

ELUCIDATING THE EFFECTS OF CERIUM OXIDE NANOPARTICLES AND ZINC
OXIDE NANOPARTICLES ON ARSENIC UPTAKE BY RICE (ORYZA SATIVA) IN A
HYDROPONIC SYSTEM

A Thesis

by

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ABSTRACT

Arsenic (As) is a toxic element widely encountered in the environment and a food safety concern. The use of engineered nanoparticles (ENPs) has grown rapidly due to the unique properties that make them beneficial in a wide range of technologies. Studies abound concerning the phytotoxicity of ENPs and their accumulation in plant tissues. However, investigations on ENPs interactions with co-existing contaminants in a plant system, especially with redox sensitive heavy metals, are rare. Two ENPs of interest are cerium oxide nanoparticles (CeO₂ NPs) and zinc oxide nanoparticles (ZnO NPs).

The goals of this study were to: (1) determine the impact of CeO₂ NPs and ZnO NPs on the As accumulation in rice, and (2) evaluate whether inorganic As species including both As(III) and As (V) may modify the plant uptake and accumulation of the metal elements of co-present CeO₂ NPs and ZnO NPs. This was done by administering either 1 mg/L of As(III) or As(V), or 100 mg/L of CeO₂ NPs or ZnO NPs or Zn²⁺, or different combinations of As and ENPs or ions at the same concentrations to rice plants. Rice (*Oryza sativa*) was utilized in this study as a model plant due to its high propensity for As uptake, and its widespread consumption as a staple food around the world. A hydroponic system was used to avoid the compounding effects of soil and the microorganisms in soil. The results indicated that CeO₂ NPs did not show significant effect on total As plant accumulation. The presence of ZnO NPs and Zn²⁺ significantly reduced total As in rice seedlings, except for the concentration of total As in rice shoots with the

co-presence of ZnO NPs and As(III). The co-presence of As significantly increased Ce in rice shoots in the CeO₂ NPs + As(III) treatment but did not affect the plant uptake of Zn from ZnO NPs or Zn²⁺. The results confirmed the active interactions between ENPs and co-existing inorganic As species and the extent to which their interactions depend on the properties of ENPs as well as the initial oxidation state of As.

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NOMENCLATURE

As	Arsenic
ENPs	Engineered Nanoparticles
NPs	Nanoparticles
CeO ₂	Cerium Oxide
ZnO	Zinc Oxide
Ce	Cerium
Zn	Zinc
NIPs	Nodulin26-like Intrinsic Proteins
Si	Silicon
PCs	Phytochelators
AR	Arsenic Reductase
Al	Aluminum
Ag	Silver
TiO ₂	Titanium Dioxide
Cu	Copper
Cd	Cadmium
Al ₂ O ₃	Aluminum Oxide
ROS	Reactive Oxygen Species
ZIP	Zn-regulated Transporters in the Iron-regulated Transporter-like
Protein	

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CHAPTER I

INTRODUCTION

Arsenic (As) is a toxic element widely present in the environment and a food safety concern due to its toxicity and carcinogenicity. It is prone to accumulate in rice grains and has mutagenic effects on humans. The earth crust is a major natural source of As, with a crustal As content ranging from 1.0 mg/kg to 490 mg/kg. However, anthropogenic activities such as mining has led to the epidemic of As contamination (Murcott 2012). Currently, As concentration in groundwater in 105 countries exceeded the limit of 10 $\mu\text{g/L}$ set by USEPA, and As concentration in soil in the U.S. is higher than the action level of 5mg/kg in several other countries (Punshon et al. 2017). The toxicity of As is related to its chemistry and oxidation state, and its oxidation state depends on the redox conditions and microbial community in the environment (Oremland and Stolz 2005). Generally, inorganic As species have higher accumulation potential in rice grains and greater toxicity than organic As species. As has a similar structure with several essential elements for rice and shows a high propensity to accumulate into rice tissues even though it is a non-essential and toxic element for rice. Because of the physiochemical similarities between silicic acid and arsenous acid, As(III) shares the nodulin26-like intrinsic proteins (NIPs) for silicon (Si) uptake (Si influx transporter *OsLsi1*) and translocation (Si efflux transporter *OsLsi2*) to transfer into rice (Ma et al. 2006, Ma et al. 2008). On the other hand, As(V) is a phosphate analogue, which usually appears under aerobic conditions, and shares the transporters with

phosphate and interfering with phosphate metabolism (Zhao et al. 2010). There is a complicated apparatus in rice root cells that could reduce As(V) to As(III) to complex it with phytochelators (PCs) as a mechanism of detoxification, and this process is carried out primarily by arsenate reductase (AR) (Duan et al. 2013). Therefore, both species will be found in rice tissues no matter what the initial As species taken up by rice roots are. The different pathways for As uptake and transportation in rice tissues indicated that As species in rice tissues depend heavily on the As speciation in rice rhizosphere.

The use of engineered nanoparticles (ENPs) has grown rapidly due to their distinctive properties, which make them beneficial in a wide range of technologies. Since ENPs allow more atoms at the grain surfaces and have higher surface-to-volume ratio, ENPs are more reactive than their bulk counterparts (Bandyopadhyay et al. 2012). However, high reactivity of ENPs can also be a concern because the rise in ENPs production has led to increasing release of these nanoparticles into the environment. CeO₂ NPs is a popular ENP with both tetravalent(Ce⁴⁺) and the trivalent(Ce³⁺) state on the surface (Cassée et al. 2011). This special characteristic has made CeO₂ NPs a common additive in a variety of industrial and consumer products (Cassée et al. 2011). ZnO NPs are another popular ENPs with strong antimicrobial properties. ZnO NPs have been reported as an effective Zn fertilizer to alleviate Zn deficiency in soils (Milani et al. 2012). Various studies have been conducted on how ENPs could affect agricultural crops, including their impact on the physiological and biochemical processes of plants (Rao and Shekhawat 2014, Rico et al. 2013, Yoon et al. 2014).

In addition to the direct impact of ENPs on plants, more and more studies have started to focus on interactions between ENPs and co-existing contaminants and corresponding bioaccumulation and risks for plants. For example, when soybeans were exposed to CeO₂ NPs and Cd²⁺, the accumulation of Ce in plant tissues significantly increased (Rossi et al. 2017). Although several studies have reported the interactions between ENPs and co-existing contaminants, very few studies have focused on the impact of ZnO NPs on the plant uptake and the bioaccumulation risks of co-existing heavy metals, especially redox sensitive metals (Rao and Shekhawat 2014, Venkatachalam et al. 2017, Yoon et al. 2014).

The potential interactions between two different initial oxidation states of As and two different ENPs in rice plant system can impact the chemical and physical processes in rhizosphere and plant cells, which together determine the fate and transport of As and ENPs in plant system. Therefore, the primary objective of this study was to assess the impact of CeO₂ NPs and ZnO NPs on the As accumulation in rice. The second objective was to determine whether inorganic As species including both As(III) and As(V) could modify the plant uptake and accumulation of the metal elements in co-existing CeO₂ NPs and ZnO NPs. These objectives were accomplished by evaluating the mutual impacts of two ENPs (CeO₂ NPs and ZnO NPs) and co-existing As(III) or As(V) on rice (*Oryza sativa*) uptake of these metal elements. A hydroponic system was used in this initial effort to avoid the compounding effects of soil and the microorganisms in soil. Rice were used due to its high propensity for As uptake, and its widespread consumption as a staple food around the world.

CHAPTER II

LITERATURE REVIEW

Arsenic (As)

Overview

As is a toxic element widely detected in the environment and in various organisms. As ranks as the 20th most abundant element in the earth's crust (Cullen and Reimer 1989). The crustal As content ranges from 1.0 mg/kg to 490 mg/kg, with the average concentration in the range of 1.5-2 mg/kg (Cutter 1992). As contamination occurs mainly due to anthropogenic activities, such as coal and petroleum mining (Murcott 2012). As concentrations in groundwater exceeded the limit (10 µg/L) set by USEPA in 105 countries. The toxicity of As is related to its chemical nature and valence state. Elemental As is generally harmless. The toxicity of organic As is generally low. Inorganic trivalent As (As(III)), such as arsenic trioxide and arsenious acid (Hughes 2006), is 25- to 60-fold more mobile and toxic than the inorganic pentavalent As, (As(V)) (Nguyen et al. 2008), which usually exist as the dominant As species in groundwater (Guo et al. 2008). People exposed to As compounds through consumption of As-tainted food or drinking water have been found to develop a wide range of multisite damages such as leucomelanosis, melanosis and keratosis (Rahman et al. 2009). Studies also showed that As increase the risk of bladder, lung, kidney, liver, and skin cancer (Ahmed et al. 2016, Martinez et al. 2011, Rahman et al. 2009, Smith et al. 1992).

As Impact on Plants

As is a non-essential nutrient to plants and is usually toxic. Many studies have been conducted to gain insight into the potential effects As could have on agricultural plants. Garg and Singla (2011) showed that As reduces root extension and reproduction of bean plant (*Phaseolus vulgaris* L., cv. Buenos Aires). This study also indicated that As can significantly inhibit bean plants growth by slowing expansion and biomass accumulation when translocated to plant shoots, resulting in low yield and fruit production (Garg and Singla 2011). Finnegan and Chen (2012) reported that As impedes with critical metabolic processes of ryegrass (*Lolium perenne* L.) and barley (*Hordeum vulgare* L.) at adequately high concentrations, which can lead to death. A lot of efforts have been undertaken to evaluate the behavior and impact of As in the food chain, especially in rice, which is the second most broadly cultivated cereal crop all over the world and provides food for more than half of the world's population (Meharg and Zhao 2012). Several physical and chemical factors of the soil or irrigation water affect the bioavailability of As in rice including the redox potential, organic matter content, pH, the sulfur, silicon, iron content, the presence of organic ligands, and inorganic (mostly phosphate) contents in plant rhizosphere (Violante 2013). Fe plays a critical role in the accumulation of As in rice, with iron oxyhydroxides on root surfaces of flooded rice or wetland plants serving as a strong adsorbent for As (Liu et al. 2006). Under a reducing environment, dissolution of iron oxyhydroxides enhanced As availability by releasing the adsorbed As (Zhao et al. 2010). Speciation and transportation of As in plants are another important content for understanding As metabolism and toxicity in plants. Rice displays constitutionally high

ability to take up and translocate As(III) in the anoxic environment such as flooded paddy soils (Su et al. 2010). Because of physiochemical similarities between silicic acid and arsenous acid, As(III) shares the nodulin26-like intrinsic proteins (NIPs) for silicon (Si) uptake (Si influx transporter *OsLsi1*) and translocation (Si efflux transporter *OsLsi2*) to transfer into rice root cell (Ma et al. 2006, Ma et al. 2008). Due to the competition between Si and As(III) for plant uptake and translocation, amending soil with Si fertilization may be an effective strategy in diminishing As level in rice grains (Fleck et al. 2013, Li et al. 2009). In contrast, Arsenate (As(V)) is a phosphate analogue, which usually appears under aerobic conditions, sharing the transporters with phosphate and interfering with phosphate metabolism (Zhao et al. 2010). Due to different uptake and transport mechanism between different As species, Xu et al. (2008) showed that growing rice aerobically decreases As accumulation, and that redox condition of As is key to affect its potential toxicity to rice. Once As enters plant root cells, both As(III) and As(V) may be transported up to the shoots and then grains. The literature suggests that the same phosphate transporters are involved in the xylem loading of As(V), however, a new set of transporters such as *OsLsi2* are more responsible for As(III) xylem loading for long distance transport (Wu et al. 2011). As a mechanism of detoxification, plants tend to sequester hazardous materials in the vacuoles of plant root cells. Only As(III) can be stored into the vacuoles in rice root cells after it complexes with phytochelatons (PCs). There is a sophisticated apparatus in rice root cells that reduces As(V) to As(III), primarily by arsenate reductase (AR), an enzyme first isolated from bacteria and yeast (Duan et al. 2013). Therefore, regardless of the forms of As species taken up by rice roots, both As(III) and As(V) are found in plant tissues even

though their relative abundance can vary. Inorganic As(III) is considered more problematic due to its high tendency for accumulation in rice grains. Due to the different uptake pathways involved for As(III) and As(V), the uptake and accumulation of different As species in rice tissues depend heavily on the As speciation in rice rhizosphere. Literature has shown that a suite of environmental parameters such as dissolved oxygen (Oremland and Stolz 2003), nitrate (Sun et al. 2008) and natural organic matters (NOMs) (Oremland et al. 2000) can alter the speciation of As in the environment.

Engineered Nanoparticles (ENPs)

Overview

ENPs are defined as materials between 1 nm and 100 nm with at least two dimensions for metal-based particles and with at least one dimension for other ENPs (Brar et al. 2010). Nanotechnology is developing rapidly and could become a trillion-dollar industry soon (Nel et al. 2006). They are used in fields such as textiles, electronics, medicines, cosmetics, and even environmental remediation (Guzman et al. 2006). ENPs have some unique properties such as very large specific surface area and high surface energy, which makes them different from their molecular and bulk counterparts. These unique properties of ENPs can lead to different environmental fate and impact compared to the bulk particles of the same composition and can be potential health hazards (Ma et al. 2014). Rapid technological advancements in nanotechnology results in increasing production and disposal of ENPs worldwide. The total global investment in ENPs was around \$10 billion in 2005 (Sellers et al. 2008) and it is estimated that over half a million tons of production levels will be reached by the year

2020 (Shah et al. 2014). This nanotechnology industry is still in the exploration stage and broad ongoing research suggests that it will continue to grow (Shapira and Youtie 2015).

ENPs Fate and Transport

Due to their rapidly development, it is expected that man-made ENPs will find their way into water, soil and air (Nowack and Bucheli 2007). As a result, there is a need for understanding the fate and transport of ENPs introduced from various applications. The transport and accumulation of ENPs in plants depend on size, physical and chemical properties, and reactivity (Rico et al. 2011). ENPs can aggregate, dissolve, precipitate and interact with other chemicals once it entered in the environment (Reddy et al. 2016). Lanphere et al. (2014) stated that roughly 80% of carbon nanotubes would be in landfills at the end. When ENPs enter soil, their movement is very slow so that they will accumulate in surface soil, increasing the chances of uptake by terrestrial plants (Schwab et al. 2016).

ENPs can transport into aquatic and terrestrial plants through sewage sludge, ENP-incorporated pesticides and fertilizers, wastewater effluent, and atmospheric sources (Schwab et al. 2016, Shah et al. 2014). Bio-solids collected at wastewater treatment plant and then applied onto agricultural lands would be the most likely pathway for ENPs to enter agricultural soil (Dahle and Arai 2014, Shah et al. 2014). Seven million dry tons of biosolids are generated by the wastewater treatment plants each year in the United States and approximately 60% is repurposed for use on agricultural land. The effects of organic compounds, metals, and microorganisms in

biosolids are not harmful to humans or the environment under correct management procedures. But there is limited information on the impacts due to ENPs in the biosolids (Jacobs and McCreary 2003). One study indicated that Ag NPs and TiO₂ NPs in the biosolids could change the bacterial richness and composition in waveling pattern, while ZnO NPs and zero-valent Cu NPs have no toxicity to soil bacterial community (Shah et al. 2014).

Impact of ENPs on Plants

Several factors, such as microorganisms in plant rhizosphere or plant root exudates, could potentially affect ENPs accumulation in plants. Some studies showed that ENP exposure rate has linear relationship with the accumulation amount in plant tissues (Schwab et al. 2016). This study also indicated that the uptake and translocation of ENPs occur in the apoplast of plant cells (Schwab et al. 2016). The interactions of ENPs with plants such as plant uptake and accumulation of ENP elements have gained more and more attentions from researchers recently. Ma et al. (2014) reported that ENPs could adhere to plant roots and exert both physical and chemical toxicity to plants. Most studies focus on the potential effects of ENPs to plants health, and results vary. Some positive effects have been reported. For example, Lin et al (2007) indicated that 100 nm aluminum nanoparticles (Al NPs) at the concentration of 2000 mg/L improved the growth of radish root. Another example is when soybean exposed to a mixture of low-concentrations of SiO₂ NPs and TiO₂ NPs, exhibited enhanced seed germination and growth (Lu et al. 2002). However, other studies have shown negative impacts of ENPs, especially at high concentrations. Silver nanoparticles (Ag NPs) at 19 mg/L have been

shown to negatively impact the germination rates of barley (El-Temsah and Joner 2012). Lee et al. (2008) showed that mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*) grown in an aqueous solution had significantly lower growth rates when exposed to 0-1000 mg/L of copper nanoparticles especially at high concentration.

Reactive Oxygen Species (ROS)

ROS is shown to be a key factor in the response of plants to stresses (Pauly et al. 2006). ROS is signaling compound for plant biotic interactions with their environments (Scheler et al. 2013), It was also found to be signaling molecule that help the plant to recognize and respond to stress factors (Pauly et al. 2006). It is reported that nodule development will be impacted in the early stage of infection if ROS production is impaired (Scheler et al. 2013). The production or release of ROS could cause by presence of ENPs which can damage cells and DNA. It could also release heavy metals which are toxic to plants (Burke et al. 2015). Recent studies indicated that ROS is not only utilized for stress responses but also during the lifespan of the plant for growth and development stages (Pauly et al. 2006).

Two nanoparticles of concern for this study are cerium oxide nanoparticles (CeO₂ NPs) and zinc oxide nanoparticles (ZnO NPs). Both nanoparticles are metal based ENPs and show potential of accumulation in the environment.

Cerium Oxide Nanoparticles

Overview

Cerium is the most abundant rare earth metal, making up 0.0046% of the crust by weight (Collin et al. 2014). CeO₂ is the most common form of cerium oxides. when

CeO₂ NPs are reduced to the nanosize (<100 nm), both tetravalent(Ce⁴⁺) and trivalent(Ce³⁺) state of Ce can exist on the surface (Casseo et al. 2011). The fate, transport and toxicity of CeO₂ NPs depend on its transformation from its originally synthesized state, which can be caused by processes such as redox reactions, dissolution, aggregation and reaction to bio-macromolecules (Maurer-Jones et al. 2013). CeO₂ NPs is popular in a variety of consumer products such as medical, food and food packaging, and agricultural products (Morales et al. 2013). However, the redox capabilities of this particle can relate directly to the level of toxicity cerium may have on the environment, therefore becoming a risk for plants, humans, and other organisms (Collin et al. 2014, Ma et al. 2014, Rickerby and Morrison 2007, Stander and Theodore 2011). Higher ratios of Ce³⁺/Ce⁴⁺ tend to result in greater toxicity to plants. One study revealed the ability of excessive Ce³⁺ on CeO₂ NP surface to produce hydrogen peroxide which is toxic to plants (Pulido-Reyes et al. 2015). Other studies showed that the reactive sites on CeO₂ NPs could scavenge free superoxide radicals and exert antioxidant effects (Celardo et al. 2011).

Impact of CeO₂ NPs on Plants

CeO₂ NPs have been shown to impact agricultural crops. Some studies indicate potential toxicity while others indicate benefits to CeO₂ NPs exposure. Ma et al. (2010) showed that 2000 mg/L CeO₂ NPs did not affect the germination and root elongation of tomato and five other plant species and slightly reduced the root elongation of lettuce. However, at the same concentration, CeO₂ NPs (7nm) were shown to reduce the germination rates of soybeans, tomatoes, and cucumbers grown in an aqueous solution

(Lopez-Moreno et al. 2010). Rico et al. (2014) showed that when dosed by CeO₂ NPs at 500 mg/kg of soil, wheat exhibited an increase in the plant biomass, height, and grain yield. When soybeans were exposed to 100 mg/kg CeO₂ NPs, net photosynthesis rates were increased, while decreasing when exposed to a higher concentration of 500 mg/kg (Cao et al. 2017). Ma et al. (2014) showed that when treated by CeO₂ NPs at 4000 mg/L, the root elongation rate of tomatoes, corn and cucumbers was inhibited. Other studies also showed that CeO₂ NPs were able to significantly increase root and stem growth for corn, alfalfa, and soybeans at concentrations of 500 - 4000 mg/L (Lopez-Moreno et al. 2010). When soybean plants (*Glycine mac*) grown in soil impregnated with CeO₂ NPs, most of the Ce stored in the soybean pods was in the form of CeO₂ NPs and a small percentage of the Ce in the pod could be changing its oxidation state from Ce(IV) to Ce(III). It is shown that CeO₂ NPs in soil can be taken up by food crops so that CeO₂ NPs can reach the food chain and the next soybean plant generation (Hernandez-Viezcas et al. 2013). Impacts of CeO₂ NPs on agricultural crops may be due to the chemical and physical transformation of CeO₂ NPs. It is reported that CeO₂ NPs could release Ce³⁺ on their surface and plants tend to uptake Ce³⁺ rather than CeO₂ NPs which might be the primary pathway for plant uptake CeO₂ NPs (Casseo et al. 2011). Studies indicated that CeO₂ NPs were converted into ionic cerium in the rhizosphere and taken up into plants through the roots due to root exudation and the reduction of Ce⁴⁺ in CeO₂ NPs is shown to be easier than in its bulk counterpart (Zhang et al. 2015). The dissolution of this ENP and its redox state is key to understanding its potential toxicity to plants and the surrounding environment (Majumdar et al. 2014).

CeO₂ NPs can significantly affect plant growth in the soil by impacting bacterial communities in plant rhizosphere. A study showed that the cerium could influence the composition of the bacterial community (Ge et al. 2014). When plants were exposed to 1000 mg/kg of CeO₂ NPs, it was shown that root exudates were reduced due to stunted growth. The adsorption of NPs on the extracellular surface might be a reason that CeO₂ NPs have negative impacts on plants. And it might change the certain protein structures of the plants.

Zinc Oxide Nanoparticles

Overview

Zinc oxide nanoparticles (ZnO NPs) are among the most commonly used ENPs in paintings, textiles, industrial coatings, antibacterial agents, and optic and electronic materials (Ju-Nam and Lead 2008). In addition, ZnO NPs display strong antimicrobial properties and have been reported as an effective Zn fertilizer to alleviate Zn deficiency in soils (Milani et al. 2012). Thus, several investigations have been conducted to understand the bioavailability and toxicity of ZnO NPs to bacteria. Studies suggested that toxicity of ZnO NPs was mainly attributed to free zinc ions and labile zinc complexes. For example, it is reported that Zn²⁺ dissolution and organic matter significantly influenced toxicity of ZnO NPs (30 nm) to *B. subtilis* and *E. coli* (Li et al. 2011). It is also reported that tannic acid decreased ZnO NP toxicity more than humic acid and fulvic acid due to its higher complexation of free Zn²⁺ ions and thereby reduced their bioavailability (Li et al. 2011). ZnO NPs could dissolve under aqueous conditions to form hydrated Zn²⁺ cations which is favored under acidic conditions and in the

presence of biological components such as amino acids and peptides (Moreau et al. 2007). Ge et al. (2014) indicated that ZnO NPs could reduce microbial biomass and diversity in soil bacterial community after 60 days of ZnO NPs exposure. It is also reported that ZnO NPs could induce significant changes in soil enzyme activities (Du et al. 2011). As a novel class of products, the environmental impacts and ecological risks of ZnO NPs are important topics that needs more understanding.

Impact of ZnO NPs on Plants

Many studies have been conducted to gain insights into the potential effects ZnO NPs could have on agricultural plants. Most studies indicate the potential toxicity of ZnO NPs. ZnO NPs (< 50 nm) have been shown to negatively impact the development of soybean growth when exposed to a concentration of 500 mg/kg in the soil, as well as negatively impact soybeans in the developmental and reproductive stages (Yoon et al. 2014). Rao and Shekhawat (2014) indicated that when dosed by ZnO NPs at 1000 mg/L, *Brassica juncea* exhibited a significant decrease in plant biomass with a gradual increase in proline content and lipid peroxidation. A wetland plant *Schoenoplectus tabernaemontani* showed significant signs of inhibition when they were exposed to 1,000 mg/L of ZnO NPs (Zhang et al. 2015). The authors also indicated that the translocation of ZnO NPs from root to shoot was limited. A recent study investigated the effect of ZnO NPs in velvet mesquite plant. When the plants were treated with ZnO NPs at concentration from 500 to 4000 mg/L, zinc was detected in vascular tissues of both roots and leaves (Hernandez-Viezcas et al. 2011). One study showed that soybean plants (*Glycine mac*) grown in soil impregnated with ZnO NPs did not accumulate these NPs in

the grains. The authors also indicated that Zn accumulated in the seeds of soybean plants is linked to O, resembling the form of Zn-citrate (Hernandez-Viezcas et al. 2013). It is shown that ZnO NPs significantly affected the root lengths of peas (*Pisum sativum* L.) and Zn²⁺ release had phytotoxic effects on the development of the peas (Huang et al. 2014). Due to the ZnO NPs and the ions it released during the early interactions between rhizobia and plant, the impact to nodule development resulted in delayed nitrogen fixation (Huang et al. 2014). The presence of these nanoparticles also produced early onset of senescence to the nodules (Huang et al. 2014). In another study, it was shown that ZnO can decrease diversity of microbial community in corn microcosm (Kim et al. 2009). ZnO NPs produce toxic effects to Gram-negative *Escherichia coli* (*E. coli*) and that it is mainly attributed to the released Zn²⁺ (Bandyopadhyay et al. 2012). Zhao et al. (2012) shown that that organic acids released by corn roots, bind Zn from the ZnO NPs attached with soil grains or clay minerals in the rhizosphere and then take up by corn roots. This study also stated that some of ZnO NPs were transported by the apoplastic pathway to the endodermis and some of them were transported to the vascular cylinder following the symplastic pathway (Zhao et al. 2012).

There are several mechanisms related to the toxicity of ZnO NPs to plants. The first is the dissolution of ZnO NPs to zinc ions. Although the dissolution of ZnO NPs has been recognized, there is usually a lack of differentiation between the effects of ZnO NPs and ionic zinc in the literature (Ma et al. 2013). One study suggested that the different action mode of ZnO NPs and dissolved Zn²⁺ could be revealed by differential gene expression profiling in *D. magna*. Therefore, using biomarker genes may be a way

to distinguish the effect to plants between ZnO NPs and dissolved Zn²⁺ (Poynton et al. 2011). Despite the origin of dissolution-related toxicity, there are several mechanism following the dissolution, such as Zn dependent ROS formation and inhibition of enzyme activity, could all have toxic effects to plants (Xia et al. 2008). Lin and Xing (2008) indicated that phytotoxicity and root uptake of ZnO NPs cannot only be explained by the dissolution of ZnO NPs from bulk materials alone. Besides dissolution of ZnO NPs, particle-induced effects via ROS-mediated may be another important mechanism for ZnO NPs toxicity (Ma et al. 2013). Photocatalytic activity of ZnO NPs might be another mechanism of ZnO NPs toxicity. ZnO is a photocatalyst and promotes generation of ROS under irradiation with energy at or above its band gap energy (3.37 eV, equivalent to 368 nm) and induce phototoxicity (Diamond et al. 2002). It is reported that natural sunlight significantly enhanced the toxicity of ZnO NPs compared to dark or laboratory fluorescent lighting (Lipovsky et al. 2011).

Impact of ENPs on Co-present Pollutants

While the intrinsic toxicity of ENPs to plants has been widely studied, limited studies have focused on the effects of interactions between ENPs and other pollutants the on overall phyto-effects. Some studies have considered the interactions of ENPs with co-existing contaminants and the subsequent bioaccumulation of these contaminants by and risks for plants. For example, it is reported that TiO₂ and CeO₂ NPs at 100 mg/L and 1000 mg/L decreased the bioavailability of soluble Cu²⁺ and lowered the inhibitive effect of 2 mg/L Cu²⁺ on the root elongation of rice (*Oryza sativa* L.) (Wang et al. 2015). Co-exposure of TiO₂ NPs (500-2000 mg/L) and tetracycline (5-20 mg/L) to rice (*Oryza sativa*

L.) significantly enhanced plant size and biomass. In addition, the co-presence of TiO₂ NPs significantly reduced the oxidative stress of plants, and prevented nutrient deficiency caused by tetracycline and reduced the levels of tetracycline in both rice roots and shoots (Ma et al. 2017). Ji et al. (2017) also reported that the co-presence of TiO₂ NPs could alleviate the toxicity of 20 mg/L Cd to the rice seedlings as indicated by the root elongation, plant height and antioxidant enzyme activities. Venkatachalam et al. (2017) found that ZnO NPs at 25 mg/L could activate several biochemical pathways to enhance plant heavy metal tolerance mechanisms, avoid cellular damage and reduce the oxidative stress caused by 50 mg/L Cd²⁺ and 100 mg/L Pb²⁺ in *Leucaena leucocephala* seedlings. The co-presence of ZnO NPs and heavy metals significantly enhanced the growth rate and biomass of *Leucaena leucocephala* seedlings and generated more antioxidant defense enzymes and satisfying genetic alterations, indicating that additional application of ZnO NPs at appropriate concentration may reduce the toxicity caused by Cd and Pb in *L. leucocephala*. (Venkatachalam et al. 2017). Wang et al. (2011) reported that Al₂O₃ NPs at 1-200 mg/L could increase the lethality of AsO₄³⁻ to *Ceriodaphnia dubia*, with the increase dependent on the Al₂O₃ NPs concentration. The mechanisms of altered toxicity response of *Ceriodaphnia dubia* include significant accumulation of As(V) on the surface of Al₂O₃ NPs and the uptake of As(V)-loaded Al₂O₃ NPs (Wang et al. 2011). Six types of NPs (kaolin, montmorillonite, hydroxyapatite, Fe₃O₄, α-Fe₂O₃, and γ-Fe₂O₃) were reported to alleviate Cd-induced root growth in four plant species (tomato, cucumber, carrot and lettuce). And the author indicated that the precipitation associated with Cd on the root surface would be the main contribution to phototoxicity reduction by

the NPs. Fe₃O₄ NPs at 2000 mg/L was reported to decrease the growth inhibition and oxidative stress caused by heavy metals (Pb, Zn, Cd and Cu) in the wheat seedlings (*Triticum aestivum* L.). The alleviating effects of Fe₃O₄ NPs on heavy metal stress could be attributed to the increase in the antioxidant activities and the high adsorption capacity of heavy metals on Fe₃O₄ NPs (Konate et al. 2017).

When soybeans were exposed to 1000 mg/L CeO₂ NPs and 0.25 and 1 mg/kg dry sand Cd²⁺, the Cd accumulation was not affected. However, the accumulation of Ce in plant tissues was significantly increased (Rossi et al. 2017). Several underlying mechanisms have been explored with regard to the alteration of Cd plant uptake and accumulation by CeO₂ NPs including the adsorption of Cd on CeO₂ NPs so that CeO₂ NPs may function as a carrier of Cd, the altered chemistry in plant rhizosphere such as elevated excretion of root exudates, and plant root anatomical structure changes in the co-presence of Cd and CeO₂ NPs (Rossi et al. 2017). TiO₂ NPs has been reported to aggravate the inhibitive effect of Cd²⁺ at the concentration of 13.2 mg/L for the ciliate *Tetrahymena thermophila* (Yang et al. 2014). Luo et al. (2018) stated that TiO₂ NPs promoted the accumulation and methylation of inorganic As in two algae species (*Microcystis aeruginosa* and *Scenedesmus obliquus*). The increased toxic effects and greater As methylation in algae species was likely due to the dissociation of inorganic As from TiO₂ NPs (Luo et al. 2018).

Although several studies have been reported that ENPs interactions with co-existing contaminants and the effects to plants, the underlying mechanisms of ENPs with co-existing environmental pollutants to plants are still unclear. In addition, the impact of

ZnO NPs with co-existing contaminants to plants, especially redox sensitive heavy metals, is rare (Rao and Shekhawat 2014, Yoon et al. 2014). The phytotoxicity of As is of great concern and its impacts on plants have already been widely studied. However, none of the previous studies has focused on the impacts of co-existing ENPs and As to plants. Some ENPs such as CeO₂ NPs could potentially have redox reaction with redox sensitive chemicals on their surface and their possible production of ROS that can also induce redox reaction with different As species. Due to these potential mechanisms between ENPs and As, it is possible the co-exposure of ENPs and As to plants would have impacts on their uptake and accumulation in plant tissues. Therefore, the primary objective of this study was to assess the impact of CeO₂ NPs and ZnO NPs on the As accumulation in rice. The second objective was to determine whether inorganic As species including both As(III) and As(V) could modify the plant uptake and accumulation of the metal elements in co-existing CeO₂ NPs and ZnO NPs. Understanding the impacts and mechanism of these particles is key to assessing potential environmental risk these particles could have on plants in the long-term.

CHAPTER III
MATERIALS AND METHODS

Nanoparticles

CeO₂ NPs dispersion (20% by weight) and ZnO NPs dispersion (20% by weight) were purchased from US Research Nanomaterials, Inc (Houston, TX). The size and morphology of NPs were determined by a Tecnai G2 F20 transmission electron microscope (TEM). The TEM image of CeO₂ NPs and ZnO NPs are shown in **Fig. 3.1**.

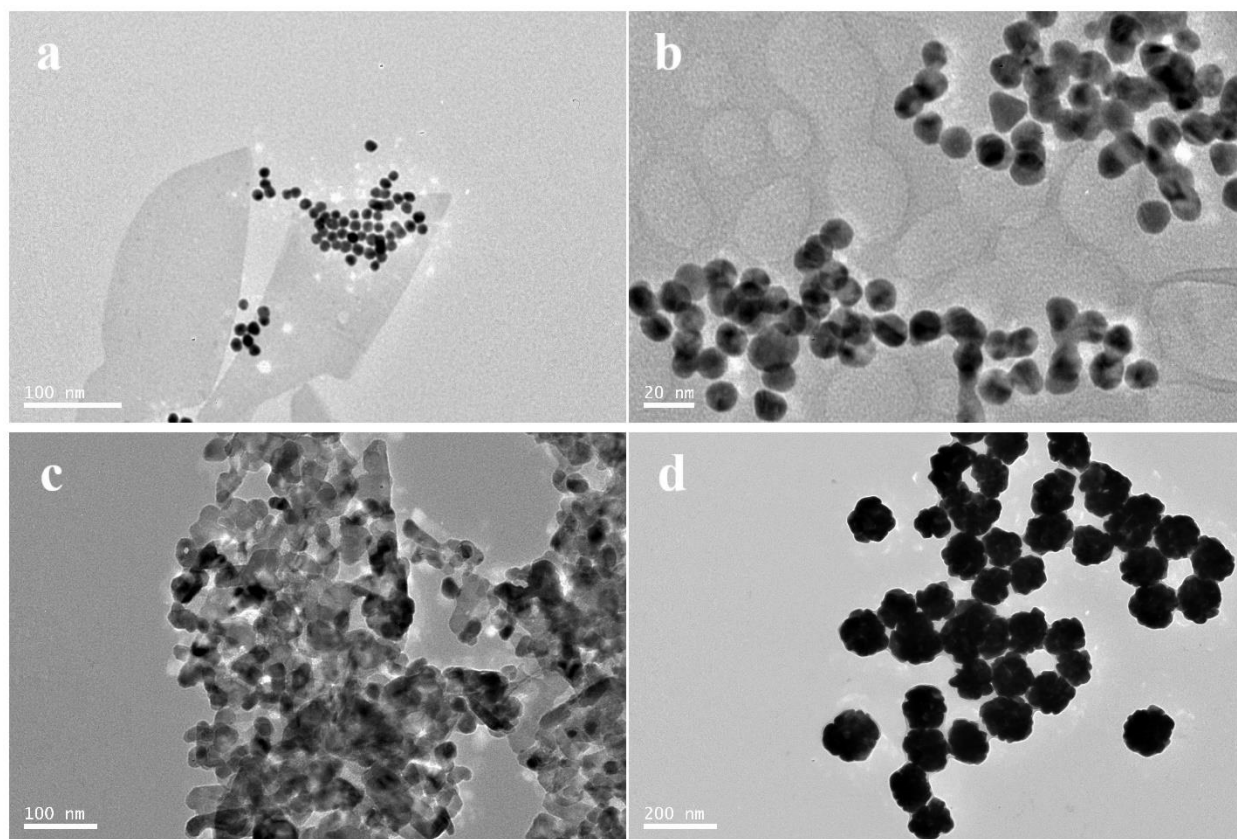


Fig. 3.1 TEM images of primary CeO₂ NPs (a)(b) and ZnO NPs (c)(d).

Most CeO₂ NPs were in the size range of 6–17 nm, with an average size of 10.48 nm. Comparatively, the size of ZnO NPs varied from 15 to 137 nm, with an average size of 68.14 nm. The average particle size was obtained by measuring the diameter of over 100 individual nanoparticles with an imaging processing software ImageJ (ver 1.51). The nanoparticles are primarily spherical and has a zeta potential of -36.55 ± 4.72 mV for CeO₂ NPs and -28.80 ± 2.04 mV for ZnO NPs. The hydrodynamic size of these two nanoparticles measured with a Dynamic light scattering (DLS) are 961.83 ± 94.39 nm for CeO₂ NPs and 621.08 ± 7.63 nm for ZnO NPs in 100 ppm water solution.

Other Reagents

High purity As(V) (Na₂HAsO₄·7H₂O >98%) and As(III) (NaAsO₂ >90%) were purchased from Sigma Aldrich (St. Louis, MO). Zinc sulfate heptahydrate (ZnSO₄·7H₂O >99%) was purchased from Acros Organics (Geel, Belgium). And Hoagland solution was purchased from PhytoTechnology Laboratories (Lenexa, KS).

Plant Cultivation and Harvest

Rice seeds were provided by the Texas A&M AgriLife at Beaumont. The procedures for seed germination and seedling development followed the established protocols in a previous study (Dan et al. 2015). Seeds were sterilized using 1.25% sodium hypochlorite solution for about 10 min and then rinsed with deionized water thoroughly. Then the seeds were germinated on filter paper in a Petri dish moistened with DI water daily for 10 days. Seedlings of similar size were picked and transferred to 15 mL polypropylene centrifuge tubes with 15 mL of quarter strength Hoagland solution because of the small size of rice seedlings. After 5 days they were transferred to 50 mL

polypropylene centrifuge tubes with 50 mL of quarter strength Hoagland solution. They were then incubated in a growth cart with a 16 h UV lighting/8 h dark cycle for seedlings development for about 35 days. The temperature was controlled at 25 °C. The Hoagland solution in the tubes was replaced every other day to avoid algae problems. After the incubation period, the plants were transferred to new 50 mL centrifuge tubes with only tap water for 2 days to remove the Hoagland solution from root surfaces. Afterward, the tap water was replaced with different treatments and replenished daily with tap water. Treatment detail and number of replicates are shown in **Table 3.1**.

Table 3.1. Summary of different treatments

Treatment	Control	100mg/L CeO ₂ NPs	100mg/L ZnONPs	100mg/L Zn ²⁺
Control	3	3	3	3
1mg/L As(III)	3	3	3	3
1mg/L As(V)	3	3	3	3

After 6 days of growth in the treatment solution, the plants were removed from the solution and rinsed with DI water thoroughly. Roots and shoots were separated and weighed to obtain their fresh weight. Rice tissues were oven-dried at 75 °C for 72 hours and weighed to obtain their dry biomass.

Total As, Ce and Zn Analysis in Plant Tissues

The total As, Ce and Zn in plant tissues were determined by strong acid digestion, following EPA method 3050b, as previously reported by Ebbs et al (Ebbs et al. 2015). Approximately 0.3 g of dry root and 0.65 g of dry shoot were added into a 5 mL solution of nitric acid (70% by volume) and sat overnight at room temperature for pre-digestion. They were then further digested using a DigiPREP MS hot block digester

(SCP science, Clark Graham, Canada) at 95 °C for 4 hours until any remaining residual tissue was fully dissolved. The digestate was then cooled to room temperature and further mixed with a 2 mL of 30% (w/v) H₂O₂ and heated in the hot block at 95 °C for another 2 hours. This solution was then analyzed through inductively coupled plasma spectrometry (ICP-MS, Perkin Elmer mod. DRCII, Waltham, MA).

Statistical Analysis

All results were subjected to the analysis of variance, means and standard deviation. Minitab 17 (Minitab Inc., State College, PA) was used to perform one-way analysis of variance (ANOVA). A one-way ANOVA determines if there are statistical differences between the means of three or more independent groups. Values were considered significant if $p \leq 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

Fresh Biomass

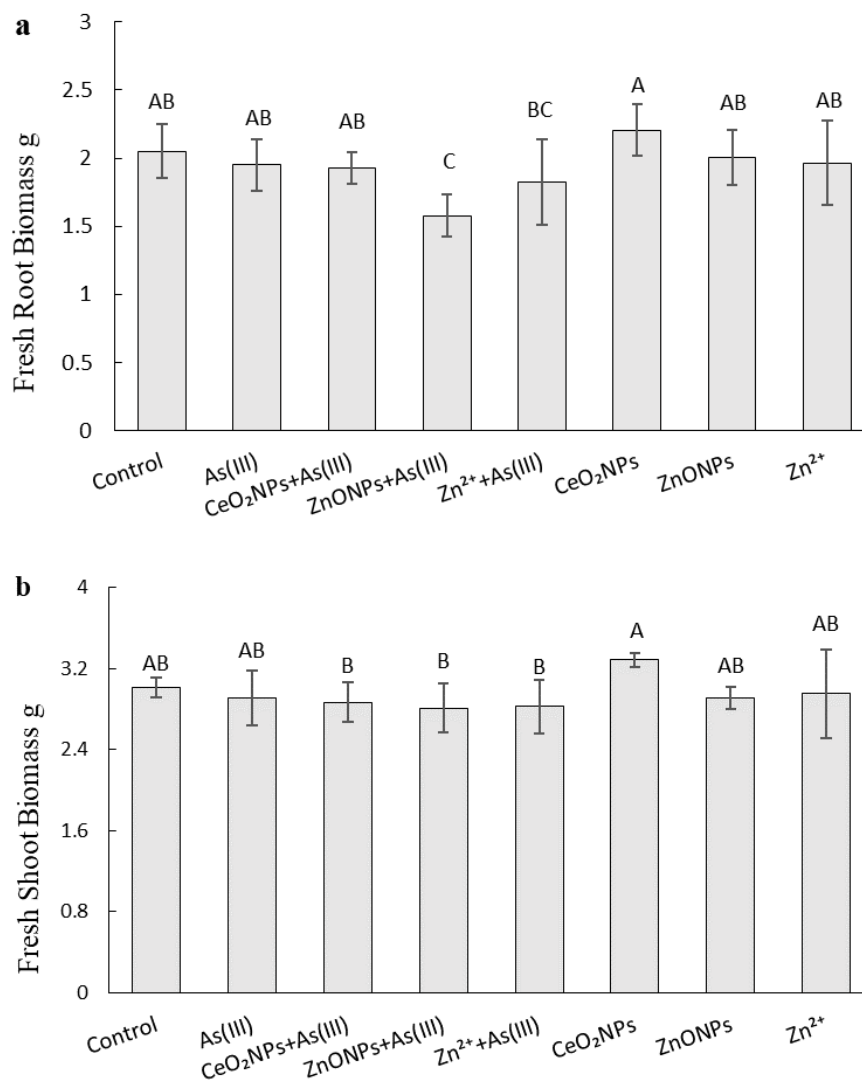


Fig. 4.1 Fresh biomass levels after As(III) exposure. Root biomass (a) and shoot biomass (b) of rice plants exposed to As(III). Rice plants were grown in two different nanoparticles (Cerium oxide nanoparticles and Zinc oxide nanoparticles) and Zinc ions. Values represent mean \pm SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA followed by Tukey's test. The letters are only reported when differences among means are statistically significant.

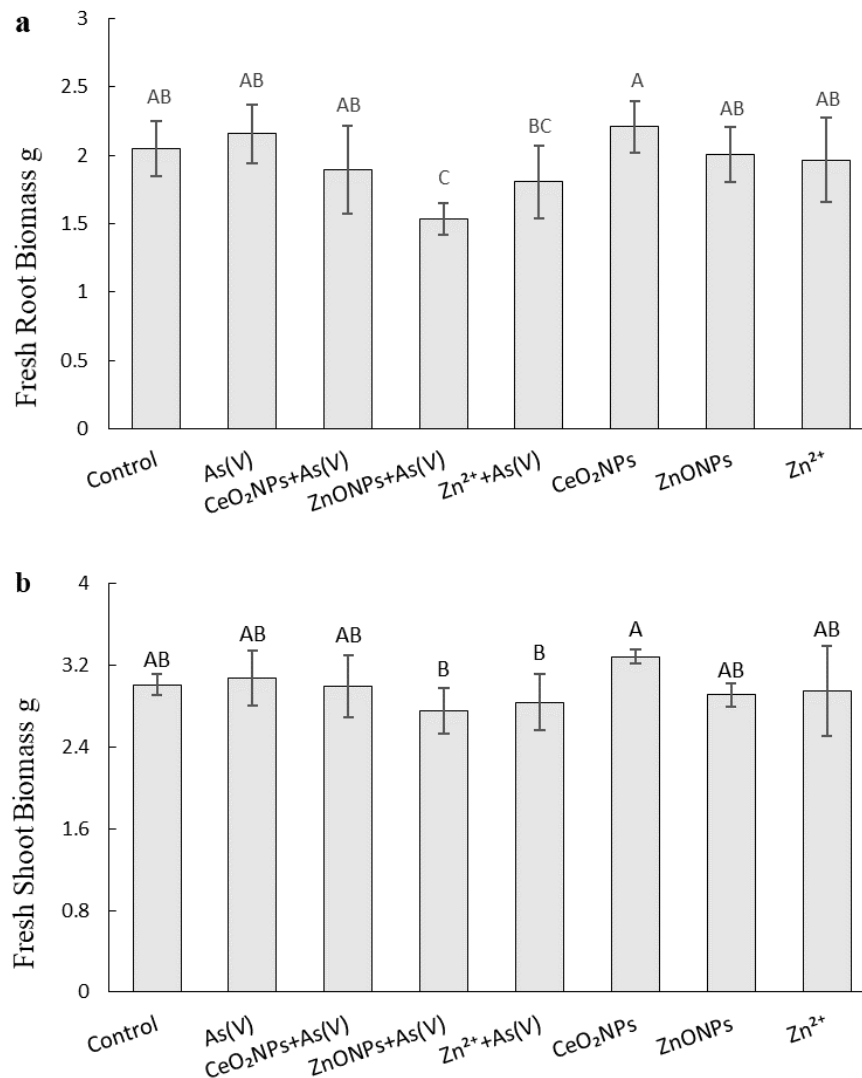


Fig. 4.2 Fresh biomass levels after As(V) exposure. Root biomass (a) and shoot biomass (b) of rice plants exposed to As(V). Rice plants were grown in two different nanoparticles (Cerium oxide nanoparticles and Zinc oxide nanoparticles) and Zinc ions. Values represent mean \pm SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA followed by Tukey's test. The letters are only reported when differences among means are statistically significant.

The fresh biomass of rice roots and shoots from different treatments are shown in

Figure 4.1 and **Figure 4.2**. Neither As nor ENPs alone at the used concentrations affected the root and shoot biomass of rice seedlings compared with the controls.

However, the co-exposure of ZnO NPs with As(III) or As(V) significantly reduced the rice root biomass compared with the control plants, or plants treated with these chemicals alone. ZnO NPs significantly decreased the fresh root biomass by 19% and 29% in the co-presence of As(III) and As(V) respectively, compared to seedlings exposed to ZnO NPs alone. Neither CeO₂ NPs nor Zn²⁺ led to any significant reduction of rice root biomass at the used concentration. The co-exposure of As(V) with CeO₂ NPs did not cause any significant changes in rice shoot biomass. However, CeO₂ NPs significantly decreased the fresh shoot biomass by 13% in the co-presence of As(III), compared to plants exposed to CeO₂ NPs alone. No significant differences were found in the fresh biomass of shoots treated with either ZnO NPs or Zn²⁺ compared with the control plants, or plants treated with these chemicals alone.

Arsenic accumulation

Concentrations of total As in rice root and shoot tissues from different treatments are shown in **Figure 4.3**. The total As concentration in rice tissues exposed to As(III) is significantly higher than those exposed to the same concentration of As(V) in all groups as expected. The accumulation of total As in rice tissues was affected by co-existing ENPs, and the extent of effect depends on the initial As oxidation state, specific plant tissue and the properties of ENPs.

The total As was significantly reduced by ZnO NPs and Zn²⁺ treatment for both As(III) and As(V) in all plants tissues compared with plants exposed to ZnO NPs or Zn²⁺ alone. CeO₂ NPs displayed little effect on the total As in plant tissues for both As(III) and As(V) exposed rice. In rice root tissues, ZnO NPs and Zn²⁺ affected the total As

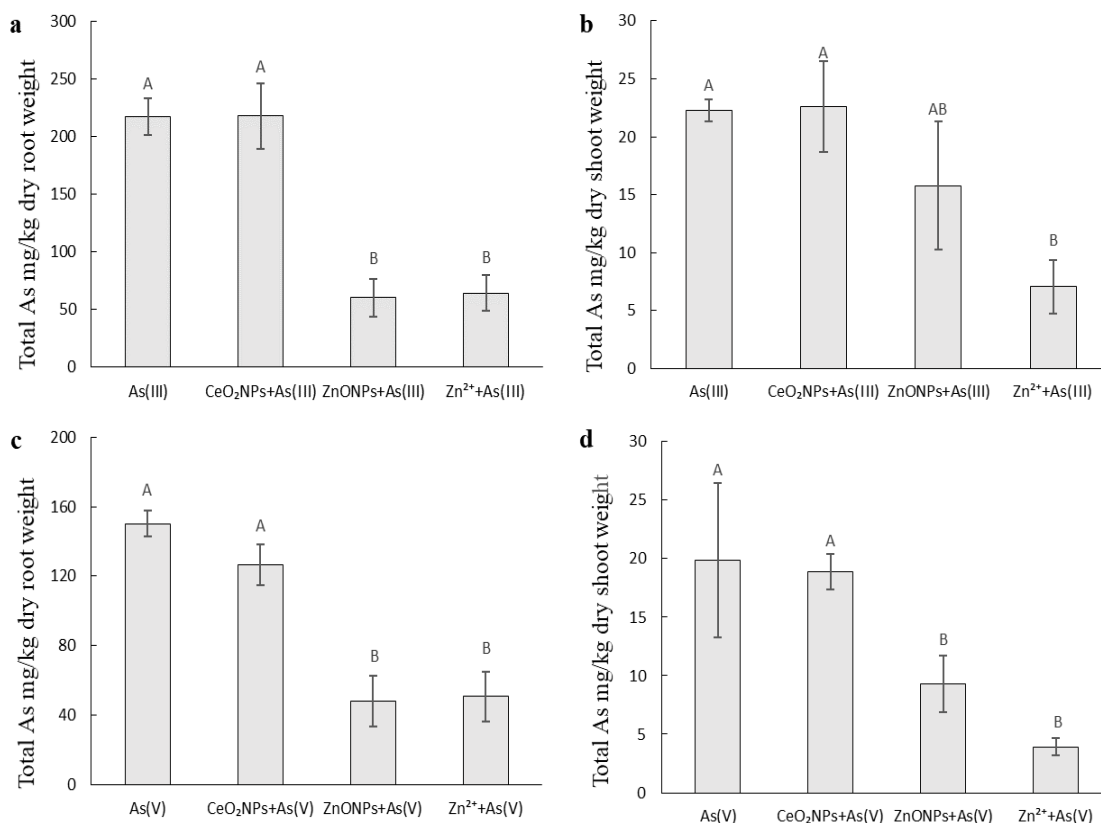


Fig. 4.3 Total arsenic in plant tissues. Accumulation levels within root biomass (a) and shoot biomass (b) of rice plants exposed to As(III) and root biomass (c) and shoot biomass (d) of rice plants exposed to As(V). Rice plants were grown in two different nanoparticles (Cerium oxide nanoparticles and Zinc oxide nanoparticles) and Zinc ions. Values represent mean \pm SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA followed by Tukey's test. The letters are only reported when differences among means are statistically significant.

accumulation to a similar extent for both As(III) and As(V). The total As in rice roots after exposure to ZnO NPs for six days was 72% and 68% lower than their respective controls for As(III) and As(V). The impact of Zn²⁺ on the accumulation of total As in rice shoots was greater than ZnO NPs for As at both oxidation states. For example, co-exposure to Zn²⁺ resulted in 55% and a 58% less total As in rice shoots than plants exposed to the same concentrations of As(III) and As(V) and ZnO NPs.

High dissolution of ZnO NPs might be a reason that ZnO NPs and Zn²⁺ both have great effects. However, this should not be the only mechanism on how ZnO NPs affect rice uptake As. Compared to Zn²⁺ treatment, ZnO NPs had a greater impact on the rice root uptake of total As, but less transport of As from root to shoot. Why did the co-presence of ZnO NPs result in stronger impact on As uptake on rice roots? It is possible that co-existing of ZnO NPs and As had negative impacts on rice growth and root development which might affect As transporters. The smaller root biomass caused by the ZnO NPs with both As species support this. This means that ZnO NPs would affect more on As(III) uptake by aquaporins other than the phosphate transporters (Rao and Shekhawat 2014, Yoon et al. 2014). Another possibility is that ZnO NPs may inhibited the reduction of As(V) to As(III) outside of rice root, so that the total As will be less in rice tissues because of more As(V) than As(III) in rice rhizosphere (Xu et al. 2008). Plant root exudates may be an important factor to consider. Plant root exudates consist of a suite of low molecular weight organic acids, amino acids, proteins, and polysaccharides (Zhalnina et al. 2018). Some of these compounds could donate electrons to convert As(V) to As(III) in the absence of ZnO NPs (Tu et al. 2004, Xu et al. 2007). With the co-presence of ZnO NPs, strong oxidants reactive oxygen species(ROS) might be induced by ZnO NPs (Dutta et al. 2012, Lipovsky et al. 2009, Luna-Velasco et al. 2011). The induction of ROS may cause oxidation of all root exudates and potentially reduced As reduction. Also, we hypothesize that the inducted ROS may cause lower As(III) inside of rice root cells by lowering the function of enzyme arsenate

reductase(AR) and interrupting enzyme catalyzing inside plant root cells. Further As speciation analysis may support our hypothesis if there is less As(III) in rice tissues. Inorganic As, especially As(III), is a primary concern in food chain safety. The dramatical reduction of total As in rice tissues indicate that co-treatment of ZnO NPs might be an effective way to reduce As in rice grains. The potential reduction of As(III) accumulation in rice by ZnO NPs is particularly intriguing due to the high accumulation of inorganic As(III) in rice grains. However, the toxicity of co-existing ZnO NPs and As at the concentration of this study should be brought into consideration. Further study is needed to find a desired concentration ZnO NPs which could significantly reduce As accumulation in rice tissues and be safe to rice plants.

Cerium Accumulation

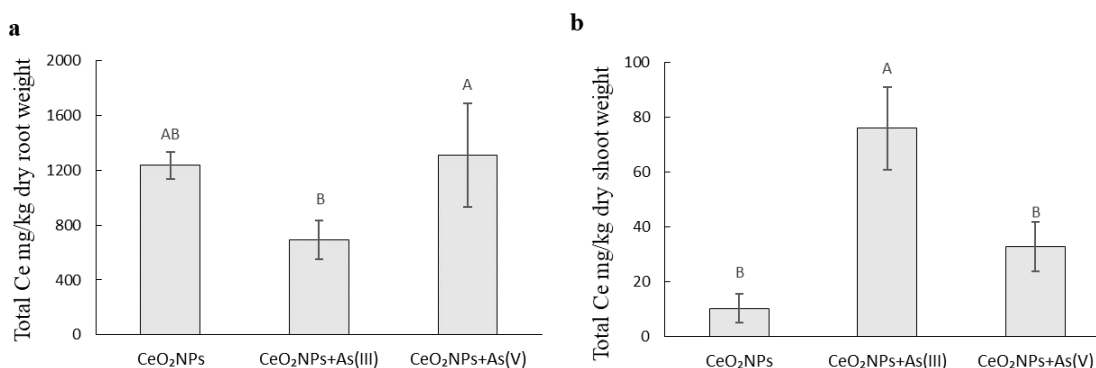


Fig. 4.4 Total cerium in plant tissues. Accumulation levels within root biomass (a) and shoot biomass (b) of rice plants exposed to CeO₂ NPs. Rice plants were grown in two different arsenic species (As(III) and As(V)). Values represent mean \pm SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA followed by Tukey's test. The letters are only reported when differences among means are statistically significant.

In addition to the accumulation of As, the accumulation of nanoparticle elements in plants have attracted some attention before. Some previous studies have shown that co-existing environmental pollutants such as heavy metals may alter the plant uptake of

nanoparticle elements (Rossi et al. 2017, Venkatachalam et al. 2017). **Figure 4.4** shows the total Ce levels in plant tissues obtained through the strong acid digestion of the dry rice tissues. Total Ce associated with both roots and shoots were significantly impacted by As(III), with a 44% decrease from in roots, and a 6-time increase in the shoots compared with plants exposed to CeO₂ NPs alone. Plants co-exposed to As(V) showed a higher Ce concentration compared to control group both in root and shoot, but this increase was not statistically significant. The co-presence of CeO₂ NPs and As have a great effect on total cerium in plants tissue. It is possible that the co-presence of CeO₂ NPs and As might have resulted in more root exudates. It was reported that the co-presence of CeO₂ NPs and Cd enhanced the excretion of root exudates (Rossi et al. 2018). Elevated root exudation could lead to greater dissolution of CeO₂ NPs. The dissolved Ce ion is much more efficiently transported from roots to shoots than CeO₂ NPs(Zhang et al. 2015). The enhanced dissolution and greater availability of dissolved Ce may be key reason for the elevated Ce in rice shoot in the co-presence of CeO₂ NPs and As, in particular, the As(III).

Zinc Accumulation

Figure 4.5 shows the total Zn levels in plant tissues obtained through the strong acid digestion of the dry rice tissues. Neither As(III) nor As(V) made a significant difference in plant Zn accumulation, regardless of the Zn source supplied ZnO NPs or Zn²⁺. Although the average accumulation in plants dosed with As(III) and As(V) were higher compared to the plants treated with ZnO NPs and Zn²⁺ alone, this was not statistically significant.

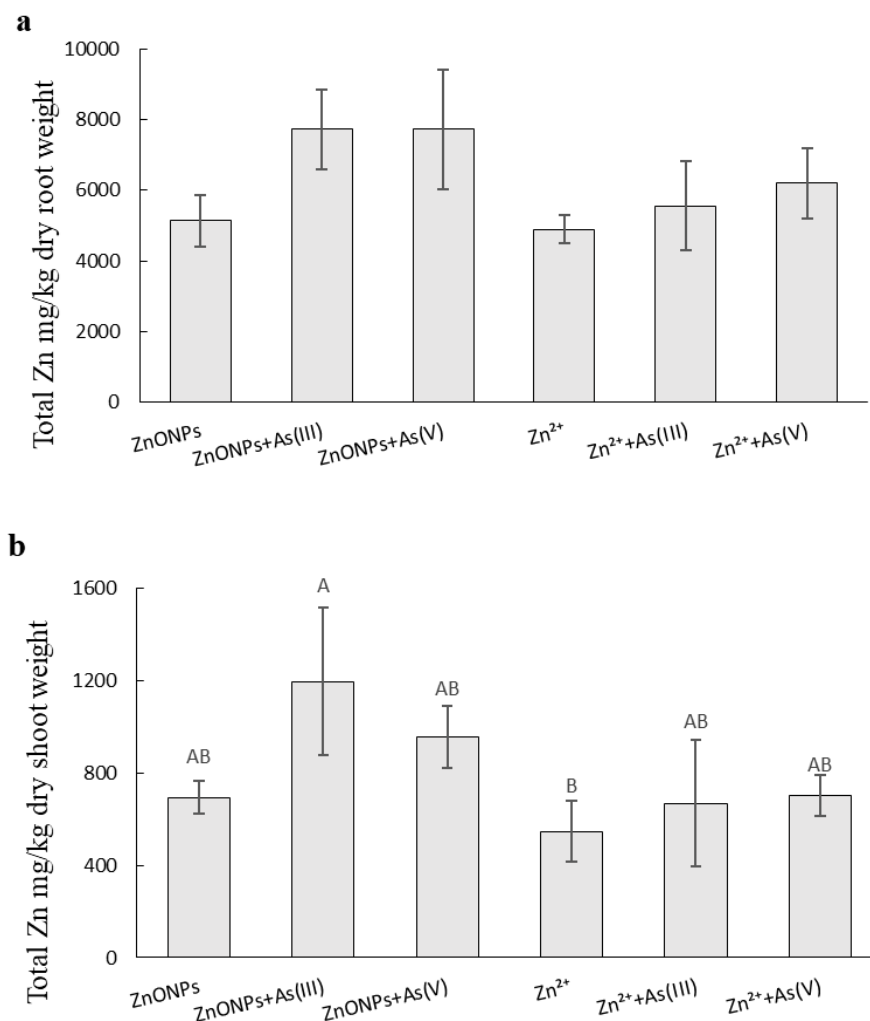


Fig. 4.5 Total zinc in plant tissues. Accumulation levels within root biomass (a) and shoot biomass (b) of rice plants exposed to ZnO NPs and Zn ion. Rice plants were grown in two different arsenic species (As(III) and As(V)). Values represent mean \pm SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA followed by Tukey's test. The letters are only reported when differences among means are statistically significant.

The results might be because of the different pathway of As uptake by rice roots.

Several members of the Zn-regulated transporters in the iron (Fe)-regulated transporter-like protein (ZIP) gene family have been characterized and shown to be involved in Zn

uptake and transport in plants (Connolly et al. 2002, Eide et al. 1996). In rice plants (*Oryza sativa*), *OsZIP4* is a Zn transporter that may be responsible for Zn translocation to the plant parts that require Zn (Ishimaru et al. 2011). Zn does not share the same transporters with As so that no competition was observed. In addition, Zn is a required nutrient for plant protein synthesis and the unaffected Zn by As indicate that co-presence of As and ZnO NPs or Zn ions would not diminish the Zn availability to plants if ZnO NPs or Zn²⁺ are used as fertilizers. The study opened new doors for potential development of ZnO NPs based nanofertilizers which could simultaneously supply Zn nutrients and inhibit As accumulation in rice.

In summary, our results showed that co-presence of ENPs and As had strong effects on total As accumulation in rice tissues. Different properties of ENPs and initial As oxidation state could have different effects on plants uptake As. According to our data, ZnO NPs might be a great supplement for plants and at the same time to reduce As accumulation in rice since Zn is a necessary nutrient for plants.

Further work is needed to understand the interactions that were seen, particularly for ZnO NPs. As speciation should be analyzed to confirm our hypothesis and to better understand the mechanism between ENPs and As. Additional experiment is also needed to verify that if a similar result in a soil system and more complicated mechanism could be observed. Also, determine the desired ZnO NPs concentration levels would be considered due to how important this is in plant health and the potential applications of ENPs in agriculture. In addition, the rice seedlings were grown for only a few weeks in this study and rice grain was not examined due to their unavailability at termination.

However, the major safety concern for As is their high accumulation in rice grains which are used as a staple food around the globe. Therefore, future studies should consider growing rice for a whole life cycle to obtain more insights into the effect of ENPs on As accumulation in rice grains and their food safety implications. Finally, more efforts should be made to determine an optimal level of ZnO NPs, that will not cause phytotoxicity in the joint exposure with As.

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