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

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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

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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