Impact of Silver Nanoparticles in Wastewater on Heavy Metals Transport in Soil and Their Uptake by Radish Plants

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Abstract

In agriculture, wastewater is widely applied in irrigation due to its accessibility and nutrient content. Meanwhile, the threat of contaminants in wastewater must not be ignored. Depending on the origin and type of wastewater, concerns about its use for irrigation differ. Currently, with nanoparticles being introduced more and more into the environment, these could have various interactions with other existing pollutants. The interaction between nanoparticles and heavy metals is of particular interest because heavy metals are major pollutants in most types of wastewater. Studies about their interaction under a simple environmental matrix have been done, but there is a lack of information on the more complicated and realistic environmental situations.

To this end, this research aimed to study the interaction between a type of commonly-used nanoparticle (silver nanoparticles) and heavy metals in wastewater; specifically, its impact on soil and on a root vegetable (radish) was analyzed. Radish was grown under controlled conditions and irrigated with different treatments (freshwater, wastewater, freshwater with nanoparticles and wastewater with nanoparticles), while synthesized wastewater was premixed with synthesized silver nanoparticles before applying. The concentration and distribution of several heavy metals (Cd, Cr, Cu, Fe, Pb, Zn) were determined both in the soil and in plant tissues after 57 days of growing. In the soil matrix, there was no significant effect of nanoparticles on the heavy metal concentrations. But the existence of silver nanoparticles significantly increased ($p<0.05$) the concentration of several heavy metals in plant tissues, namely Cr (126.6%), Cu (30%), Fe (63.7%), Pb (71.9%) and Zn (81.1%). Moreover, the plant leaf performance (greenness, NDVI and photosynthesis) improved with the interaction of silver nanoparticles, while the plant biomass (radish) decreased by almost half. This is possibly due to the increasing concentrations of heavy metals in plant tissues, which caused toxicity to the plant growth.

These results proved that there was an interaction between silver nanoparticles and heavy metals and indicated that they could adversely affect crop growth by increasing the plant uptake of heavy metals. However, the mechanism of this interaction is still unclear and needs further research.

Résumé

Les eaux usées sont souvent utilisées dans l'agriculture grâce à leur accessibilité et aux substances nutritives. En même temps, on ne devrait pas ignorer la menace des contaminants dans les eaux usées. Les concernes diffèrent et dépendent des régions et des pollutions divers. En tant que contaminant nouveau, les nanoparticules sont introduites à l'environnement de plus en plus actuellement. Elles pourraient interagir avec les autres polluants qui existent déjà dans les eaux usées. Particulièrement, l'interaction entre la nanoparticule et les éléments-traces métalliques est d'intérêt, car l'éléments-trace métallique porte la majorité de pollution dans n'importe quel genre des eaux usées. Il y a des études disponibles sur l'interaction dans les matrices simples, mais on manque encore la connaissance sur les situations plus compliquées.

À cette fin, cette recherche avait visé l'interaction entre un type de nanoparticule (les nanoparticules d'argent) qui est utilisée assez souvent dans l'industrie et les éléments-traces métalliques dans les eaux usées. Notamment, leurs influences sur le sol et un légume-racine (le radis) ont été analysées. Les radis ont été cultivés sous les conditions contrôlées, avec les irrigations des eaux usées synthétisées et les nanoparticules d'argent avant d'application. La concentration et la répartition des certains éléments-traces métalliques (Cd, Cr, Cu, Fe, Pb, Zn) ont été analysées après 57 jours de cultivation, autant pour le sol que pour les radis. Dans le sol, il y n'avait pas d'effet des nanoparticules d'argent sur les concentrations des éléments-traces métalliques. Pourtant, l'existence des nanoparticules d'argent a augmenté ($p<0.05$) les concentrations des éléments-traces métalliques dans les radis. Les accroissements étaient presque 126.6%, 30%, 63.7%, 71.9% et 81.1% pour Cr, Cu, Fe, Pb et Zn. De plus, la biomasse des radis a diminué malgré l'amélioration du fonctionnement des feuilles. Cela est probablement à cause de l'accroissement des concentrations des éléments-traces métalliques dans les radis qui a provoqué une toxicité.

D'une part, ces résultats ont démontré l'interaction entre les nanoparticules d'argent et les éléments-traces métalliques. D'autre part, ils pourraient menacer les croissances des plantes sous cette condition. Cependant, le mécanisme de cette interaction n'est pas encore clair. Plus de recherches sont nécessaires dans le futur.

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Contribution of Authors

The author of this thesis was responsible for the field experiment conduct, data collect and analysis, and manuscript preparation. Dr. Shiv Prasher is the thesis supervisor and gave such advice on experiment conduct. He was also involved in editing and reviewing this thesis.

Dr. Ramanbhai Patel provided advice during the field experiment, he was also involved in editing and reviewing the manuscript.

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Acronyms

Ag NP: silver nanoparticle

BAF: bioaccumulation factor

CCME: Canadian council of ministers of the environment

CMC: carboxymethyl cellulose

CNT: carbon nanotube

DTPA: diethylenetriaminepentaacetic acid

EU: European union

LMWOA: low molecular weight organic acid

nHAP: nano-hydroxyapatite

nZVI: zero-valent iron nanoparticles

NDVI: normalized difference vegetative indexes

NM: nanomaterial

NMO: nano-sized metal oxide

NP: nanoparticle

USEPA: United States Environmental Protection Agency

Chapter 1: Introduction

Due to the growth of world population and industrialization, the demand for freshwater is growing while the production of wastewater is also increasing. As there is an acute shortage of freshwater for irrigation of crops, use of wastewater in agriculture is becoming more and more common in many countries. The nutrients contained in wastewater could be a good source for improving soil fertility and crop growth, but the contaminants such as heavy metals and other organic pollutants also bring up safety and health concerns. Furthermore, with modern industries releasing large amounts of nanoparticles (NPs) into the wastewater, there is concern about the interaction of NPs in wastewater with other pollutants, such as heavy metals, and their effects on soil and, in turn, the influence on crops when used for irrigation.

1.1. Silver nanoparticles in agriculture

According to the Woodrow Wilson Database, among all the nanomaterial (NM) incorporated consumer products, over 25% are composed of silver nanoparticles (Ag NPs) (Fabrega et al., 2011). These products are commonly used in personal or health care products, and the textile industry.

During the synthesis of Ag NPs, as well as manufacture and use of nanoparticle-incorporated products, there is an increased risk that Ag NPs will be released into the environment. Once NPs are released, they could be transformed into more stable chemical forms in the wastewater treatment plant or they could either end up in the sewage sludge or reach water supplies. Several studies have reported on various pathways of Ag NPs entering and existing in the environment (Benn and Westerhoff, 2008; Farkas et al., 2011; Kaegi et al., 2010). Gottschalk et al. (2009) predicted, using a model study, that the exposure concentration of Ag NPs in sludge-treated soils could be as high as 1581 ng kg^{-1} yr^{-1}. In many regions where wastewater irrigation or sludge amendments are commonly applied in agriculture, the risk of Ag NPs existence would increase. Although according to the United States Environmental Protection Agency (USEPA) guidelines, the maximum permissible concentration for Ag ions is 3.2 and 1.9 μg/L for freshwater and salt water, respectively, currently there is no criteria for Ag

NPs. This is partly because their toxicity and influence on the environment are still little known.

Ag NPs' morphology partly contributes to its uptake and transport in living cells, and results in cell aberrations or DNA damage (Fabrega et al., 2011). Due to its small size, the direct contact between particles and plants allows them to penetrate cell membranes, either through stomatal pores by aerosol particles or through root uptake. While the small particles tend to agglomerate and thus, become trapped in the root zone, they, in turn, could interact with the rhizosphere microbiome and influence plants' nutrient uptake, thereby affecting plant growth in an indirect way.

It is widely agreed that the toxicity of Ag NPs is not only NPs' morphological and dose dependent, but also Ag speciation dependent (Koser et al., 2017), because the release of Ag ions induces most of the influence on organisms (Lodeiro et al., 2017). Ag ions are known to have an antibacterial and anti-fungal capacity and therefore are often utilized in the pharmaceutical industry. Nevertheless, it could exhibit toxicity to many other living organisms. A gene expression study by Kaveh et al. (2013) found that changes in the plant's gene expression caused by Ag NPs overlapped with those caused by Ag ions, suggesting part of the effects of Ag NPs are initiated by the release of Ag ions. In general, the pollution of Ag NPs in the environment could cause different phytotoxicity to plants, including seedling growth, root and shoot growth (Lee et al., 2012; Nair and Chung, 2014; Thuesombat et al., 2014), etc.

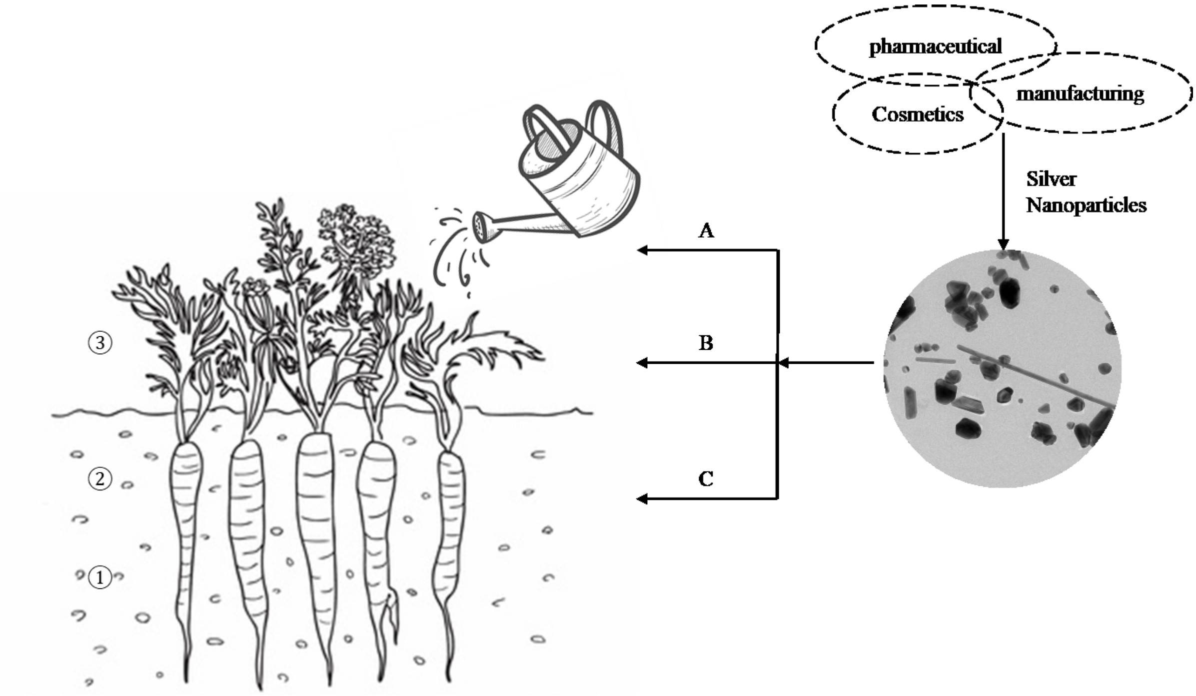

Figure 1 Silver nanoparticles transport in agricultural scenario

Note A: through irrigation application; B: through evaporation-transpiration in the form of aerosol particles; C: through soil transport; ①: enter plants root and affect the according zone; ②: affect the rhizosphere microbiome; ③: transport and accumulate in the above ground part. Picture of radish from https://www.education.com/ ; picture of watering can from https://www.vectorstock.com/.

1.2. Heavy metals in wastewater irrigation

Wastewater (either treated, partially-treated or untreated) irrigation is exploited worldwide. Not only because wastewater is more accessible, but also because it contains many elements that could provide a nutrient source for the crops. Depending on the type of wastewater used for the irrigation, there could be different effects on the environment and crops.

Studies (Ahmed and Al-Hajri, 2009; Kiziloglul et al., 2007) have proved that wastewater irrigation would alter some soil properties, such as decreasing pH, thus, increasing electrical conductivity and organic matter (Maldonado et al., 2008). It may increase the plant biomass by restoring nutrient content in soil, but seldom impedes crop growth.

According to some field studies in developing areas where wastewater pollution is a serious problem, wastewater irrigation introduces large amounts of heavy metals into soil which eventually translocate to crops. It could alter the physiological and morphological properties of the plants by decreasing the dry matter content or changing the nutrient portion (Keser, 2013). The significant increase of heavy metal concentration in the edible parts of the plants also poses health risks (Asgari and Cornelis, 2015; Khan et al., 2013; Ma et al., 2015). Under prevailing freshwater scarcity, the use of wastewater for irrigation is inevitable, and therefore it is highly necessary to consider associated health risks with its use.

1.3. Interaction of silver nanoparticles and heavy metals

As Ag NPs are being produced and released into the environment, another concern arises with wastewater irrigation and that is the possible interaction of Ag NPs and heavy metals.

Studies have shown that under aqueous solution, Ag NPs could interact with heavy metals. For example, Zuo et al. (2015) proved that Ag NPs increased the removal capacity of bioavailable Cd from the solution. Other research showed that different kinds of NPs or NMs could interact with heavy metals under aqueous solution or even in a complicated soil environment (Ahmadi et al., 2017; Bhowmik et al., 2017; Ghorai et al., 2012; Hughes et al., 2018). These findings shed light on the possible interaction between Ag NPs and heavy metals, but interaction may vary depending on the physicochemical and environmental conditions. Nevertheless, information on the interaction of NPs with heavy metals in wastewater, and the effects of using such wastewater on agricultural soil and crops is still sparse. Therefore, it is important to understand the effect of NPs on the transport of wastewater borne heavy metals in soil and their translocation into different parts of the plants, especially root vegetables which come in direct contact with irrigation water.

1.4. Objectives

Although there are some studies that focused on the interaction of NPs and heavy metals, they were mostly done under an aqueous solution. Current research tends to be more laboratory-based; there is little information available for more realistic scenarios. Also, when it comes to the soil environment which is more complicated, and even the possible influence on plants and soil, a lot of work still needs to be done.

This study aimed to determine if there is an interaction between Ag NPs and heavy metals contained in irrigation wastewater, and the effect of NPs on the transport of heavy metals in soil and plants. The influence of NPs on plant growth was also of interest.

The goal of this study was achieved through the following specific objectives:

1. To determine the effect of Ag NPs on heavy metal movement in soil due to wastewater application;
2. To determine Ag NPs' impact on heavy metal uptake by plants from irrigation with wastewater;
3. To determine if the plant growth would be affected by these two categories of chemicals and their possible interactions.

Correspondingly, the hypotheses of the study were:

1. H0: Ag NPs existence in wastewater irrigation does not change the transport of heavy metals in soil;

   H1: Ag NPs existence in wastewater irrigation does change the transport of heavy metals in soil.

2. H0: Ag NPs existence in wastewater irrigation does not change the accumulation of heavy metals in plant tissues;

   H1: Ag NPs existence in wastewater irrigation does change the accumulation of heavy metals in plant tissues.

3. H0: under wastewater irrigation, Ag NP does not affect the plant growth;

   H1: under wastewater irrigation, Ag NP does affect the plant growth.

1.5. Thesis organization

This dissertation is comprised of 6 chapters as follows:

Chapter 1 Introduction: this chapter provides a general background of Ag NPs and the existence of heavy metals in the agricultural scenario, specifies the knowledge gap and research objectives.

Chapter 2 Literature review: a synopsis of current knowledge regarding NPs and the existence of heavy metals. Their interactions in various environments are also discussed.

Chapter 3 Impact of Ag NPs in wastewater on heavy metals transport in soil and their uptake by radish plants: this chapter presents a field research and discusses the results.

Chapter 4 Conclusion: this chapter presents general conclusions and conclusions corresponding to each of the objectives drawn from the study; directions for future studies are also suggested.

Chapter 5 Bibliography: all references in this thesis are included in this chapter.

Chapter 6 Appendix: information pertinent to this research, not included in preceding chapters, is given in the Appendix.

Chapter 2: Literature Review

With wastewater irrigation being applied in agriculture worldwide, several studies have documented the presence of contaminants in wastewater, their transport to soil and translocation to agricultural crops which could cause health risks.

Among the contaminants in wastewater, NP is a special type because of its small dimension and different properties than that of its bulk counterpart. Studies about the NPs' presence and influence in agriculture are of interest due to the development of nanotechnology; both the advantages (e.g. wastewater treatment) and disadvantages (e.g. toxicology of NPs) have been widely investigated in different situations, except in agricultural field conditions.

However, nanotechnology is still an industry in its early development; the knowledge about NPs' field application needs to be enriched. There is some published literature regarding heavy metals and NPs interaction in water or soil, but little is known about the interaction in agricultural soil-water-plant system. Such environment is present in many parts of the world where NP-contaminated wastewater irrigation is applied. The fate and transport of various contaminants in soil and their effects on plants, especially heavy metals as affected by NPs, need thorough investigation.

This literature review introduces NP and heavy metal in the context of agriculture and focuses on the interaction between NPs and heavy metals in light of the current knowledge gap.

2.1. Nanoparticles

2.1.1. Definition and sources of nanoparticles

In nanoscience, NPs refer to the ultrafine particles that have at least one dimension under 1-100 nm in size (Ghosh and Pal, 2007). According to this definition, NPs could be found everywhere in the environment and in our daily life. Classified by their origins, NPs could be divided into non-engineered or natural NPs and engineered NPs, where natural NPs are generated by natural events such as incomplete combustion or even the metabolic pathways of microorganisms. Compared to natural NPs, engineered NPs are of importance, as they are produced by human activities and exist in various fields, such as agriculture, physics, chemistry, medicine and other sciences or engineering fields. Engineered NPs include different types of NPs ranging from inorganic (metals, metal oxides, salts, and aluminosilicates) to organic (fullerenes and carbon nanotube (CNT)) (Table 1) (Nowack and Bucheli, 2007), and are found in many materials or products such as cosmetics, sunscreens, clothing, wood stains or sports equipment (Nel et al., 2006).

Table 1 Classification of nanoparticles

Classification		Examples	Reference
Non-engineered nanoparticles	Inorganic	Biogenic/geogenic/atmospheric metals/oxides	(Nowack and Bucheli, 2007)
	Organic	Humic, fulvic acids, fullerenes, CNT	
Engineered nanoparticles	Inorganic	Metal (Ag, Au, Fe…)	(Subbenaik, 2016)
		Metal oxides (TiO_2, SiO_2, CeO_2…)	
		Non-metals (silica)	
	Organic	Carbons (fullerene, CNT)	
		Polymers (alginate, chitosan…)	
		Lipids (lecithin, stearic acid)	

2.1.2. Nanoparticle characteristics

2.1.2.1. Physical and chemical characteristics

The size of the NP is the most essential parameter that determines its properties, predominantly, because the small dimension gives the NP a very high area to volume ratio. According to surface catalytic analysis, higher surface area particles are much more active in general, and they possess excess energy at the surface. NMs are more unstable than their bulk counterpart, and are often used as catalysts for various chemical reactions (Bowker, 1995). Other than their size, the shape of the NP also contributes to its unique physical properties. For instance, under the same size, the larger the percentage of edge and corner atoms a NP has, the more active it would be (Narayanan and El-Sayed, 2004).

Unique size and shape also cause NPs to have very interesting electronic and optical properties. When the particle size is reduced to fewer atoms compared to its bulk counterpart, the electronic properties, such as conductivity and collective magnetism, would change dramatically since the number of electrons is determined by the particle size. This could even lead to the metal to non-metal transition depending on the metallicity criterion (Banin and Millo, 2005). Due to their special optical properties, many NPs exhibit a different color than their bulk counterparts. For example, Ag NPs appear greyish green in solution, and they are often very photosensitive, so they are used as photocatalysts (Yu et al., 2012).

As defined by its size range, NPs follow the colloid (which include particle size from 1 nm to 1 μm) theories of aggregation (i.e. irreversible) and agglomeration (i.e. reversible). Because of their high reactivity and colloidal instability, the particles tend to interact and attach to each other, and in some cases even scale up to lose their nano-size, especially in solution (Zhang, 2014). Many parameters, such as size, shape, surface coating characteristics of NPs, and other environmental parameters (pH, organic matter) could have an impact on the aggregation process of NPs.

As the dispersion and aggregation of NPs in the liquid phase is due to their surface interaction, the surface chemistry and structure is another essential property of NPs. Small size and high surface area contribute to the activity and instability of NPs, while for engineered NPs there exists more ways to manipulate them, including surface modification and structure control. During NP synthesis, different surface coating methods are often used to prevent or slow down the aggregation process and maintain NP stability. The other reason is to give the NP different surface functionalities (Kittler et al., 2009; Levard et al., 2011). For example, an application by Iijima (2018) used a silane coupling agent to modify TiO_2 NPs surface, significantly improving its stability in redispersion. All particles have surface and internal structures, while engineered NPs have an active state for the surface layer, whereas they could be very different when it comes to their internal structures. Microporous material such as zeolite has been used as a powerful adsorbent due to its unique structure; many nanocomposites are also used as catalyst supports to maintain control during catalyzing because of their porous structures (2018b). Depending on their characteristics, different combinations of surface and internal structures could be suitable for various pharmaceutical applications, such as transporting different types of drugs (2018a).

2.1.2.2. Nanoparticle toxicity

For the past two decades, NPs have entered the environment following the inception of NMs and nanotechnology. There have been many studies about the influence of NPs on the organism and the surrounding environment. While NPs or NMs are indeed very promising technological tools in many fields, and sometimes could even boost the performance of organisms in certain situations, there is evidence of side effects or toxicity of NPs when they enter the environment.

Compared to their bulk counterparts, NPs' toxicity often exhibits in a different way. Because they are small enough to easily enter plant cells and cause cellular damage (Love et al., 2012), they are more active with stronger ability to release bio-toxic ions.

The toxicity of NPs is determined by multiple factors. First, with the decrease in size and the increase in surface area as well as reactivity, toxicity increases. Aggregation also affects their toxicity, since it changes not only the size of the particle but also the properties and mobilities in the environment. According to some in-vitro studies, the agglomerated NPs could be more toxic since they accumulate more in cytosol and lysosomes (Monteiro-Riviere et al., 2005; Wang et al., 2011; Wick et al., 2007). On the other hand, engineered NPs present more toxicity biologically and ecologically than natural NPs, because the surface functionalization (e.g. capping agents) would also influence the property by altering their hydrophobicity, bioavailability or solubility. Other factors such as concentration and dispersing media could contribute to its toxicity as well (Singh, 2015). In the field of agriculture, NPs could cause toxicity to soil microbiome and plants.

A) Soil microbiome

Many NPs have anti-microbial properties, some due to the toxicity and anti-bacterial characteristics resembling parent heavy metals and the release of ions (Ag and Au); others could be due to the reaction with peroxides and the generation of highly toxic free radicals that kill soil microbiomes (Saliba et al., 2006). Some incubation studies found that Ag NPs, Zn NPs and ZnO NPs had effects on dehydrogenase activity, and they inhibited colony growth of some plant beneficial bacteria (Kim et al., 2011; Murata et al., 2005). This impact on soil microbiome could alter the soil eco-cycle, affect soil quality and indirectly influence crop growth (Dinesh et al., 2012).

B) Plants

The large amount of engineered NPs released into the environment could pose a serious problem in agriculture, especially for plants which are at the base trophic level of the food chain and have direct contact with soil that may contain engineered NPs from wastewater irrigation or sludge amendment.

To better understand engineered NPs' influence on plants, the toxicity of engineered NPs for plants and its possible transport pathway should be assessed. NPs impact on plants varied depending on its concentrations, sizes, and even the plant species and external environment (Rico et al., 2011). In some studies, NPs decreased the germination rate, inhibited photosynthesis and hydro conductivity, and hampered plant growth and reproduction. While in other cases NPs increased root growth (Arruda et al., 2015). Although mechanisms causing toxicity to plants are unclear, engineered NPs cause not only cellular toxicity but also genotoxicity, causing an adverse impact on plants.

The concerns about NPs existence in the agricultural system focus not only on its possible toxicity to plants, but also on its uptake and accumulation in plant tissues. Since the accumulated NPs in edible plants would end up being consumed by animals, including humans. NPs could enter the plant cells through different pathways, depending on its properties. So far, it is known that they could bind to carrier proteins, go through aquaporins, ion channels, through endocytosis, or even bind to organic chemicals to form chemical complexes in the environment. CNTs are able to interact with the proteins and polysaccharides on the cell wall and create new pores to enter the cell and thus, be responsible for more damage; while metal oxide NPs usually tend to be larger in size, easily aggregated and attached to the soil, thus, more difficult for plant uptake (Rico et al., 2011). Metallic NPs could damage plants by releasing more toxic ions (Stampoulis et al., 2009). After entering the plant, NPs would accumulate and translocate inside plant tissues. Studies showed that Ag NPs could accumulate on the surface of root cell organelles and also be transported into stems (Gardea-Torresdey et al., 2003; T. Harris and Bali, 2008). Lin et al. (2009) demonstrated that NPs could be inherited by the next generation.

2.1.3. Nanoparticles in the environment

The large number of NPs present in the environment is alarming. A model-based estimation showed that human activities generate TiO_2 NPs, Ag NPs and CNTs up to 5000, 500, 350 t/year, respectively (Mueller and Nowack, 2008).

The amount and pathway of the NPs released from different products depend on the properties of the products. In general, longer lifetime, higher usage, strong fixation or binding of NPs to the product would reduce the amounts of NPs released into the environment when the product is disposed. Mueller and Nowack (2008) predicted that for Ag NPs, a large portion would be released into the soil and sewage treatment plants, TiO_2 mainly end up in sewage treatment plants, while CNTs end up in water isolation plants.

After being released into the environment, the fate of NPs is mainly determined by some basic physicochemical and electronic properties including solubility, hydrophilicity, and lipophilicity. Under a hydro environment, NPs could either dissolve, settle down or bind into other particles; they can also escape from water treatment processes and enter natural water bodies. A case study (Praetorius et al., 2012) using models predicted that TiO_2 NPs could aggregate with suspended particle matter in rivers, making it possible to be removed quickly. In the soil which is a more complex matrix, there are many factors, such as pH, soil organic matter, and soil type that could affect the fate of NPs (Singh, 2016). The characteristics of NP such as size, surface charge and aggregation also play important roles in its transport in soil. For example, aluminum NPs transport in soil is in reverse proportion to its agglomerated size; particles with the same charge as the environmental matrix are easier to transport (Darlington et al., 2009).

2.2. Heavy metals in agriculture

Although heavy metals naturally exist in the soil, human activities aggravate heavy metal contamination, especially in the mining industry, in biosolid amendment and wastewater irrigation. Wuana and Okieimen (2011) stated that heavy metals like Pb, Cr, Zn, Cd, Cu and Hg persist in the soil for a very long time once introduced; they could change their chemical forms and become more or less bioavailable; later they affect soil microbiome and plants, and sometimes are gradually washed into the groundwater system. But most alarming is that they end up accumulating in the food chain.

In the agricultural system, the toxicity of heavy metal to crops and their accumulation in edible parts are mainly considered, because they would enter the food chain. As plants uptake the bioavailable form of heavy metals from soil, they could directly hamper plant metabolism by affecting enzyme activity or causing oxidative damage (Nagajyoti et al., 2010). Just like NPs, heavy metals could also affect plant growth by killing the microorganisms that are beneficial and essential for plants; the soil microbiome would be changed and no longer be an advantage for the plants (Chibuike and Obiora, 2014). It is worth noting that the uptake of heavy metals by plants is determined not only by their bioavailability in soil solution, but also by the plant species. To this end, phytoremediation is widely employed using heavy metal tolerant plants for heavy metal removal; these plants could tolerate heavy metal accumulation in their cells (Thakur et al., 2016).

There are several methods for remediation of heavy metal contaminated soil, including isolation, immobilization, toxicity and mobility reduction, physical separation and extraction (Wuana and Okieimen, 2011). Among these methods, bioremediation or phytoremediation is commonly used due to its cost effectiveness. Microorganisms (e.g. bacteria) and plants could uptake heavy metals or transform them into another speciation that is less bioavailable and less toxic, but these methods are time consuming because this is a natural process. Biochar, as a powerful sorbent, has also been introduced for soil remediation because of its capacity to immobilize heavy metals (Ahmad et al., 2014).

2.3. Nanoparticle and heavy metal interaction

2.3.1. Aqueous solution

NPs and various related nano-sized products are widely used as adsorbents for heavy metal removal in aqueous solutions, such as in wastewater treatment plants.

There are many factors that could contribute to NPs' adsorption or removal capacity for heavy metals. Internal parameters such as the morphology of NPs, and external factors such as the pH, the temperature of the environment and the contact time, can affect removal capacity of NPs. It is also widely accepted that different NPs have distinctive properties that enable them to remove heavy metals from an aqueous solution.

2.3.1.1. Nanoparticles

Nano-sized metal oxides (NMOs) have a high capacity and selectivity to adsorb heavy metals due to their large surface area. They possess complex structures which allow for quick adsorption of metal ions and further intraparticle diffusion. It should also be noted that their high surface energy could lead to instability, aggregation and thus, a decrease in those favorable adsorption properties (Hua et al., 2012). Nevertheless, the inevitable instability of NPs necessitates research on more stable nano-composites.

Other than NMOs, CNT is another promising adsorbent due to its physical characteristics. CNT combines the advantages of active carbon and NP, having high porosity and loosely layered structures. With the addition of functional groups which could interact actively with metal ions, CNTs are widely used as adsorbents for heavy metals (Gupta et al., 2016).

2.3.1.2. Nano-composites

Because of the intrinsic instability of NPs, more studies were directed towards synthesizing nano-composites or functionalized NPs which combined multiple properties of different materials to better achieve the goal of heavy metal removal.

Apart from the adsorption ability, separation and regeneration are also important factors to determine the applicability of a nano-based adsorbent. For this reason, magnetic or zero-valent iron nanoparticles (nZVI) are usually considered in wastewater treatment with complementary functionalization. For example, chitosan magnetic nano-composites are effective in removing Cu^{2+}, Pb^{2+}, and Cd^{2+} from aqueous solutions and are powerful adsorbents; not only because chitosan would interact with heavy metals, but also due to its reversibility which enables quick and easy regeneration of the adsorbent by simply using external magnets (Liu et al., 2009). Ahmadi et al. (2017)

optimized the condition for chitosan coated nZVI to remove Cd; they also proved the reusability of this kind of NMs without secondary pollution.

Different supporting materials could also boost the NPs adsorption performance, by impregnating NPs in porous materials, by decreasing the aggregation/agglomeration possibility, and even by co-functioning as an adsorbent due to their unique structures. Other than natural materials, such as bentonite or porous polymer materials, engineered biochar (Yang et al., 2018) is also considered by many researchers as a suitable support material, owing to their hierarchical pores which lead to precipitation and reduction of heavy metals, thereby, complementing the adsorption process.

2.3.2. Soil environment

2.3.2.1. Mechanism of heavy metal removal by nanoparticles

Apart from the aqueous solution, there are several studies on heavy metal removal by NPs in the soil environment. Different kinds of NMs have been investigated, including simple NPs and their functionalized or composited counterparts. The various characteristics give them distinct mechanisms for heavy metal removal.

While the mechanism for single NPs is simply the adsorption of metal ions by the particles, nano-composites depend more on additional properties, such as support materials and functionalization. A summary of some studies on different functionalization or augmentation of NMs is presented in Table 2.

Organic acids in the soil could release heavy metals and make it easier for NMs' adsorption. After being released from the soil with the help of acids, metal ions could be precipitated and reduced to a more stable form by NMs, rather than stay in insoluble states (Taghipour and Jalali, 2016). For example, Wang et al. (2014) found that when citric acid was combined with nZVI, Pb removal efficiency was increased by nearly 60%-80%. Furthermore, when fluvic acid is adsorbed into nano-hydroxyapatite (nHAP), it could bind Cd ions and facilitate the removal efficiency by providing a negative charge and decreasing the possibility of NP aggregation (Li et al., 2019). Yet, there are many other materials such as starch, carboxymethyl cellulose (CMC), silica fume, diethylenetriaminepentaacetic acid (DTPA) that could work as a stabilizer or facilitator in removing heavy metals from the soil.

Table 2 Summary of different functionalized nanoparticles

Category		Influence	Reference
Organic acid	Low molecular weight organic acid (LMWOA), e.g. citric acid, malic acid	LMWOA could extensively increase the Pb removal efficiency by nZVI	(Wang et al., 2014)
	Citric acid, oxalic acid	Organic acid release Cr from soil and increase the efficiency of its adsorption to NMs	(Taghipour and Jalali, 2016)
	Fulvic acid	Fulvic acid facilitate the transport of nHAP through soil	(Li et al., 2019)
CMC		CMC functions as a stabilizer for FeS NP	(Wang et al., 2018)
Silica fume		Silica fume supports nZVI and inhibits Fe/Cr precipitation forming on NPs' surface	(Li et al., 2011)
DTPA		Capable of extract a wide range of mixed heavy metals	(Hughes et al., 2018)

2.3.2.2. Influence on heavy metal speciation

It is well understood that NPs could affect metals speciation in the soil matrix, which is also the main mechanism for metal removal. Wide ranging studies proved that NPs could decrease the exchangeable, carbonate fractions of metals in the soil. They transfer the metals into iron-manganese oxides, organic matter and residual fractions which are more stable, less soluble and bioavailable to plants (Liu and Zhao, 2007a, b; Mohamadiun et al., 2018).

Use of NMs for heavy metal removal in soil is becoming a trend, due to their capacity, selectivity, and reusability, but the possible toxicity and secondary pollution of NMs should also be taken into consideration. The complex matrix effect of the soil environment on the removal process of NMs should also be considered. To this end, more studies about NMs' fate and interaction in the soil need to be performed.

2.3.3. Mixed environmental matrix

The individual effects of NPs and heavy metals on agriculture have been studied, as well as their interaction in a simple environmental matrix (water, soil). But little is known about the way in which all of these factors add up in the agricultural scenario. NPs are released into the environment, enter waterbodies and the soil matrix, and interact with heavy metals present in the environment. How this whole process would affect the environment, crops and consumers requires more attention and investigations.

In the current study, NPs and heavy metals were combined in the agricultural environment in which crops were introduced. The goal of this study was to find out if any interaction exists between NPs and heavy metals, and how it would affect the plant.

Chapter 3: Impact of Silver Nanoparticles in Wastewater on Heavy Metals Transport in Soil and Their Uptake by Radish Plants

Abstract

Engineered nanoparticles are being released into the agricultural environment without a full understanding of their ecosystem toxicity; there is no clear knowledge of their possible interactions with other chemical compounds. In a pot study, radish was grown under controlled environmental conditions and irrigated with synthesized wastewater containing various heavy metals, with or without silver nanoparticles. Soil samples were collected 30, 45, 51 and 56 days after seeding, along with plant samples after harvesting (Day 57) for heavy metal analysis. Analysis showed that there was no significant difference in heavy metal (Cd, Cr, Cu, Fe, Pb, Zn) concentration in soil collected from pots irrigated using wastewater, with or without silver nanoparticles. However, the concentrations of heavy metals (Cr, Cu, Fe, Pb, Zn) significantly increased ($P<0.05$) in different plant tissues under wastewater irrigation with silver nanoparticles; a decrease in radish weight was also noticed for this treatment. The concentration of Cd in radish exceeded acceptable level (0.1 mg/kg) in both treatments with no significant difference. Other than the possible health risk posed by nanoparticles and heavy metals, very little is known about the interaction mechanisms and their environmental impact; thus, more studies are needed in this area.

Keywords: silver nanoparticles, heavy metals, wastewater irrigation, radish

3.1. Introduction

It has been two decades since nanotechnology was introduced to industrial uses. NPs and NMs are being produced and exploited in various industries because of their novel properties. Among organic NPs (i.e. CNT) and inorganic NPs (TiO_2 NPs, Ag NPs, etc.), Ag NPs is one of the most commonly used NPs in consumer products and the fastest growing group of NMs (Fabrega et al., 2011). They are used in textile industry, in the medical field for diagnosis and drug delivery, for personal products such as cosmetics, in the food industry such as food packaging, catalysis in the chemical field and also the environmental scenario (Keat et al., 2015). Because of their wide industrial use, large quantity of Ag NPs is released into the environment. According to an estimation from a model study (Mueller and Nowack, 2008), human activities generate Ag NPs by 500 t/year, and most of the Ag NPs end up in the soil or in sewage treatment plants.

With the alarming existence of Ag NPs in the environment, concerns about their influence are increasing. Just like other NPs, Ag NPs have very distinct physical and chemical properties due to their small size, including optical, electrical and catalytical properties (Frattini et al., 2005). Specifically, Ag NPs are known for their antimicrobial characteristics, which are mainly due to the release of Ag ions. This biotoxicity could damage cell membranes and DNA; there is serious concern that they could profoundly affect the soil microbiome and possibly plants and animals (Durán et al., 2016). A study by Oukarroum et al. (2013) proved that Ag NPs toxicity inhibited the growth and viability of an aquatic plant. The combined influence on the soil microbiome and plants could have damaging effect on agriculture.

Irrigation with wastewater that contains NPs is another threat. Despite the high concentration of heavy metals and NPs in wastewater, they are widely used for irrigation due to the shortage of freshwater and disposal problem of wastewater. Effect of heavy metals alone on soil and plants have been investigated through years. After introduced to the agricultural land by irrigation with wastewater or application of sewage sludge, heavy metals are absorbed by plants, and they can translocate and accumulate in every part of the plant. Consumption of such crops pose health risks (Amin et al., 2013). Meanwhile, the uptake of heavy metals by plants is selective. For example, Roy and McDonald (2015) reported higher accumulations of Pb and Zn in radish root as compared to other heavy metals. This is indicative of selectivity of plants for translocation and accumulation of different heavy metals, and hence, the health risk assessment needs to be evaluated for different crops. Root vegetables come in direct contact with pollutants present in irrigation water or soil, and therefore are highly prone to the danger of pollutants.

With the introduction of NPs, there could be interaction with heavy metals and complex effect on soil microorganisms and the plants. The interaction between heavy metals and NPs has been studied under different matrices. In a simple aqueous solution, the research target is mainly heavy metal removal by NPs. Zuo et al. (2015) found that the biological removal capacity of Cd was in direct proportion to the concentration of Ag NPs introduced into the solution. Other studies investigated the possibility of heavy metal removal by surface-functionalized nano-composites (Ahmadi et al., 2017; Liu et al., 2009), proving that they could be more stable and powerful as adsorbents in solutions. In the soil environment, NPs affect metal speciation and contribute to heavy metal removal. By transforming more soluble and bioavailable fractions to stable residuals, the heavy metals tend to stay in the soil rather than being absorbed by plants (Liu and Zhao, 2007a, b; Mohamadiun et al., 2018).

Studies on the impact of heavy metals and NPs, and their interaction under some simple matrices are available. But one of the main bottlenecks in this field is the understanding of soil-plant-heavy metal interactions when irrigation water also contains NPs, in addition to heavy metals. Therefore, the current study was carried out to understand the interactions between heavy metals and NPs in wastewater used for irrigation, and their effects on the transport of heavy metals in soil and their translocation to different parts of the plant.

3.2. Material and Methods

A pot experiment was conducted in the summer of 2018. The site was located at the Macdonald campus farm of McGill University (45°24'47.9"N 73°56'30.3"W), Ste. Anne de Bellevue, QC, Canada. Radish was planted and irrigated with synthetic wastewater and silver nanoparticles; after harvesting, the soil and different plant tissues were analyzed in the lab for heavy metals.

3.2.1. Synthetic wastewater

The wastewater used in this experiment was synthesized in the laboratory. All constituents and their corresponding concentrations were based on the worst-case environmental scenario reported in literature. Basic wastewater constituents (nitrogen, carbon, phosphorus sources and minerals) and heavy metals (Cu, Zn, Cd, Pb, Cr, Fe) were included in the synthetic wastewater (Table 3). All the analytical chemicals and standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientific (Waltham, MA, USA). Stock solution was prepared using these constituents and stored in laboratory. To remove chlorine from irrigation water, tap water was filled in a container and left open for one day before preparing the irrigation wastewater. Predetermined volume of stock solution was thoroughly mixed in fixed volume of tap water to prepare the synthetic wastewater.

Table 3 Components and concentrations in synthetic wastewater

Purpose	Substance/ compounds	Concentration (mg/L)	Source
C source	Na Acetate	79.37	(Nopens et al., 2001)
	Milk powder	116.19	
	Soy Oil	29.02	
	Starch	122	
	Yeast Extract	52.24	
N Source	NH_4Cl	12.75	
	Peptone	17.41	
	Urea	91.74	
P Source	$Mg_3O_8P_2$	29.02	
Minerals	CaCl2	60	(LaPara et al., 2006)
	NaHCO3	100	
Heavy Metals	Chromium (Cr)	2	(Ahmad et al., 2011)
	Cadmium (Cd)	5	
	Lead (Pb)	16	
	Iron (Fe)	120	
	Zinc (Zn)	3	
	Copper (Cu)	8	

3.2.2. Synthesis of silver nanoparticles

Ag NPs were synthesized using silver nitrate and sodium citrate dihydrate, following a modified Turkevich method (Kimling et al., 2006). Briefly, 1 mM aqueous solution of silver nitrate and 10 mM trisodium citrate solution were prepared and mixed at a 2: 1

(v: v) ratio. The mixture was then vortexed for 2 minutes, wrapped with aluminum foil and incubated in 70°C water bath for 3 hours until reaching a greenish yellow color.

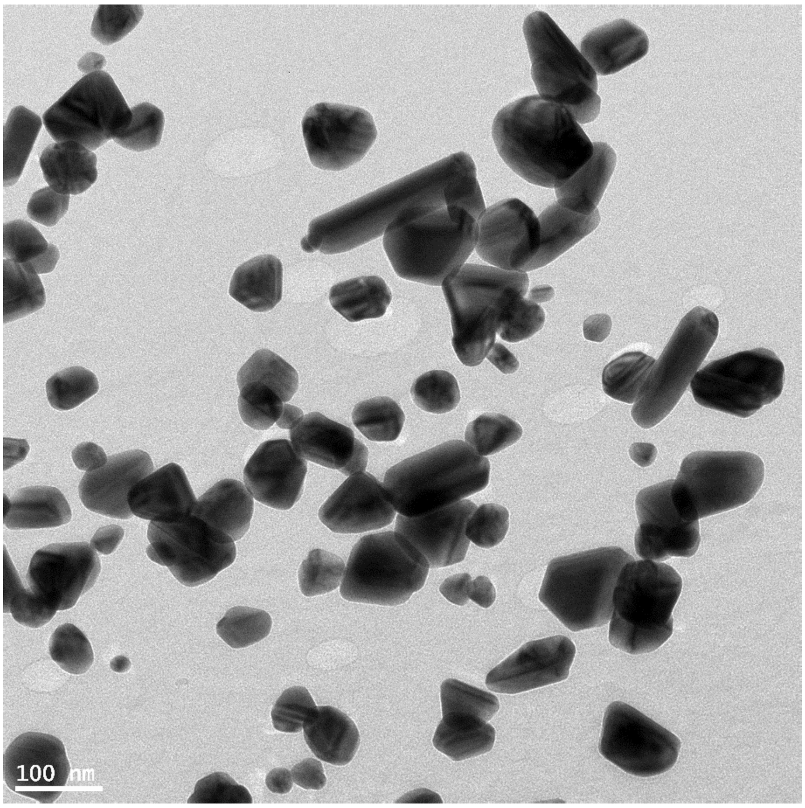

Figure 2 Synthesized silver nanoparticles characterized by transmission electron microscopy

Note TEM FEI Tecnai G2 F20 200 kV Cryo-STEM

Table 4 Silver nanoparticles properties

Properties of silver nanoparticles	
Initial Ag^+	112.67ppm
Conc. [Ag^+]	74.79±2.29ppm
Conc. [nAg]	59.72±1.87ppm
Size	59.21±0.81nm
Recovery (Ag^+%)	66.38%

Note Single-particle ICP-MS (Azodi et al., 2016) *was applied to determine the size and the effective nanoparticle concentration in the synthetic silver nanoparticles solution; size distribution was analyzed by imageJ* (Schneider et al., 2012). *Where applicable, the values are presented as mean ± standard error of 3 replicates.*

3.2.3. Experimental setup

The experiment was carried out under a tent in the field to avoid rainwater interference. Sixteen plastic pots (36 cm in diameter, 30 cm in height) were evenly packed with sandy soil (ρ_b =1.45 Mg m^{-3}), with the soil properties shown in Table 5. A randomized complete block design was followed for the experiment to account for sunlight gradient under the tent where the pots were placed. The pots were arranged in four rows with four pots in each row. Each column of the setup represented a block. The factors considered in the experimental design were irrigation water and Ag NPs. There were two levels for each factor, i.e., Freshwater/Wastewater irrigation and with/without Ag NPs. Accordingly, the following four treatments were randomly assigned to the pots within each block: Freshwater irrigation (FW); Wastewater irrigation (WW); Freshwater irrigation with Ag NPs (FW+NP); Wastewater irrigation with Ag NPs (WW+NP).

Given that there would be no appreciable effect on heavy metal concentrations in soil with the application of freshwater, the freshwater treatments (FW, FW+NP) were used to determine only the effect of NPs on plant performance. The FW treatment also provided baseline information on plant performance. The wastewater treatments (WW, WW+NP), however, were intended to understand the interaction effect of NPs and heavy metals in aqueous solution and the subsequent influence on soil transport and on plant uptake. Background heavy metal concentration in soil is given in Table 6.

Table 5 Soil properties

Type	Sandy
Sand	87.5%
Silt	8.9%
Clay	3.6%
pH	7.04±0.087
Soil Organic Matter (%)	3.28±0.42
P (mg/kg)	125.36±9.71
K (mg/kg)	121.40±35.68
Ca (mg/kg)	1602.33±99.81
Mg (mg/kg)	85.73±21.95
Al (mg/kg)	1453.47±12.89
NO_3 (mg N/kg)	15.2±7.65
NH_4 (mg N/kg)	4.59±0.54
Cation Exchange Capacity (cmol(+)/kg)	8.19±0.39
Percent Base Saturation (%)	99.68±0.32

Note Soil properties measurements were adapted from Nzediegwu et al. (2019). *Where applicable, the values are presented as mean ± standard error of 3 replicates.*

Table 6 Heavy metals background in soil before starting experiment

Heavy metal	Concentration (mg kg^{-1})	Recovery accuracy (%)
Ag	0.05±0.01	103.55
Cd	0.28±0.04	110.00
Cr	33.43±2.75	105.43
Cu	8.37±3.59	107.95
Fe	11958.89±535.70	99.14
Pb	8.67±0.92	111.56
Zn	46.91±6.25	100.81

Note Heavy metal concentration was determined by ICP-MS (ICP820-MS Varian, CA, USA) in the Bioresource Engineering laboratory, Macdonald Campus of McGill University; recover accuracy was calculated based on reference material SED90-03 (Environment Canada). Where applicable, the values are presented as mean ± standard error of 3 replicates.

Fertilizers (2.01 g ammonium phosphate, 0.607 g ammonium sulphate, and 1.74 g potassium sulphate) were applied and the soil was brought to field capacity 1 day before seeding. On 26th June (Day 0), 10 winter radish (*Raphanus sativus*) seeds were evenly sowed into each pot at a depth of 1 cm. On the 20th day of planting, when two sets of true leaves grew, sprouting plants were thinned to 1 plant per pot.

The first treatment irrigation of 2 L per pot was applied on Day 31, when the plants were established, and subsequent four irrigations were applied on 6-day intervals.

Background soil was sampled one day before the treatment irrigation (Day 30). Top soil (0-2 cm) samples were collected two days after each irrigation, starting from the third irrigation. Soil cores were sampled at 2-cm intervals, from 0-10 cm depth immediately after harvesting. Plant performance parameters were recorded one day before each irrigation and they included greenness, photosynthesis and crop reflectance expressed as Normalized Difference Vegetative Indexes (NDVI), using SPAD 502 Chlorophyll Meter, LI-6400/XT and Crop Circle ACS-430, respectively.

After 57 days of planting, radishes were harvested. Above-ground parts (stem and leaves) were cut and weighed on-site. Radish taproots were then carefully harvested.

All plant parts were washed with deionized water and separated into peel, flesh, stem and leaves. All samples were cut into 1-cm lengths and oven-dried at 60℃ for 48 hours; the dried samples were ground into powder form with a coffee grinder and stored for further analysis.

3.2.4. Heavy metal analysis

3.2.4.1. Soil heavy metal analysis

Soil heavy metal was recovered following a hot nitric acid extraction method (Kargar et al., 2013; Stephan et al., 2008). A 0.16 g air-dried and sieved soil sample was weighed into a 15 mL ignition tube (No.9860). Next, 2 mL concentrated nitric acid was added, and the sample was left overnight in a fume hood. Next day, the mixture was placed in a block digester (Isotemp Dry Bath Incubator, Fisher Scientific, USA) at a temperature of 120°C for 5 hours. After digestion, the solution was diluted with 48mL of HPLC-grade water. Quantification was conducted using ICP-OES (Vista-MPX CCD Simultaneous, Varian, CA, USA). Reference material SED90-03 (Environment Canada) was used for recovery, and the recovery percentages were 97.2% for Cr, 137.7% for Cu, 80.2% for Fe, 112.1% for Pb, and 108.8% for Zn.

3.2.4.2. Plant heavy metal analysis

The ground plant tissue samples were digested following the same procedure as soil, and the extracted solution was quantified using ICP-MS equipment (ICP820-MS Varian, CA, USA). Standard reference material peach leaves (NIST1547) were used as a quality control, and the recovery percentages were 131.4% for Cd, 89.1% for Cr, 98.2% for Cu, 83.2% for Fe, 95.2% for Pb and 99.6% for Zn.

3.2.5. Data analysis

3.2.5.1. Transportation factor

Transportation factor (T_i), indicating the tendency of the heavy metal translocation from root to shoot, was defined as the concentration of heavy metal in leaves/ concentration of heavy metal in roots (Ghosh and Singh, 2005). Different heavy metals transportation factors were calculated for each treatment using the following equation:

$$T_i = \frac{Conc.[Leaves]\ (mg/kg)}{Conc.[Roots]\ (mg/kg)} * 100$$

3.2.5.2. Bioaccumulation factor

The bioaccumulation factor (BAF) was calculated as the ratio of concentration of heavy metal in plant tissue and concentration of heavy metal in soil (Zhuang et al., 2009):

$$BAF = \frac{Conc.[plant\ tissue]}{Conc.[soil]}$$

Weighted average soil concentration was used for calculating BAFs of different heavy metals in all plant tissues.

3.2.5.3. Statistical analysis

The concentration of heavy metals in soil samples was analyzed based on both repeated measures in time and one-way ANOVA (WW, WW+NP) comparison for final concentrations. For plant tissue samples, one-way ANOVA (WW, WW+NP) was applied. Plant performance analysis was conducted following the repeated measurements model and a two-way ANOVA (FW, FW+NP, WW, WW+NP) analysis. Outliers were identified by the robust-fit-outliers method and capped by the average of the remaining data. Statistical tests were performed using SAS (SAS 9.4).

3.3. Results and Discussion

3.3.1. Heavy metals transport in the soil profile

Heavy metals are not degradable; they would either stay in the soil, move down with the leachate or be absorbed by plants. After five irrigations of wastewater, the total mass of heavy metals in the soil for Cr, Cu, Fe, Zn were 647, 430, 255839 and 843 mg, respectively. At the end of season, the uptake percentage of heavy metals by plants verses the total mass were Cr (0.0011%), Cu (0.0189%), Fe (0.0004%), and Zn (0.094%) for WW treatment, and Cr (0.0025%), Cu (0.02%), Fe (0.0006%), Zn (0.097%) for WW+NP treatment. The uptake of Fe significantly increased in WW+NP treatment, which is in accordance with the Fe concentration in plant tissues and will be discussed later. In general, heavy metal uptake by plants was little compared to the soil heavy metal mass, because they exist in soil in much higher concentration. This is common for other studies as well (Nzediegwu et al., 2019).

Heavy metal (Cr, Cu, Fe, Zn) concentrations in the top soil (0-2 cm) are shown in Figure 3. All heavy metals were detected in the top soil, because they are ubiquitous in the environment (Alloway, 2013). They existed already in the soil profile even without their introduction from wastewater irrigation (Table 6). Regardless of the wastewater irrigation, the concentration of heavy metals did not change significantly as compared to the background concentration. With the application of each irrigation, 4, 16, 240 and 6 mg of Cr, Cu, Fe and Zn were added in each pot, respectively. Had all metals stayed in the top 10 cm of soil, the concentration increase would be only 0.39 mg/kg (Cr), 1.56 mg/kg (Cu), 23.35 mg/kg (Fe), 0.58 mg/kg (Zn). These increases are low in light of the initial metal concentrations in soil. Also, heavy metal uptake by plants would cause lower soil concentrations. It must, however, be noted that there could be a heavy metal buildup in soil if wastewater irrigation is practised year after year.

Overall, there was no treatment effect. Heavy metal concentration in soil did not change significantly ($p<0.05$) between WW and WW+NP treatments. It is, however, worth noting that although not significant, Cr and Cu had lower concentrations in the top soil in WW+NP treatment as compared to the WW treatment, especially towards the end of the experiment.

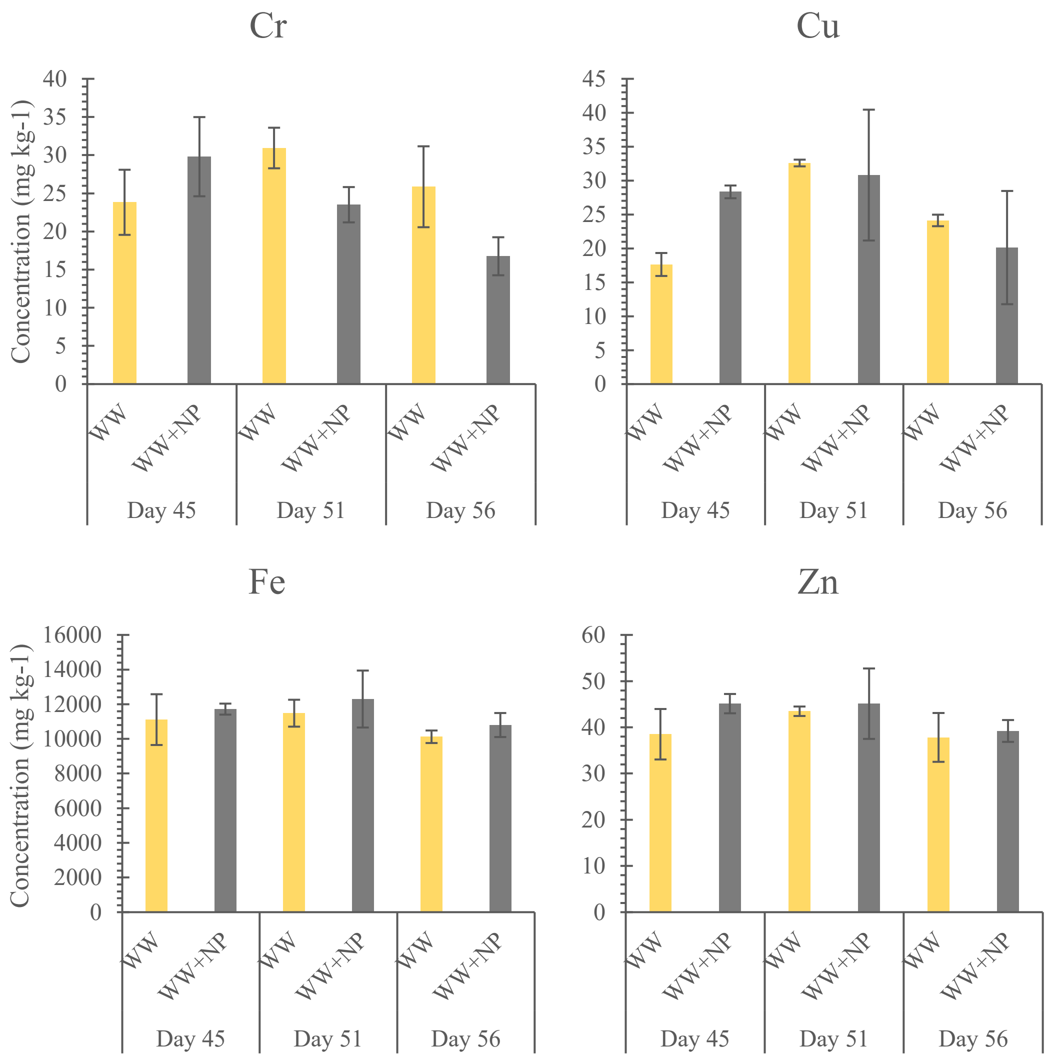

Figure 3 Heavy metal concentration in top 0-2 cm soil depth

Note Error bars represent standard errors of 4 replicates (when there is no block effect, under Cauchy k=3). The vertical scales are variable.

Heavy metal concentrations in different soil depths (0-10 cm) at the end of the season are shown in Table 7, along with the different guidelines for permissible limits of heavy metals in agricultural soils. Compared to soil quality guidelines, none of the heavy metals exceeded the regulatory concentrations under the wastewater irrigation. However, the experiment represented only one season of wastewater irrigation. Since heavy metals do not degrade but tend to accumulate, repeated wastewater irrigation may cause problems in the long run. Irrespective of treatment, only the concentrations of Cr and Cu showed significant differences ($p<0.1$ and $p<0.01$, respectively) between different layers within the top 10-cm soil, although there was no consistent trend (Table 8). With a longer experiment period, the trend of higher heavy metal concentrations in soil might be found. There was no block effect (Table 8). There was no apparent difference between treatments, indicating that Ag NPs did not affect the heavy metal distribution in soil. However, there is a trend that Ag NPs increased downward movement of Cr and Cu.

Table 7 Heavy metal concentration (mg kg^{-1}) in different soil layers after harvesting

		CCME	China-SEPAC	EU	0-2 cm	2-4 cm	4-6 cm	6-8 cm	8-10 cm
Cr	WW	64	90	200	25.9±9.73	22.7±6.07	21.7±3.38	26.1±2.82	26.4±6.16
	WW+NP				16.7±0.97	25.1±3.70	23.3±1.23	24.9±5.33	26.0±1.80
Cu	WW	63	35	100	24.1±3.93	17.7±1.58	15.6±0.54	16.2±1.99	18.2±4.16
	WW+NP				20.1±2.97	18.5±3.02	15.7±1.75	19.7±1.17	16.3±1.38
Fe	WW	n.a.	n.a.	n.a.	10122.6±1145.54	11915.5±1616.82	11705.5±1356.80	12413.4±567.57	12466.5±1226.46
	WW+NP				10799.2±651.66	11686.1±330.33	11783.9±282.48	11427.2±1727.53	20382.1±16776.04
Zn	WW	250	100	250	37.8±3.21	39.8±5.12	41.3±1.67	39.8±0.67	40.9±0.81
	WW+NP				39.2±4.50	37.3±3.89	37.5±2.32	40.1±5.97	38.7±3.11

Note CCME: Canadian council of ministers of the environment (Environment, 2007); China-SEPAC (1995); EU: European union (Meng et al., 2016). Concentrations are presented in mg kg^{-1}. Where applicable, the values are presented as mean ± standard error of 4 replicates (when there is no block effect, under Cauchy k=3).

Table 8 Statistical analysis of heavy metal concentrations in soil layers

	Repeated ANOVA						
	Univariate Approach					Treatment effect	Depth effect
	0-2 cm	2-4 cm	4-6 cm	6-8 cm	8-10 cm		
Cr	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
Cu	n.s.	n.s.	n.s.	*	n.s.	n.s.	***
Fe	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Zn	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.

*Note ***p<0.01, **p<0.05, *p<0.1, n.s.: not significant, n.a.: not applicable*

3.3.2. Plants uptake of heavy metals

The concentrations of heavy metals (Cd, Cr, Cu, Fe, Pb, Zn) in different radish plant tissues are given in Table 9. The uptake and accumulation of heavy metals by plants differ among plant tissues. Irrespective of treatment, peels generally accumulated more heavy metals than the flesh, possibly because of their direct contact with irrigation water and soil. It should be noted that the leafy part contained the highest concentrations of heavy metals (except Cr), as compared to the other plant tissues. However, Roy and McDonald (2015) found that radish roots accumulated more metals (Zn, Pb, Cd, Cu) than other tissues; this contradiction is possibly due to the different solubilization and complexation of heavy metals under the two different experimental conditions. In the meantime, another study in China (Ai et al., 2016) concluded that heavy metals have a higher bioaccumulation tendency for radish shoots (stem and leaf) as compared to radish roots (peel and flesh), which is in accordance with the current study results. They explained that concentrations for these heavy metals reached their peak at the slow growth period of radish (0-28 days) and then started to decrease, with the exception of the leafy part. This dynamic transfer is probably due to the translocation of heavy metals from root to stem and leaf as the plant grows and consequently leads to a higher final concentration of heavy metals in the leafy part. As defined by Ghosh and Singh (2005), transportation factor (leaf/root heavy metals concentrations) indicates the translocation tendency of heavy metals from root to leaf, while larger numbers indicate the tendency of transport and higher concentration in the plant leaves. The transportation factors of different heavy metals are shown in Table 10 for each treatment. It is evident that transportation factor is relatively higher for Cd, Fe, and Pb, suggesting that these heavy metals are more prone to translocating to radish shoots. Among the heavy metals analyzed, the transportation factor of Zn significantly increased ($p<0.05$) with the participation of Ag NPs, while Cd, Cu and Fe also showed the same trend, indicating that NPs existence increased their translocation to the leafy parts. While the effect was reversed for Cr and Pb, according to Yoosaf et al. (2007), comparing to other metal cations, Pb^{2+} ions tend to accelerate Ag NPs aggregation through complexation and lead to immobilization. This could explain that with Ag NPs presence, the transportation factor of Pb decreased.

Table 9 Heavy metal concentration (mg kg^{-1}) in different plant tissues

Heavy metals	Standard Limit	Peel		Flesh		Stem		Leaf	
		WW	WW+NP	WW	WW+NP	WW	WW+NP	WW	WW+NP
Cadmium	0.02	1.6±0.34a	2.3±1.02a	1.2±0.15a	1.2±0.19a	2.6±0.18a	3.4±1.43a	3.6±0.53a	5.4±2.00a
Chromium	1.3	**0.4±0.09b**	**0.8±0.20a**	0.3±0.01a	0.9±0.72a	0.3±0.13a	0.2±0.01a	0.4±0.07a	0.4±0.02a
Copper	10.0	4.1±0.92a	4.4±0.38a	3.1±0.36a	3.1±0.05a	**2.1±0.33b**	**2.7±0.03a**	6.3±0.34a	6.8±1.48a
Iron	20.0	**48.6±8.16b**	**79.6±1.03a**	**30.8±4.48b**	**40.6±1.29a**	43.2±6.78a	41.3±2.47a	107.9±10.99a	150.8±50.76a
Lead	2.0	**0.5±0.25b**	**0.9±0.31a**	0.3±0.24a	0.4±0.29a	1.1±0.55a	0.8±0.41a	2.7±1.73a	2.7±1.69a
Zinc	50.0	37.8±2.99a	33.3±5.82a	37.5±10.70a	36.8±6.07a	36.7±12.33a	45.2±0.80a	**26.7±3.62b**	**48.4±4.80a**

Note Values are shown as mean ± standard error of 4 replicates (when there is no block effect, under Cauchy k=3, α=0.05); different letters indicate significant difference between treatments under each plant tissue category ($p<0.05$). Standard limit by WHO (Nazir et al., 2015).

Table 10 Transportation factor for different heavy metals

	Cd	Cr	Cu	Fe	Pb	Zn
WW	3.0±0.64a	1.2±0.23a	2.0±0.27a	3.6±0.52a	9.1±5.22a	**0.7±0.17b**
WW+NP	4.3±1.52a	0.9±0.76a	2.2±0.50a	3.7±1.25a	6.8±4.56a	**1.3±0.25a**

Note Values are shown as mean ± standard error of 4 replicates (when there is no block effect, under Cauchy k=3, α=0.05)

Meanwhile, the uptake of different heavy metals agrees with the findings of Gaw et al. (2008), where the concentration of heavy metals in the radish leaf followed the order of: Fe>Cu>Cd>Pb. The order is relevant to the loading concentration, soil concentration and plants selective uptake of nutrients (Fe, Cu), although the loading concentration of Pb (16 mg/L) was higher than that of Cd (5 mg/L). This is also in accordance with the findings of Samsøe-Petersen et al. (2002) that Cd uptake by plants did not show a correlation with soil concentration. Because the bioavailability of Cd is more dependent on soil properties such as pH and organic matter rather than simply soil supply, compared to other heavy metals (Loganathan et al., 2003; Luo et al., 2014). Also, for the most consumed parts of the radish (flesh with or without peel), only Cd exceeded the permissible limits for concentration in plants (0.02 mg kg^{-1}) recommended by WHO (Nazir et al., 2015).

It is evident from our results that there was a significant effect of treatment on heavy metal accumulation in different plant tissues (Table 9). In the peel, Pb, Fe, Cr concentrations were significantly higher ($p<0.05$) in the treatment WW+NP as compared to WW. Fe concentration was also significantly higher ($p<0.05$) in the radish flesh with the existence of NPs in wastewater. NPs presence in the irrigation wastewater also resulted in significantly more Cu accumulation in the radish stem. In the leaves, there was a significant increase of Zn concentration in WW+NP compared to WW.

Many parameters that influence plant uptake of heavy metals have been studied previously. The uptake is plant species and heavy metal dependent because they all have different pathways to enter plants, and the plants' tolerance for each heavy metal is different (Peralta-Videa et al., 2009). Soil parameters, such as pH and clay content, affect the uptake by altering the solubility and availability of heavy metals to plants (Golia et al., 2008). But other external factors are seldom investigated, such as the influence of NP existence.

Although no similar experiment of wastewater irrigation with NPs was undertaken before, there were some studies that showed that NPs often behave as an adsorbent for heavy metals or other trace contaminants in the soil matrix because of the higher surface area (Pachapur et al., 2016). In the case of engineered NPs with specific functional groups or support materials, the capacity of adsorption for various heavy metals (Cd, Cr, Fe, Pb, etc.) was greatly enhanced (Li et al., 2011; Taghipour and Jalali, 2016; Wang et al., 2014). After adsorption, NPs could transport into the plant tissues along with the adsorbed heavy metals. This provides a possible explanation for the increase in heavy metal concentration in plant tissues when NPs are presented. Other studies also stated that NPs could affect the macro or micro nutrient uptake and accumulation in plants. These effects are dependent on the NPs' characteristics and plant species. According to Zuverza-Mena et al. (2016), both macro (Ca, Mg) and micro (B, Mn, Cu, Zn) nutrient content reduced with an increased concentration of Ag NPs (up to 500 mg/L) in radish seedlings. This is not consistent with the current study where NPs increased the uptake and accumulation of certain heavy metals (Pb, Fe, Cr, Cu, Zn), possibly because of the different doses of NPs used (the dosage range used in their study was much higher than the current research, which was only 1 mg/L). Also, there were different environmental factors and plant growing periods studied. More studies regarding NPs impact on nutrient content in different crops are available, but the differences of NP type, dosage and environmental factors make it difficult to compare with the current study.

Bioaccumulation factor (BAF) is an index showing the ability of different plant tissues to accumulate certain heavy metals in comparison to the concentration in soil (Zhuang et al., 2009). A BAF<1.0 indicates that the heavy metal has stronger affinity to soil than plants, while BAF>1.0 could raise concerns since the uptake and accumulation of heavy metals would be high (Nzediegwu et al., 2019). Among the heavy metals analyzed (Figure 4), only the BAF in the stem and leaf for Zn exceeded 1.0. However, there is little health concern because Zn has relatively low biotoxicity and the BAF was lower in the most edible parts. But it should be noted that although Cd's BAF is not available because of lower soil concentration than the detection limit, Cd existed in a relatively higher concentration in the plant tissues. Therefore, it is reasonable to conclude that the BAF for Cd was also high. A study in Canada (Murray et al., 2009) also proved that Cd had a very high BAF, compared to other heavy metals, resulting in high concentrations in plant tissues and soil concentrations that were below the detection limit. In general, the BAFs followed the order of Zn>Cu>Cr>Fe for all parts of the plant tissues. As compared to a study by Qureshi et al. (2016), the BAF for radish showed a decreasing order of Cu>Zn>Cr>Fe. In their study, BAF was based on the whole plant rather than different plant tissues, so minor differences were discovered. Notice that with the existence of NPs, the leaf BAF showed a general trend of increasing for Cr, Cu, Fe and Zn. When it comes to the most edible parts, NPs elevated the BAF for all heavy metals especially Cr, which could pose a health risk at higher loading concentrations.

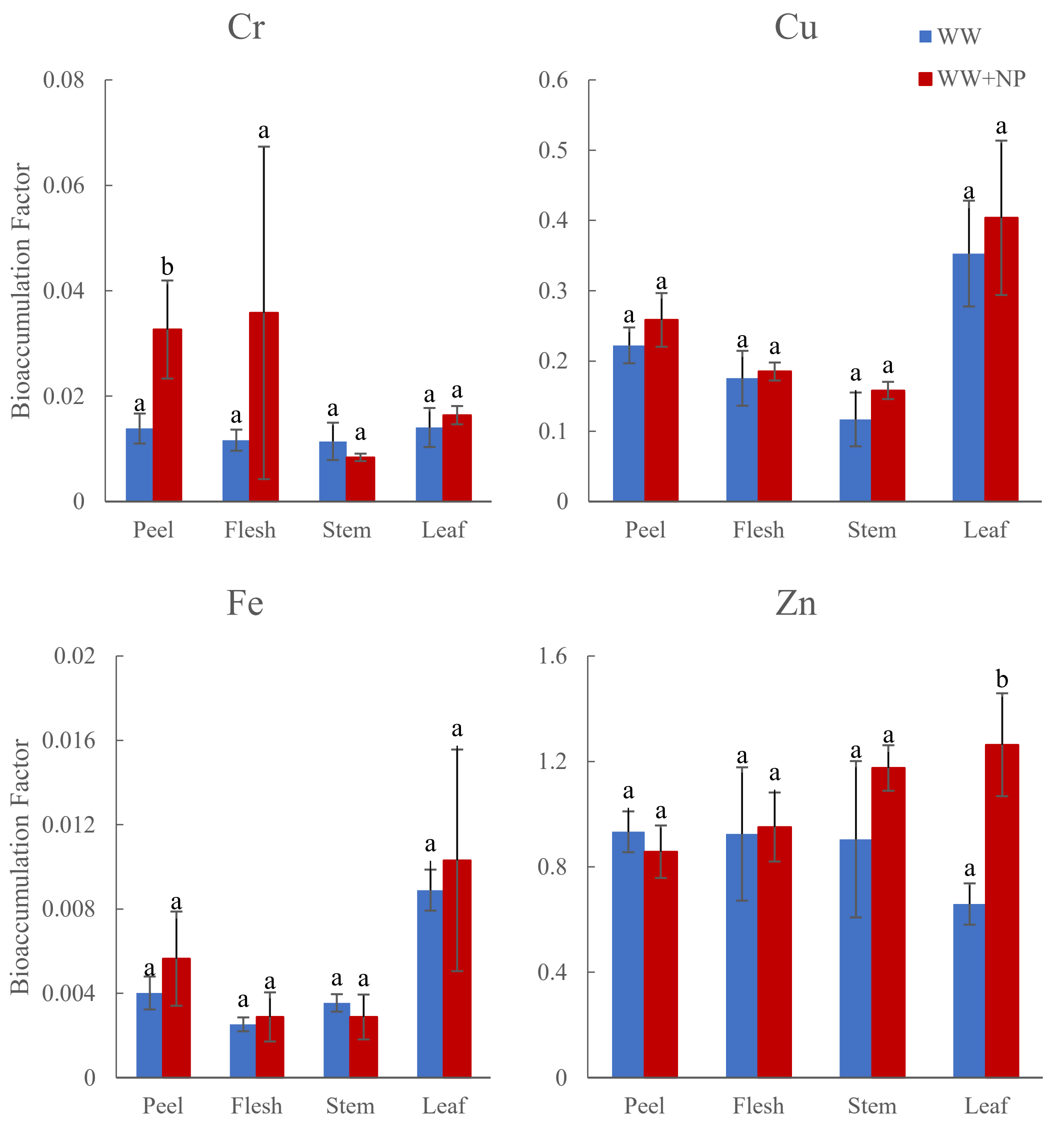

Figure 4 Bioaccumulation factor

Note Error bars represent standard errors of 4 replicates (when there is no block effect). The vertical scales are variable.

Combining different indexes, it is evident that NPs' existence increased the accumulation of certain heavy metals in different plant tissues, but it did not affect the general trend of how heavy metals (Cu, Cd, Pb and Fe) tend to accumulate in different parts of the plants.

3.3.3. Plant performance and growth under different situations

3.3.3.1. Growth parameters

The growth parameters of greenness, NDVI and photosynthesis are given in Figure 5. SPAD is an indirect indicator of leaf chlorophyll content and nitrogen sufficiency (Marchiol et al., 2004). In this study, SPAD measurements were statistically analyzed by repeated ANOVA measurements. According to the results summarized in Table 11, there was no overall treatment effect from Ag NPs, but there was a time effect. The SPAD was the lowest on Day 30 then increased by Day 42 and remained almost the same throughout the remaining growth period (Figure 5). On the 42^{nd} day, treatment FW+NP presented the highest SPAD; in the end of the growing period, treatments with wastewater performed better than those with freshwater regarding the SPAD, or that is to say, leaf health. In conclusion, wastewater could increase the leaf chlorophyll content and improve leaf performance, which is probably due to the nutrient content in the wastewater. Other studies also proved heavy metal exposure could increase the chlorophyll content, which is possibly a mechanism to counter phytotoxicity (Keser, 2013). But no NPs influence was found regarding this parameter.

NDVI is another indicator for plant canopy health or vigor. Similar to SPAD, there was no significant difference between treatments, except that the measurement near the harvesting day (Day 54) showed a significant decrease in treatment WW+NP, implying a poor canopy performance or less leaf area produced. Although this is counter to the results of SPAD where WW and WW+NP had better and greener leaves; the decrease only showed while wastewater and NPs both existed in the system, indicating the reason for which is possibly their interaction.

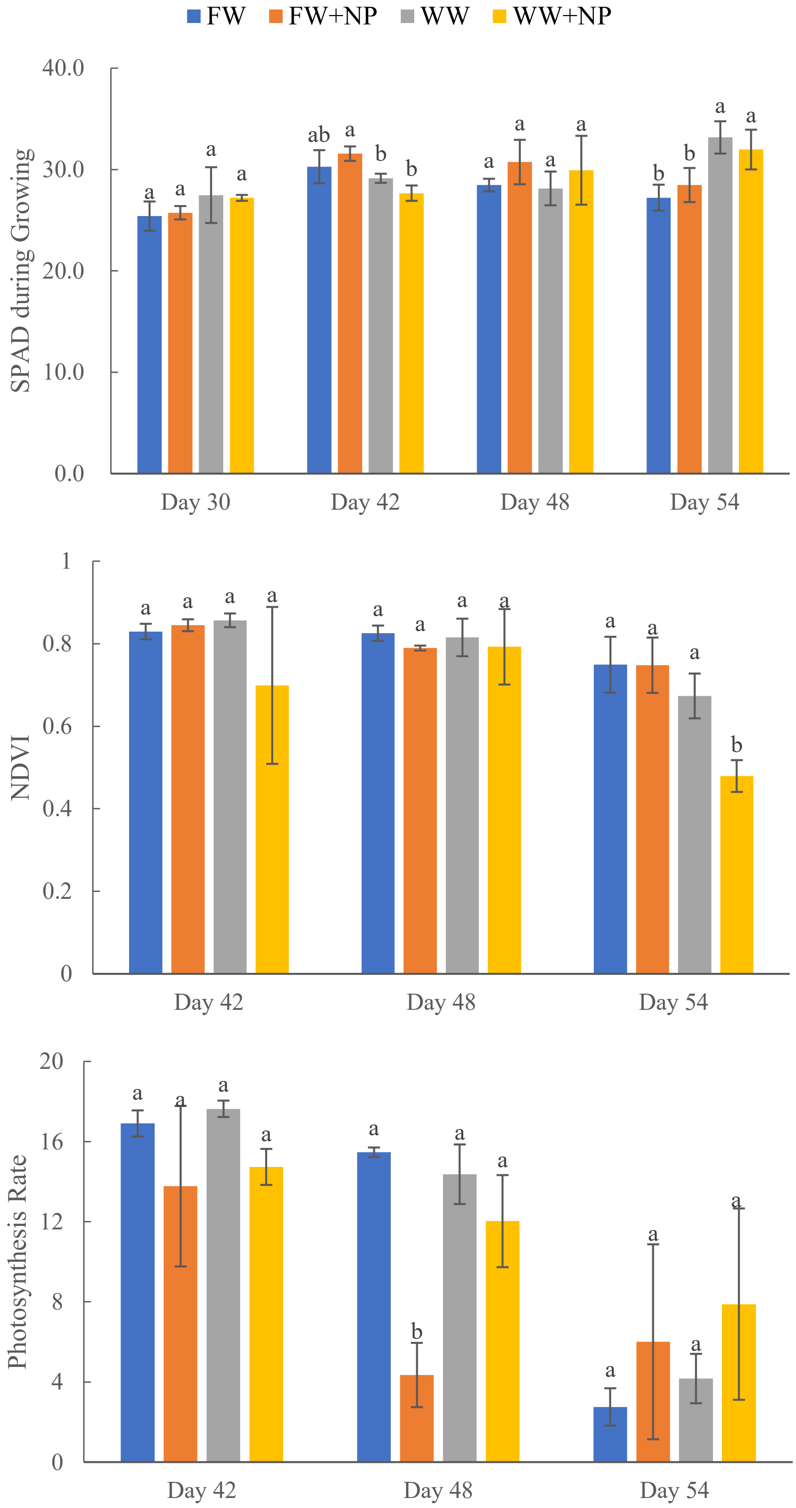

Figure 5 SPAD, NDVI, Photosynthesis Rate (μ mol CO_2 m^{-2} s^{-1})

Note Error bars represent standard errors of 4 replicates (when there is no block effect, under Huber k=1.2); significance level is only compared within each measuring date.

Table 11 Statistical analysis of plant performance parameters

	Repeated ANOVA					
	Univariate Approach				Treatment effect	Time effect
	30th	42nd	48th	54th		
SPAD	n.s.	***	n.s.	***	n.s.	***
NDVI	n.a.	n.s.	n.s.	***	***	***
Photosynthesis Rate	n.a.	*	***	n.s.	**	***

*Note ***p<0.01, **p<0.05, *p<0.1, n.s.: not significant, n.a.: not applicable*

Photosynthesis rate (Figure 5) significantly decreased from Day 42 to Day 54 (Table 11). It appears that the photosynthesis rate decreased gradually as radish matured. Only on the 48th day, FW+NP treatment showed a dramatic decrease. Towards the end of the growing period, although not statistically significant, there is a trend that the wastewater treatments had higher photosynthesis rates than the freshwater treatments, which concurs with the measurements made with SPAD. Also, the photosynthesis activity improved with the participation of NPs for both freshwater and wastewater irrigation. The enhancement of photosynthetic efficiency is also reported by Govorov and Carmeli (2007). They explained the mechanism in two ways: one is the improvement of chlorophyll light absorption because of the resonance effect of metal NPs, the other is the efficiency of energy transfer.

Moreover, a review by Liu and Lal (2015) concluded that some engineered NPs could be used as fertilizer to enhance plant growth, indicating that many metal-based NPs could enhance the plant leaf performance. For example, superparamagnetic Fe NP could increase the chlorophyll content in soybeans (Ghafariyan et al., 2013), TiO_2 NP also enhances the chlorophyll content for certain plant species. These studies concur with the current finding that NPs existence enhanced the leaf performance and activity. In Liu and Lal's study, they also defined nanomaterial-enhanced fertilizers, which referred to those NMs that could enhance plant performance when accompanied by nutrients, but do not contain any nutrient themselves. This also provides a probable explanation for the current study, since NMs might carry nutrients as well as the contaminants.

3.3.3.2. Plant biomass

Figure 6 below shows the radish (taproot) length and weight at harvesting. Compared between the wastewater treatments, the existence of NPs caused a significant decrease of both length and weight. Within the freshwater group, NPs presence also led to a lower fresh weight, but the radish tended to grow longer with freshwater and NPs (FW+NP), producing a "skinnier" radish.

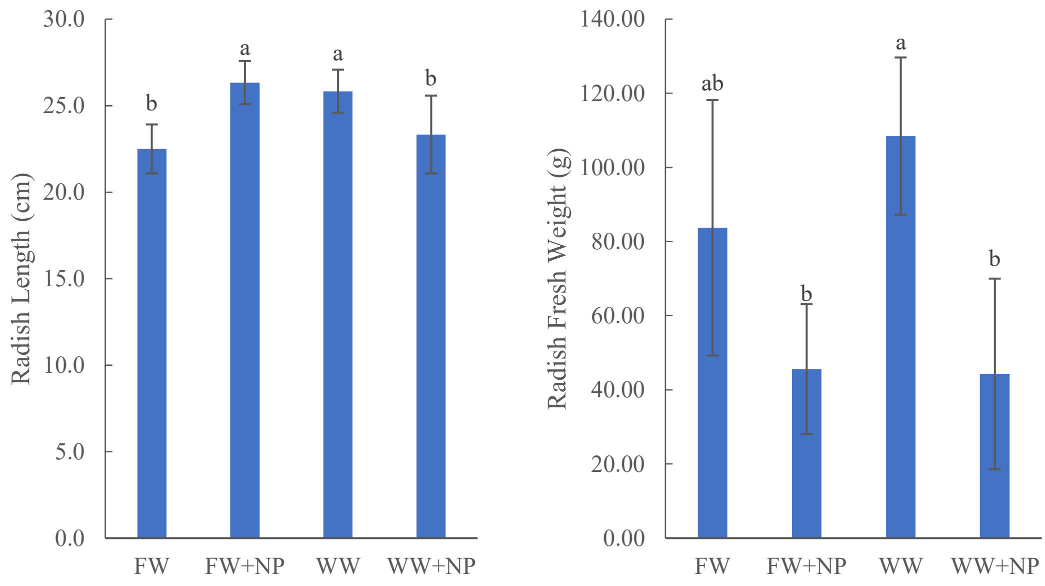

Figure 6 Radish biomass

Note Error bars represent standard errors of 4 replicates (when there is no block effect, under Huber k=1.2).

There was no significant difference (P=0.3673) when it comes to the shoot (stem and leaf) fresh weight (Figure 7), but a slight increase of weight could be noticed in the wastewater treatment with NPs.

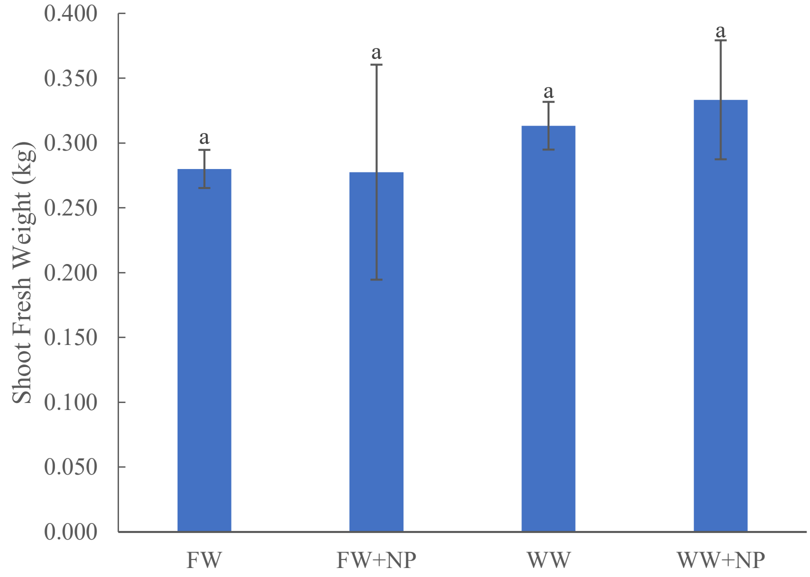

Figure 7 Radish biomass

Note Error bars represent standard errors of 4 replicates (when there is no block effect, under Huber k=1.2).

There are many studies related to heavy metals or NPs influence on different plant growth, but a few of them involved the real environmental scenario where they exist simultaneously. When talking about Ag NPs only, the influence could be both negative and positive, depending on the plant species and NPs' properties. Ag NPs could reduce both the root and shoot biomass of rice and soybeans, with a positive relation to the dosage (Li et al., 2017). During germination and the seedling growth period, radish root and shoot elongation were also negatively affected by the increasing concentration of Ag NPs (Zuverza-Mena et al., 2016). Another study by Thuesombat et al. (2014) investigated Ag NPs (in different sizes and dosage) impact on rice germination and seedlings. Within the dosage range (0.1-1000 mg/L) they determined a decreasing trend of biomass and length for both root and shoot. In this study, there was no significant reduction compared to the control. These findings concur with the current results that NPs existence could reduce the root biomass of radish, possibly due to Ag NPs' capability of altering gene expression and protein production, and further acting as an inhibitor of plant growth (Siddiqui et al., 2015). But the mechanism of this reduction is still unclear. Also, more attention should be paid to the role of the interaction of NPs and heavy metals in decreasing biomass.

Under wastewater irrigation group, an increasing trend in plant heavy metal concentration showed when NPs were included in the irrigation. The uptake of heavy metals by plants affected the plant's metabolic process, evidently indicated by the changes of SPAD, NDVI and photosynthesis rate. This eventually led to an impact on plant biomass, causing the biomass reduction of the major part of the radish that is consumed.

3.4. Conclusions

Heavy metal concentrations (Cr, Cu, Fe, Zn) in the surface soil did not change because of the wastewater/freshwater irrigation but showed a slight decrease with the existence of NPs. Also, there was no significant difference in heavy metal concentrations in subsoil between WW and WW+NP treatments.

The existence of NPs facilitated the transport of several heavy metals (Cr, Cu, Fe, Pb, Zn) to different plant tissues although plants uptake of heavy metals followed the same order of: Fe>Zn>Cu>Cd>Pb>Cr. The Cr, Fe, and Pb concentrations were about two times higher in the peel in WW+NP treatment. In the flesh, only Fe showed a higher concentration ($p<0.05$) in WW+NP treatment while Cu and Zn concentration significantly increased ($p<0.05$) in the stem and the leafy parts, respectively. Indicated by the transportation factor, NPs' presence increased the tendency of translocating from root to shoot for Cd, Cu, Fe, and decreased that of Cr and Pb. Zn's BAF for stem and leaves were above 1.0 for both WW and WW+NP treatments, indicating increased transport of Zn to the plant tissues. NPs influence on the BAF was not consistent.

The NP interference and accumulation of heavy metals in plants also affected the plant performance. Plants had healthier leaves and higher photosynthesis activity in both WW and WW+NP treatments. However, plant biomass (primarily the radish taproot weight) decreased with the existence of NPs, in spite of better leaf performance, possibly due to the increased heavy metal concentrations in plants.

Chapter 4: Summary and Conclusions

4.1. General summary

Wastewater irrigation is one of the main reasons for heavy metal accumulation in crops. Depending on the concentration of the wastewater applied, various consequences could appear. For example, in some severely heavy metal polluted areas, plants could accumulate high concentrations of heavy metals that might exceed acceptable limits. On the other hand, external factors such as the introduction of NPs could also affect heavy metal transport and impair plant growth.

Ag NP is one of the most exploited NMs in the nano-industry, due to its unique properties and outstanding antimicrobial characteristics. Once released into the environment, Ag NPs not only interfere with the performance of living creatures, but also interact with other opponents in the environment, especially heavy metals.

A pot study was undertaken to study the impact of wastewater irrigation on radish production. Additional investigations were also made to determine the influence of Ag NPs presence in wastewater. The summary of the results obtained in this study is as follows:

A) Heavy metal concentrations in the soil profile were tested, showing no significant difference between treatment (WW, WW+NP), indicating an even distribution of heavy metals in the soil matrix. But there was a general trend of concentration decreasing as plants grew, and WW+NP treatment showed a more rapid decrease than WW, suggesting NPs possibly increased the mobility of heavy metals in soil.

B) Plant tissues were sampled and analyzed by four parts (Peel, Flesh, Stem, Leaf). The peel accumulated more heavy metals than flesh because of its direct contact with soil, while different heavy metals showed different trends of uptake and translocation in plants. Fe and Zn are usually regarded as necessary nutrients for plants. Accordingly, they demonstrated the highest concentration among other metals and accumulated mostly in the shoot. For the consumable part (radish), Cd's concentration exceeded the permissible limit of 0.02 mg kg^{-1}. With the use of Ag NPs, several heavy metals showed a significantly ($p<0.05$)

higher concentration in different plant tissues. In the peel, Cr, Fe, Pb increased in WW+NP treatments; for flesh, stem and leaf, Fe, Cu, and Zn increased under WW+NP, respectively. This trend also corresponds with the differences in concentrations between different treatments in soil.

C) Plant performance was monitored during the growing season by measuring the greenness of the leaves, canopy development, and photosynthesis rate. Biomass was taken right after harvesting. According to the leaf greenness and photosynthesis rate, NPs' existence slightly improved the leaf performance, but the canopy did not benefit. In the meantime, biomass decreased significantly with NPs, under both freshwater and wastewater irrigation, which happened to be a reverse correlation with plant heavy metal concentration.

4.2. Conclusions

The following conclusions were drawn from the current study and corresponding to the objectives:

Objective 1: To determine the effect of Ag NPs on heavy metal movement in soil due to wastewater application.

Ag NPs existence in wastewater did not significantly affect heavy metal distribution and transport in soil, although a minor decrease in metal concentration in the top 0-2 cm soil was observed in the presence of Ag NPs.

Objective 2: To determine Ag NPs' impact on heavy metal uptake by plants from irrigation with wastewater.

Ag NPs in the wastewater irrigation enhanced the plant's uptake of certain heavy metals. Cr, Fe, Pb showed significantly higher concentrations ($p<0.05$) in peel while accompanied by NPs; in flesh, Fe also accumulated significantly more ($p<0.05$) in WW+NP than in WW; Cu and Zn showed significantly higher accumulation ($p<0.05$) in the stem and leaves.

Objective 3: To observe if the plant growing performance would be affected by these two categories of chemicals or their interaction.

According to the growing parameter measurements taken during the experiment, plants had a better leaf performance with the existence of NPs, while wastewater also enhanced plant performance. But when it comes to the taproot part of radish, NPs in wastewater decreased its size and weight.

4.3. Recommendation for further studies

A) Future research could focus on other NMs to provide more specific information and suggestions.

B) The mechanism of NPs and heavy metals interaction needs to be studied more. In the current study, the mechanism of the interaction was unclear; more lab experiments could be done in the future concerning this interaction. The plant physiological reaction in the presence of NPs should also be investigated.

C) In this study, radish, a root vegetable, was studied. Other types of plants and vegetables, such as leafy vegetables, should be studied.

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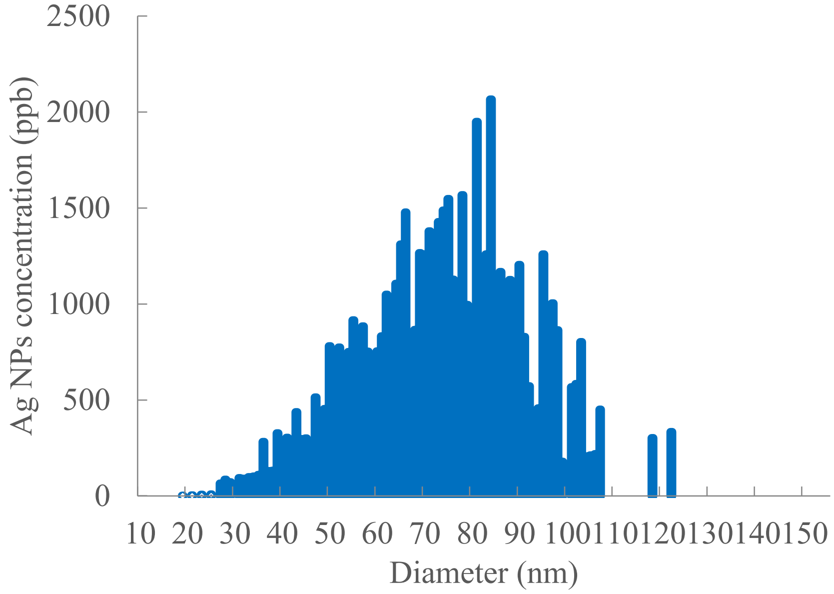
Chapter 6: Appendix

1. Ag NPs size distribution



2. Conductance rate for radish