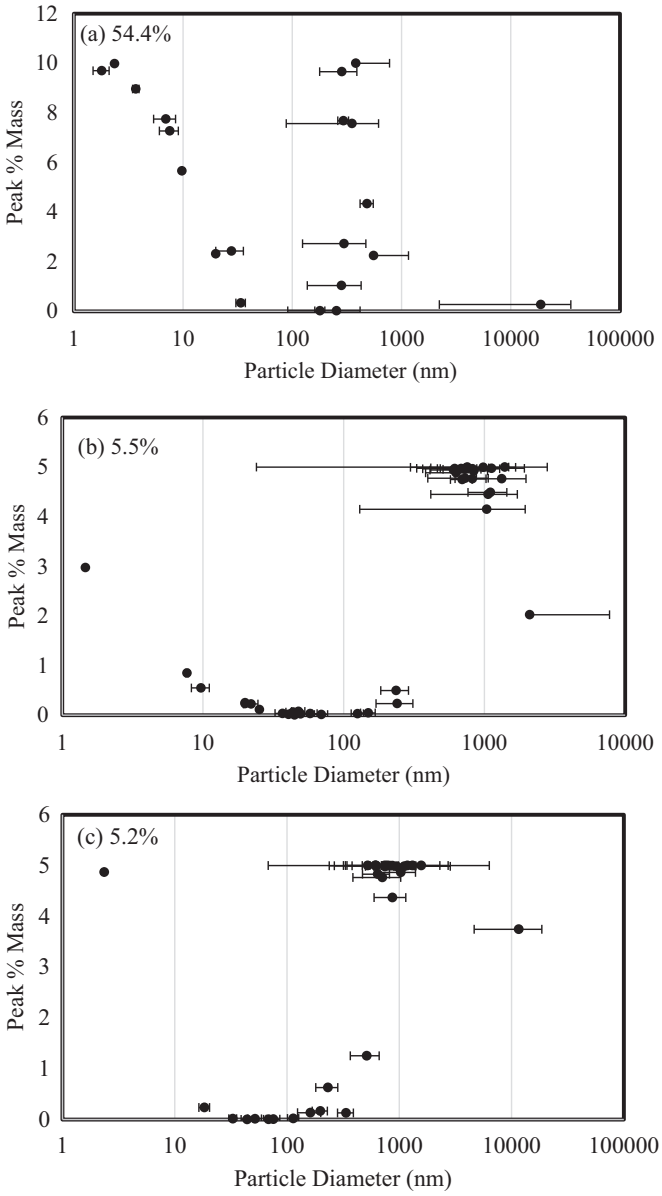


**Fig. 2.** Particle size distributions (as measured via dynamic light scattering) in wastewater effluent (a), in soil extracts from wastewater irrigation (b), and in soil extracts from deionized water irrigation (c). Percentages of particles (by mass) less than 100 nm are indicated for each graph.

amount of particles less than 100 nm comprised over half of all the particles (by mass) present in the WE (Fig. 2a). Moreover, the pH of the soil (6.43 ± 0.05) irrigated by WE was significantly lower than that irrigated by DIW (6.56 ± 0.02) (Table 1).

Soil extracts contained only a small amount of NPs - ~5.5% and ~5.2% by mass, in the range of ~2–70 nm and ~2–80 nm in diameter, by WE and DIW irrigation, respectively. Most of the particulate matter in the extracts belonging to the microscale regime. Considering the measurement uncertainties, these differences may be not significant. Extractable Ti, Zn, and Ag concentrations were increased from 161.1 ± 2.1 mg/kg, 4.0 ± 0.1 mg/kg and 64.2 ± 14.8 μg/kg, respectively, of the control soil to 439.2 ± 24.4 mg/kg, 9.0 ± 0.5 mg/kg and 73.3 ± 13.7 μg/kg, respectively, of the soil irrigated by WE (Table 1). The *t*-test showed that the increase was significant ($p < 0.05$) for Ti and Zn but insignificant for Ag ($p = 0.14$).

### 3.2. Effects on *A. thaliana* and *E. fetida*

The fresh weights (biomasses) of the shoots, roots, and seeds slightly increased from 1.44 ± 0.35, 0.02 ± 0.01, and 0.04 ± 0.01 g, respectively, for the plants irrigated by DIW to 1.53 ± 0.22, 0.02 ± 0.01, and 0.05 ± 0.02 g, respectively, for the plants irrigated by WE (Table 1), although these increases were not significant (with respective *p* values of 0.37, 0.42, and 0.08). However, *A. thaliana* turned yellowish one week earlier when irrigated with WE than the control. Corrected total cell fluorescence (CTCF) was highly significant, much higher ($2.4 \times 10^5 \pm 2.4 \times 10^4$) in the roots of WE-irrigated plants compared to the control ($2.7 \times 10^4 \pm 0.4 \times 10^4$) (Table 1). The weight of *E. fetida* was reduced in WE-irrigated soil over the 95-day study period but it was slightly increased in DIW-irrigated soil (Table 1): The weight ratio (after exposure/before exposure) of *E. fetida* decreased from 102.4 ± 16.5% to 73.7 ± 13.0% for the specimens harvested from DIW-irrigated soil compared to those in WE-irrigated soil, but these weight changes are within the uncertainty ranges (and are not statistically significant).

### 3.3. Effects on soil microbiome

The microbial biomass carbon was slightly reduced from 10,600 ± 200 mg/kg in DIW-irrigated soil to 10,100 ± 200 mg/kg in WE-irrigated soil, and the microbial biomass nitrogen was reduced from 117.7 ± 19.0 to 83.4 ± 1.2 mg/kg (Table 1). However, these minor differences were not individually significant. Nevertheless, WE did appear to affect the composition of the microbial community. The number of identifiable bacteria species increased in the WE-irrigated soil from 732 to 773. Moreover, shifts in relative abundances of different types of bacteria in the soil were triggered by WE irrigation (Fig. 3a). Relative abundance of Cyanobacteria was increased from 0.9% to 13.4% (Fig. 3a) by WE irrigation. Among all the Cyanobacteria, the percentage of the species *Trichodesmium spp*. increased from 0.1% in the control to 12.8% in the WE-irrigated soil. With the introduction of more Cyanobacteria to the soil, the abundances of Proteobacteria, Verrucomicrobia, Acidobacteria, Actinobacteria, and Fusobacteria were relatively reduced. By contrast, the abundances of Bacteroidetes, Planctomycetes, Firmicutes, Gemmatimonadetes, and Chloroflexi were slightly increased (Fig. 3a). For archaea, the relative abundances of

**Table 1**
pH of soil; extractable metal concentrations in soil; fresh weights of shoots, roots, and seeds; corrected total cell fluorescence of root of *Arabidopsis thaliana*; weight ratio of earthworms; microbial biomass carbon and nitrogen in soil, after 95 days' irrigation by wastewater effluent or by deionized water (control). CTCF: corrected total cell fluorescence. *: significant.

| | **Soil** | | | | **Microorganism** | |
|---|---|---|---|---|---|---|
| | pH | Ti (mg/kg) | Zn (mg/kg) | Ag (μg/kg) | Biomass Carbon (mg/kg) | Biomass Nitrogen (mg/kg) |
| Control | 6.56 ± 0.02 | 161.1 ± 2.1 | 4.0 ± 0.1 | 64.2 ± 14.8 | 10,600 ± 200 | 117.7 ± 19.0 |
| Wastewater | 6.43 ± 0.05 | 439.2 ± 24.4 | 9.0 ± 0.5 | 73.3 ± 13.7 | 10,100 ± 200 | 83.4 ± 1.2 |
| *p* value | .05* | $1.1 \times 10^{-6}$* | $7.0 \times 10^{-4}$* | 0.14 | 0.21 | 0.15 |
| | ***A. thaliana*** | | | | ***E. fetida*** | |
| | Shoot Weight (g) | Root Weight (g) | Seed Weight (g) | CTCF | Weight Ratio (%) | |
| Control | 1.44 ± 0.35 | 0.02 ± 0.01 | 0.04 ± 0.01 | $2.7 \times 10^4 \pm 0.4 \times 10^4$ | 102.4 ± 16.5 | |
| Wastewater | 1.53 ± 0.22 | 0.02 ± 0.01 | 0.05 ± 0.02 | $2.4 \times 10^5 \pm 2.4 \times 10^4$ | 73.7 ± 13.0 | |
| *p* value | .37 | 0.42 | 0.08 | 0.03* | 0.07 | |

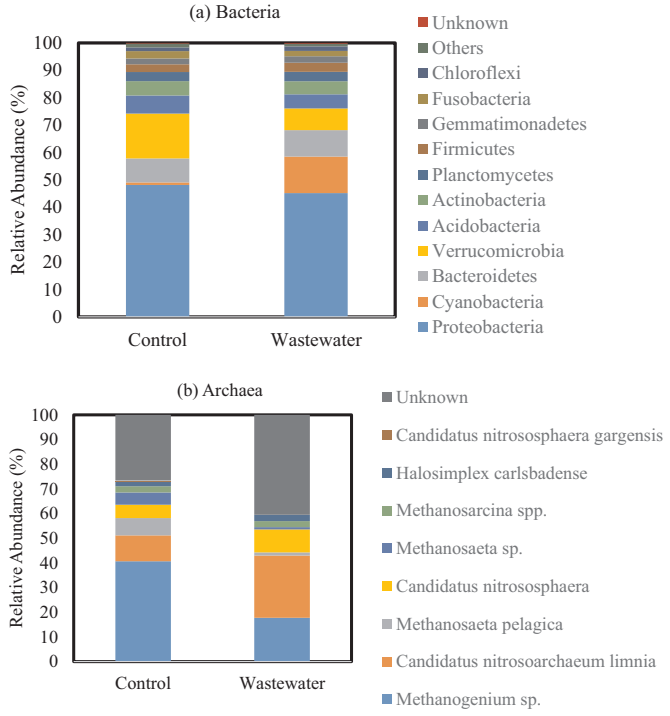


**Fig. 3.** Relative abundances of (a) bacteria and (b) archaea in wastewater effluent-irrigated soil compared to the abundances measured for DIW-irrigated soil (control).

*Candidatus Nitrosoarchaeum limnia* and *Candidatus Nitrososphaera* were increased from 10.5% and 5.4% to 25.2% and 9.3%, respectively, by WE irrigation, while the abundances of all the other species were reduced or slightly increased (Fig. 3b). Up to 26.7% and 40.5% of archaea remained unknown in both types of soils (Fig. 3b).

## 4. Discussion

### 4.1. Wastewater effluent and experimental soil

The total concentrations of Ti, Zn, and Ag in the WE were contributed by both metal-containing NPs and free metal ions. Ti, Zn, and Ag-containing NPs, as well as their dissolved ions have been detected in the effluent from this WWTP (Zuluaga, 2016). The Ti concentration was higher than the comparison (25 μg/L) reported by Westerhoff et al. (2011). The Ag concentration after subtracting the error bar was comparable to the reported value of 700 ng/L, which included both Ag NPs and dissolved Ag ions ($Ag^+$) (Mitrano et al., 2012). The Zn concentration was higher than the simulated result of 0.7 μg/L in U.S., 1.4 μg/L in Europe, and 1.3 μg/L in Switzerland reported by Gottschalk et al. (2009). NPs less than 100 nm in WE could be taken up into plant roots through root hair cells and root cap cells (Bao et al., 2016). The pH reduction of soil irrigated by WE might be due to the released hydrogen ion from ammonia nitrification introduced by the WE irrigation (Mohammad and Mazahreh, 2003), in spite of the fact that the original pH of the WE was 8.6 ± 0.2. The reduced pH still fell within the range allowable for crop irrigation, which is ~ 6.0–9.0 (Bedbabis et al., 2014).

The NPs in soils introduced from the WE may be heteroaggregated with soil particles, or might transport to plants and earthworms (Klitzke et al., 2015; Lee et al., 2010; Pérez-de-Luque, 2017). To date, this is the first report on extractable NPs from soil irrigated by WE. Other components in the WE such as organics might reduce the extractability of NPs originally present and introduced in the soil. Elevated metal concentrations in soils were likely introduced from WE. The extractable (bioavailable) Zn concentration in the WE-irrigated soil was slightly higher than that (i.e., 1.5 mg/kg) extracted by ethylenediaminetetraacetic acid mixed with ammonium acetate in calcareous soil irrigated by treated wastewater for 17 years (Belaid et al., 2012). So far, no extractable Ti and Ag concentrations can be found in the literature for the soil irrigated by WEs. These metal concentrations were the total extractable metal concentrations in the soil, which included both the metallic NPs and dissolved metal ions. Given the increased metal concentrations and the introduction of NPs into soils by WE irrigation, it was speculated that metallic NPs containing Ti, Zn, and Ag existed in the WE-irrigated soil. As $TiO_2$, ZnO, and Ag are three of the most common engineered NPs, the metallic NPs in the WE-irrigated soil would probably originate from these materials.

### 4.2. Effects on A. thaliana and E. fetida

The shortened life-cycle of *A. thaliana* may be caused by the metallic NPs in the WE; metallic NPs, such as Ag NPs at concentrations from ~5 μg/L − 500 μg/L, were previously observed to promote root growth of 4-day-old seedlings of *A. thaliana* (Syu et al., 2014) while shortening lifespan of their reproductive growth by 3–4 days when the first generation was irrigated by 75 μg/L of Ag NPs (Geisler-Lee et al., 2014). Another study on *Escherichia coli* bacteria showed hormetic (i.e., a low dose beneficial effect and a high dose toxic effect) response to stimulate bacteria growth at 3 – 8 μg/L of Ag NPs under anaerobic environment to prevent the release of $Ag^+$ ions (Xiu et al., 2012). Higher CTCF indicated a higher reactive oxygen species (ROS) level present in the WE-irrigated samples. The elevated ROS concentration was consistent with the elevated presence of NPs such as $TiO_2$, ZnO, or Ag; the ROS reacts with the H2DCF-DA to form highly green fluorescent 2′,7′-dichlorofluorescein for fluorescence detection. These NPs could be taken up into the roots during WE irrigation through root hair cells and root cap cells, and then probably transported through plasmodesmata and through intercellular transport (Geisler-Lee et al., 2014). Although the NPs present in the roots introduced by WE irrigation apparently did not affect the plant weight in this study, the translocation of these NPs to plant shoots and transformation of these NPs in soils as well as in plants warrant further investigation.

So far, there is no specific report on the impact on soil earthworms such as *E. fetida* present in soils treated by domestic WE. Growth of earthworms might be affected by the potential toxic components in the WE, such as soluble ions from metallic NPs. In previous work, it was found that metal ions such as Zn, Ni, and Pb were toxic to *E. fetida*; additionally, up to 10 mg/kg of cerium salt affected the survival and reproduction of *E. fetida*, whereas $CeO_2$ NPs/microparticles and $C_{60}$ nanomaterials did not apparently affect the growth and reproduction of the earthworms (Lahive et al., 2014; Li et al., 2010; Neuhauser et al., 1985). Absorption of toxins by worms might actually be helpful for the plant's growth by reducing the effective concentration of these substances exposed to plants (Sinha et al., 2010). Given that WE is complex – containing a variety of low concentrations of organic compounds, metal ions, and NPs – any potential impact on earthworms would be a combination of all the aforementioned factors. Thus in a soil microecosystem such as the subject of the present study, it is also necessary to study the contaminant (e.g., metallic NP and metal ions) accumulation in earthworms, which may reduce the risks to surrounding plants and soil microorganisms and benefit plant growth.

### 4.3. Effects on soil microbiome

Components in WE, including aged NPs, did not pose an apparent effect on soil microbial biomass. In a previous study, aged Ag, ZnO, and $TiO_2$ ENMs in biosolids were reported to significantly reduce the microbial biomass of gram-positive bacteria, gram-negative bacteria, actinomycetes, and fungi as indicated by total phospholipid fatty acid in soil samples compared to bulk/dissolved metal treatments and control treatments (Judy et al., 2015). Although all the mentioned aged elements were similar and came from the same source (i.e., WWTP),

sediments in biosolids might concentrate the aged elements in contrast to WEs. Thus, a discrepancy of significant and insignificant impacts to soil microorganisms between biosolids (Judy et al., 2015) and WEs (this study) may occur.

Different microorganisms were introduced into the soil during WE irrigation. Cyanobacteria are normally an important component in the WWTP's biological treatment process, reaching levels approaching 100% of the total phytoplankton density in some systems (Vasconcelos and Pereira, 2001). The occurrence of Cyanobacteria can change the ecology of microbial communities (Martins et al., 2011). Cyanobacteria can fix atmospheric nitrogen into ammonia, nitrite, or nitrate, which are essential nutrients for plants. An increase in the abundance of these bacteria can benefit plant growth, especially economically important crops that demand synthetic fertilizers. *Trichodesmium* is a diazotroph, which is capable of fixing atmospheric nitrogen gas into more usable nitrogen such as ammonia. Normally *Trichodesmium* is found in ocean waters; thus it was interesting to find it present in WE-irrigated soil, particularly given the location of the study. Introduction of a significant amount of this species by WE irrigation could provide *A. thaliana* a good source of fixed nitrogen as a nutrient for growth. To our knowledge, this is the first study demonstrating the introduction of *Trichodesmium* to soil environments from WE irrigation. Although the plant biomass was apparently not improved with the enhanced nitrogen fixation ability, this could be attributed to the simultaneous introduction of other chemical species such as the aged NPs as well as their possible dissolved ions, or to the fact that the error bars were large for the plant biomass measurements. *C. Nitrosoarchaeum limnia* and *C. Nitrososphaera* are two ammonia-oxidizing archaea (AOAs) that are widespread in nitrifying bioreactors in WWTPs. The increase in AOAs might provide the plants with a greater ratio of $NO_3^-/NH_4^+$ for their nitrogen sources (Briones et al., 2003). Compared to bacteria, the unknown percentage of archaea is very high, which showed that many types of archaea species from commercialized soil and from treated wastewater are still unknown.

## 5. Conclusions

Based on this study, four important findings can be concluded. First, aged nano-sized particles and metals of Ti, Zn, and Ag were present in the WE used for irrigation. This indicated that the most used ENMs of $TiO_2$, ZnO, and Ag were possibly present in the reclaimed wastewater. Second, lifespan of *A. thaliana* was shortened by about a week but its biomass was not significantly increased. The shortened life cycle may have been caused by the NPs present in WE. Third, the mass of *E. fetida* was not significantly reduced due to WE irrigation. Fourth, microbial biomass was not significantly reduced, but the total number of the bacterial species was increased in the WE-irrigated soil; moreover, the relative abundances of species comprising the soil microbiome were shifted through WE irrigation. Indeed, the abundance of Cyanobacteria rose as manifested by the increased occupation of *Trichodesmium* spp. – a species that could elevate nitrogen fixation needed for *A. thaliana* growth. The NPs and metals in WE did not negatively affect the growth of *Trichodesmium* spp. in the soil. Given the trace amounts of a variety of components in the WE, besides the existence of metallic NPs in the water, it was difficult to correlate the biological activities to specific components in the WE. Finally, the interactions among different organisms – plants, earthworms, and soil microbiome in the soil microecosystem – warrant future investigations to decipher the influence of aged metallic NPs in reclaimed wastewater.

## Acknowledgements

This research is sponsored by the Startup (to Jia Liu), and the Elevated research 2.0 grant, Southern Illinois University Carbondale. Thanks are given to Dr. Honglan Shi, Missouri University of Science and Technology, for providing suggestions for NP soil extraction, and to Dr. Karen Renzaglia, Southern Illinois University for assisting reactive oxygen species detection. B.M.G. acknowledges support from the National Science Foundation (CHE 1416432).

## References

Ahmadi, L., Merkley, G.P., 2009. Planning and management modeling for treated wastewater usage. Irrig. Drain. Syst. 23, 97–107.
Allison, S.D., 2008. Chloroform fumigation direct extraction (CFDE) protocol for microbial biomass carbon and nitrogen.
Aristi, I., von Schiller, D., Arroita, M., Barceló, D., Ponsatí, L., García-Galán, M.J., Sabater, S., Elosegi, A., Acuña, V., 2015. Mixed effects of effluents from a wastewater treatment plant on river ecosystem metabolism: subsidy or stress? Freshw. Biol. 60, 1398–1410.
Auvinen, H., Kaegi, R., Rousseau, D.P.L., Du Laing, G., 2017. Fate of silver nanoparticles in constructed wetlands—a microcosm study. Water Air, Soil Pollut. 228, 97.
Bao, D., Oh, Z.G., Chen, Z., 2016. Characterization of silver nanoparticles internalized by Arabidopsis plants using single particle ICP-MS analysis. Front. Plant Sci. 7, 32.
Becerra-Castro, C., Lopes, A.R., Vaz-Moreira, I., Silva, E.F., Manaia, C.M., Nunes, O.C., 2015. Wastewater reuse in irrigation: a microbiological perspective on implications in soil fertility and human and environmental health. Environ. Int. 75, 117–135.
Bedbabis, S., Ben Rouina, B., Boukhris, M., Ferrara, G., 2014. Effects of irrigation with treated wastewater on root and fruit mineral elements of Chemlali olive cultivar. Sci. World J. 2014, 973638.
Belaid, N., Neel, C., Lenain, J.F., Buzier, R., Kallel, M., Ayoub, T., Ayadi, A., Baudu, M., 2012. Assessment of metal accumulation in calcareous soil and forage crops subjected to long-term irrigation using treated wastewater: case of El Hajeb-Sfax, Tunisia. Agric., Ecosyst. Environ. 158, 83–93.
Briones, A.M., Okabe, S., Umemiya, Y., Ramsing, N.-B., Reichardt, W., Okuyama, H., 2003. Ammonia-oxidizing bacteria on root biofilms and their possible contribution to N use efficiency of different rice cultivars. Plant Soil 250, 335–348.
China, M.o.E.P.o.t.P.s.R.o., 2012. National environmental protection standard of the Peoples' Republic of China. In Water quality determination of total nitrogen alkaline potassium persulfate digestion UV spectrophotometric method.
Council, N.R., 2012. Water Reuse: Potential for Expanding the Nation's Water Supply Through Reuse of Municipal Wastewater (In). National Academies Press.
Drury, B., Rosi-Marshall, E., Kelly, J.J., 2013. Wastewater treatment effluent reduces the abundance and diversity of benthic bacterial communities in urban and suburban rivers. Appl. Environ. Microbiol. 79, 1897–1905.
Durenkamp, M., Pawlett, M., Ritz, K., Harris, J.A., Neal, A.L., McGrath, S.P., 2016. Nanoparticles within WWTP sludges have minimal impact on leachate quality and soil microbial community structure and function. Environ. Pollut. 211, 399–405.
EPA, 2012. 2012 Guidelines for Water Reuse. U.S. Environmental Protection Agency, Washington, D.C.
Geisler-Lee, J., Brooks, M., Gerfen, J.R., Wang, Q., Fotis, C., Sparer, A., Ma, X., Berg, R.H., Geisler, M., 2014. Reproductive toxicity and life history study of silver nanoparticle Effect, uptake and transport in Arabidopsis thaliana. Nanomaterials (Basel, Switzerland) 4, 301–318.
Geisler-Lee, J., Wang, Q., Yao, Y., Zhang, W., Geisler, M., Li, K., Huang, Y., Chen, Y., Kolmakov, A., Ma, X., 2013. Phytotoxicity, accumulation and transport of silver nanoparticles by Arabidopsis thaliana. Nanotoxicology 7, 323–337.
Gottschalk, F., Sonderer, T., Scholz, R.W., Nowack, B., 2009. Modeled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag, CNT, fullerenes) for different regions. Environ. Sci. Technol. 43, 9216–9222.
Harris, E., 2015. Israel Bets On Recycled Water To Meet Its Growing Thirst (Parallels Many Stories, One World: NPR WSIU Radio).
Judy, J.D., McNear, D.H., Chen, C., Lewis, R.W., Tsyusko, O.V., Bertsch, P.M., Rao, W., Stegemeier, J., Lowry, G.V., McGrath, S.P., et al., 2015. Nanomaterials in biosolids inhibit Nodulation, shift microbial community composition, and result in Increased metal uptake relative to bulk/dissolved metals. Environ. Sci. Technol. 49, 8751–8758.
Kaveh, R., Li, Y.S., Ranjbar, S., Tehrani, R., Brueck, C.L., Van Aken, B., 2013. Changes in Arabidopsis thaliana gene expression in response to silver nanoparticles and silver ions. Environ. Sci. Technol. 47, 10637–10644.
Klitzke, S., Metreveli, G., Peters, A., Schaumann, G.E., Lang, F., 2015. The fate of silver nanoparticles in soil solution — Sorption of solutes and aggregation. Sci. Total Environ. 535, 54–60.
Lahive, E., Jurkschat, K., Shaw, B.J., Handy, R.D., Spurgeon, D.J., Svendsen, C., 2014. Toxicity of cerium oxide nanoparticles to the earthworm Eisenia fetida: subtle effects. Environ. Chem. 11, 268–278.
Laposata, M.M., Dunson, W.A., 2000. Effects of treated wastewater effluent irrigation on terrestrial Salamanders. Water Air, Soil Pollut. 119, 45–57.
Lee, W.-M., Kim, S.W., Kwak, J.I., Nam, S.-H., Shin, Y.-J., An, Y.-J., 2010. Research trends of ecotoxicity of nanoparticles in soil environment. Toxicol. Res. 26, 253–259.
Li, D., Fortner, J.D., Johnson, D.R., Chen, C., Li, Q., Alvarez, P.J.J., 2010. Bioaccumulation of 14C60 by the earthworm Eisenia fetida. Environ. Sci. Technol. 44, 9170–9175.
Madela, M., Neczaj, E., Grosser, A., 2016. Fate of engineered nanoparticles in wastewater treatment plant. Eng. Prot. Environ. 19, 577–587.
Martins, J., Peixe, L., Vasconcelos, V.M., 2011. Unraveling cyanobacteria ecology in wastewater treatment plants (WWTP). Microb. Ecol. 62, 241–256.
Maupin, M.A., Kenny, J.F., Hutson, S.S., Lovelace, J.K., Barber, N.L., Linsey, K.S., 2014. Estimated Use of Water in the United States in 2010 (U.S. Geological Survey Circular), pp. 56.
Miller, E.L., Nason, S.L., Karthikeyan, K.G., Pedersen, J.A., 2016. Root uptake of

pharmaceuticals and personal care product ingredients. Environ. Sci. Technol. 50, 525–541.
Mitrano, D.M., Lesher, E.K., Bednar, A., Monserud, J., Higgins, C.P., Ranville, J.F., 2012. Detecting nanoparticulate silver using single-particle inductively coupled plasma–-mass spectrometry. Environ. Toxicol. Chem. 31, 115–121.
Mizyed, N.R., 2013. Challenges to treated wastewater reuse in arid and semi-arid areas. Environ. Sci. Policy 25, 186–195.
Mohammad, M.J., Mazahreh, N., 2003. Changes in soil fertility parameters in response to irrigation of forage crops with secondary treated wastewater. Commun. Soil Sci. Plant Anal. 34, 1281–1294.
Neuhauser, E.F., Loehr, R.C., Milligan, D.L., Malecki, M.R., 1985. Toxicity of metals to the earthworm Eisenia fetida. Biol. Fertil. Soils 1, 149–152.
Pal, S., Tak, Y.K., Song, J.M., 2007. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the Gram-negative bacterium Escherichia coli. Appl. Environ. Microbiol. 73, 1712–1720.
Pérez-de-Luque, A., 2017. Interaction of nanomaterials with plants: what do we need for real applications in agriculture? Front. Environ. Sci. 5.
Qureshi, A.S., Hussain, M.I., Ismail, S., Khan, Q.M., 2016. Evaluating heavy metal accumulation and potential health risks in vegetables irrigated with treated wastewater. Chemosphere 163, 54–61.
Schwertfeger, D.M., Velicogna, J.R., Jesmer, A.H., Scroggins, R.P., Princz, J.I., 2016. Single particle-inductively coupled plasma mass spectroscopy analysis of metallic nanoparticles in environmental samples with large dissolved analyte fractions. Anal. Chem. 88, 9908–9914.
Sinha, R., Valani, D., Sinha, S., Singh, S., Herat, S., 2010. Bioremediation of contaminated sites: a low-cost nature's biotechnology for environmental clean up by versatile microbes, plants & earthworms. In: Faerber, T., Herzog, J. (Eds.), Solid Waste Management and Environmental Remediation. Nova Science Publishers, Inc, pp. 1–72.
Syu, Y.-y., Hung, J.-H., Chen, J.-C., Chuang, H.-w., 2014. Impacts of size and shape of silver nanoparticles on Arabidopsis plant growth and gene expression. Plant Physiol. Biochem. 83, 57–64.
Vasconcelos, V.M., Pereira, E., 2001. Cyanobacteria diversity and toxicity in a Wastewater Treatment Plant (Portugal). Water Res. 35, 1354–1357.
Wakelin, S.A., Colloff, M.J., Kookana, R.S., 2008. Effect of wastewater treatment plant effluent on microbial function and community structure in the sediment of a freshwater stream with variable seasonal flow. Appl. Environ. Microbiol. 74, 2659–2668.
Wang, P., Menzies, N.W., Dennis, P.G., Guo, J., Forstner, C., Sekine, R., Lombi, E., Kappen, P., Bertsch, P.M., Kopittke, P.M., 2016. Silver nanoparticles entering soils via the wastewater-sludge-soil pathway pose low risk to plants but elevated Cl concentrations increase Ag bioavailability. Environ. Sci. Technol. 50, 8274–8281.
Westerhoff, P., Song, G., Hristovski, K., Kiser, M.A., 2011. Occurrence and removal of titanium at full scale wastewater treatment plants: implications for $TiO_2$ nanomaterials. J. Environ. Monit. 13, 1195–1203.
Xiu, Z.-m., Zhang, Q.-b., Puppala, H.L., Colvin, V.L., Alvarez, P.J.J., 2012. Negligible Particle-specific antibacterial activity of silver nanoparticles. Nano Lett. 12, 4271–4275.
Yadav, R.K., Goyal, B., Sharma, R.K., Dubey, S.K., Minhas, P.S., 2002. Post-irrigation impact of domestic sewage effluent on composition of soils, crops and ground water—A case study. Environ. Int. 28, 481–486.
Zuluaga, N.L., 2016. Impacts of Engineered Nanoparticles ($TiO_2$, ZnO, Ag) on Aquatic Microbial Communities In Civil and Environmental Engineering. Southern Illinois University Carbondale.