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ENGINEERING ZINC OXIDE NANOPARTICLES TO BE USED AS NANOFERTILIZERS

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ENGINEERING ZINC OXIDE NANOPARTICLES TO BE USED AS NANOFERTILIZERS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By
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Lexington, Kentucky
2018

ABSTRACT OF DISSERTATION

ENGINEERING ZINC OXIDE NANOPARTICLES TO BE USED AS NANOFERTILIZERS

Zinc deficient soils, or soils with low Zn bioavailability, are widespread, which exacerbates Zn deficiency in human as crops grown on these soils have low Zn content. Often crop yields are also compromised. Fertilizers based on soluble Zn salts often have limited efficacy in such soils. In this research, we evaluate the performance of polymer coated and bare ZnO nanoparticles (NPs) in an attempt to overcome limitations of soluble Zn salts in alkaline soils. We first synthesized 20-30 nm bare ZnO NPs with different surface chemistries to impart colloidal stability to the particles. Bare ZnO were treated in phosphate solution under certain conditions leading to the formation of a core made of ZnO NPs that is covered by a shell of amorphous $Zn_3(PO_4)_2$ (core-shell NPs). This confers a negative charge to the particles over a wide pH range. The addition of nonionic (neutral dextran) and polyelectrolyte (negatively charged dextran sulfate ($DEX(SO_4)$) during the synthesis resulted in the formation of DEX and $DEX(SO_4)$ ZnO NPs. Dextran has a minimal effect on the surface charge of ZnO but dextran sulfate confers a net negative charge. Bare and core-shell ZnO NPs were both electrostatically stabilized whereas DEX and $DEX(SO_4)$ ZnO NPs were sterically and electrosterically stabilized, respectively. We investigated the effect of treating seeds with ZnO NPs on the growth and accumulation of Zn in wheat (*Triticum aestivum)* seedlings in comparison to $ZnSO_4$. All ZnO NPs stimulated seedling growth. Seedlings accumulated higher Zn concentrations when treated with ZnO NPs than with $ZnSO_4$. Zinc sulfate was toxic even at the lower exposure concentrations, which was demonstrated by significantly lower germination success and seedling growth. In the second experiment, we investigated the effect of pH on the attachment and dissolution of ZnO NPs in soil, as compared to $ZnSO_4$. Soil pH was adjusted to 6 and 8, then the soil was spiked with 100 mg Zn/kg soil in the form of $ZnSO_4$, bare, DEX, $DEX(SO_4)$, and core-shell ZnO NPs. The results showed that DEX and core-shell ZnO NPs had significantly higher total Zn in soil solution compared to $ZnSO_4$ at pH 8, with little dissolution. Dissolved Zn was similar among treatments except $ZnSO_4$ at pH 6, indicating little dissolution of the ZnO NPs at either pH value. We also found that the engineered coatings dictate the behavior of the particles in simple aqueous systems, but their properties are altered in natural soil solutions because of the dominant effect of natural organic matter (NOM) on their surface chemistry. Based on the outcomes of the previous two experiments, we selected DEX and bare ZnO NPs to test the efficacy of ZnO NPs in delivering Zn to the grain of wheat under greenhouse conditions. We performed two independent studies where seeds were either treated with the NPs or grown in a soil spiked with Zn at pH 6 and 8 and spiked with Zn treatments (nano and ionic). We found that treating seeds with bare ZnO NPs significantly enhanced grain Zn concentrations as compared to the control, DEX-

ZnO NPs, and $ZnSO_4$. There were no differences in grain Zn concentration of plants treated with ionic or nano Zn treatments regardless of the soil pH. This work has elucidated important principles which will help carry forward efforts at developing effective ZnO NP-based fertilizers. It also suggests that treatment of seeds with ZnO NPs is more effective than amending soil or treating seeds with $ZnSO_4$.

KEYWORDS: zinc oxide nanoparticles, surface chemistry, zinc malnutrition, partitioning,

soil pH, natural organic matter.

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ENGINEERING ZINC OXIDE NANOPARTICLES TO BE USED AS NANOFERTILIZERS

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DEDICATION

For there are not enough words to describe my love for my dedicated loving parents, family at home, and my wonderful sister Nour, with whom I shared this wonderful journey of six years, I dedicate this work to all of you.

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Chapter 1: General Introduction

1.1 Zinc Bioavailability for Human and Plants

1.1.1 Zinc Biological Functions

The biochemical importance of Zn has been widely addressed in the literature. Zinc is an essential micronutrient required for plant and animal growth and development[1]. About 10% of the human proteome consists of metalloproteins which require Zn for proper structure and function[2]. Human and animals require Zn in trace amounts as a co-factor in more than 300 enzymes involved in key reactions associated with the immune, reproductive, and nervous systems[3]. Likewise, in plants, Zn has a catalytic role in numerous enzymes (e.g. dehydrogenases) and a structural role in other proteins (e.g. Zn finger domain proteins)[4].

1.1.2 Zinc Malnutrition in Human

Micronutrient malnutrition is a worldwide problem[5]. Zinc is among the most deficient micronutrients in the human diet. Due to the involvement of Zn in hundreds to thousands of enzymes and proteins which encompass a myriad of biological functions, Zn deficiency could lead to a multitude of health conditions, such as dwarfism, skin lesions, cognitive and immunological dysfunction, delay in skeletal maturation, anorexia, hypogonadism, diarrhea, and pneumonia [6-9].

The first case that addressed Zn deficiency in human was in the 1960s in Iranian adult males[10]. In 1991, the United Nations dropped Zn from its list of "deficient micronutrients" due to the non-specific clinical conditions associated with Zn deficiency in human[11], and the lack of reliable markers[12]. However, in the wake of around one million deaths of infants and children who suffer from severe Zn deficiency per year, the

world health organization (WHO) has published several reports focusing on Zn deficiency as an epidemic that needs to be resolved, especially in developing countries[8]. As a result, more attention has been given to Zn deficiency since the mid-1990s.

Zinc deficiency in human is mainly attributed to dependence on cereals in the diet with, very low consumption of animal food sources[5]. In developing countries, the major sources of Zn are legumes and cereals[13]. In cereals, processing (polishing and milling) often removes the embryonic tissues and the aleurone layer which accumulate most of the micronutrients, including Zn[14]. Moreover, cereals are rich with antinutrient molecules such phytic and tannic acids[12]. The complexation of Zn with phytic acid results in an insoluble form of Zn inside plant tissue. Human lacks the enzyme phytase which is responsible for breaking down phytate molecules within the digestive system[15]. The high consumption of cereals, which are rich in phytic acid, also lowers the absorption and bioavailability of Zn inside the human body.

1.1.3 Factors Affecting Zinc Bioavailability to Plants

Zinc deficiency in soils is a major reason for low Zn in crops[16, 17]. Zinc deficiency significantly reduces yields and nutritional values of staple crops such as wheat[18]. About half of the arable lands around the world have soils that are Zn deficient[19, 20]. Soils may have low plant available Zn for many reasons, such as low geogenic Zn levels[21], high organic matter content, high soil pH, and high carbonate and/or phosphate contents which make Zn less available for plant uptake[7]. Higher soil pH results in the fixation of the positively charged Zn ions on the negatively charged soil surfaces including humic substances, clay minerals, and Al/Fe oxohydroxides[22]. Moreover, the precipitation of Zn

in carbonates and hydroxides of very low solubility renders Zn less bioavailable to plants[23].

1.1.4 Limitations to Crop Enrichment with Zinc

Numerous efforts have been made to overcome Zn deficiency using many approaches. While plant genetics and breeding are among the most promising approaches[24], fortifying food with micronutrients and the use of pharmaceutical supplements have been of limited success in developing countries as a result of their relatively high costs[13]. Also, the use of genetically modified crops is not acceptable in some countries, and metal hyperacumulating plants may have limited success in areas of low geogenic Zn. Diversification of the diet, including incorporation of animal sources to gain the required micronutrients, is also economically challenging in developing countries[3]. Agrochemical fortification using conventional Zn fertilizers is one popular approach to circumvent Zn malnutrition. Unfortunately, in some cases, large amounts of relatively expensive Zn fertilizers are required to meet crop needs. In addition, undesirable Zn binding and precipitation as insoluble minerals is likely in some soils, especially those with high carbonate, phosphate, and pH[25]. Another important issue that should be accounted for is the partitioning of Zn in plant tissue where the edible tissue should be targeted for Zn enrichment to succeed in human dietary fortification. The efficiency of Zn enrichment is also species-dependent, cereals and legumes which are the major sources of Zn in developing countries can suffer from high phytate content, a P storage molecule which binds Zn ions, and since human lacks the required enzyme to metabolize phytate, Zn bound to phytate is not bioavailable to human regardless of Zn concentrations in the cereals grains[26].

One study showed that the use of P fertilizer increased phytate content which bound Zn in wheat grain, a phenomenon that was counteracted by foliar application of Zn instead of adding Zn fertilizer to soil[27]. Starchy crops such as potatoes have high phytate contents in the roots making up 0.1% of root dry weight[28], which decreases Zn bioavailability[29].

Zinc is tightly regulated in plants[7, 30]. Several physiological bottlenecks reportedly limit the success of Zn fortification of crops[31]. The transport of Zn ions from the soil through the root epidermis and the subsequent cell to cell movement from the xylem to the phloem are all orchestrated in a manner that will ensure internal Zn homeostasis[32]. Cell walls are loaded with proteins and ATPases which regulate the entry of Zn ions from/to the cells. Also, excess Zn is more likely to be stored in vacuoles, and in anti-nutrient molecules (e.g. phytic acid), which limits the mobility, translocation, and bioavailability in edible tissue[32]. Also, localization of Zn ions in the fruit is important. In wheat grain, Zn partitions more to tissues in the embryo and the aleurone layer[33], which are removed during grain polishing, whereas less Zn is localized in the endosperm which is more often consumed[14]. One possible way to avoid this problem is by alternatively consuming unpolished whole grain wheat.

1.2 Application of Nanotechnology to Agriculture

Agriculture is constantly under pressure to provide adequate food for a rapidly growing population. The world population is expected to reach an estimated 10 billion by the middle of the 21st century[34]. However, limitations in water, energy, land, and the constant deterioration of soil and environmental quality will widen the gap between food

supply and demand[34, 35]. The precision application of agrochemicals such as fertilizers and pesticides is critical to increasing crop yields[36, 37].

The current production and use of fertilizers are not sustainable[38, 39]. Indeed, nitrogen use efficiency (NUE), for example, was estimated to be as low as 30-50% due to runoff and denitrification losses encountered when using conventional fertilizers[40]. Likewise, efforts to fortify staple crops with Zn fertilizers often fall short due to the confounding effects of soil properties which limit the efficacy of Zn fertilizers. Inorganic Zn salts such as $ZnSO_4$ and chelated forms (Zn-EDTA) are the most widely used soluble Zn fertilizers. While the latter is more effective and successful in enhancing Zn bioavailability, especially on calcareous soils, it is relatively more expensive[41].

Nanotechnology involves manipulation of matter at the nanoscale, which is between 1-100 nm[42]. At the nanoscale, materials manifest different chemical and physical properties compared to their atomic/molecular and bulk counterparts[43]. Many of these unique properties can be attributed to their high specific surface area to volume ratio[44]. Considering their novel properties, nanoscale materials have become widely utilized in the manufacturing of many products and materials[45]. Recently, interest in the use of nanomaterials in agriculture has grown, including the use of nanomaterial-based pesticides[46] and fertilizers[47]. In 2104, an American Chemical Society (ACS) report projected a revolutionary advancement in agriculture through nanotechnology[48]. The number of research patents pertinent to incorporating nanomaterials in agrochemical products and applications has exponentially increased, although commercialization of such products is still lagging [49].

Targeted delivery of nutrients and pest control products using nano-delivery systems could provide more efficient and sustainable tools to enhance crop nutrition and protection against diseases[39, 50]. Indeed, nano-delivery systems complying with the nutrient stewardship framework of the "four Rs", right source, right amount, right time, and right place to ensure fertilizer application is efficient[51], offer a promising and more economically and environmentally sound alternative to conventional fertilizers.

There are an increasing number of studies investigating the possibility of using engineered ZnO nanoparticles (NP) as a source of Zn to enhance the yield of crops such as maize (*Zea mays*)[52], foxtail millet (*Setaria italica*) [53], wheat (*Triticum aestivum*)[54], and peanut (*Arachis hypogaea*)[55]. Also, one study have suggested that ZnO NPs may have enhanced Zn bioavailability to crops relative to conventional Zn sources[56]. Prasad et al. reported that fertilizer made of bare ZnO NPs that were used as seed treatment resulted in a marked yield increase in peanut when compared to both chelated Zn and bulk $ZnSO_4$ fertilizers[55]. However, the vast majority of these studies have focused on bare ZnO NPs and have not explored the use of coatings to tune their behavior for specific applications. The use of coatings to tune the surface chemistry of particles can have a dramatic effect on their behavior.

1.2.1 Transformation of Nanoparticles

One of the major knowledge gaps in the field of nanotechnology is the lack of understanding of the behavior, transformation, bioavailability, fate, and toxicity of NPs as they enter an environmental media such as soil[57, 58]. Thus, a comprehensive investigation that covers the effects of environmental variables (e.g. pH, ionic strength, NOM content, presence of inorganic ligands, redox potential, etc.), and the intrinsic properties of NPs

(size, shape, surface chemistry), should be performed in order to engineer ZnO NPs with appropriate physicochemical properties. In the past, research into the behavior of NPs in extremely complex media such as soil has been difficult because of the lack of specific and sensitive analytical techniques for their detection and characterization[59]. The introduction of NPs into complex media will alter their properties as a result of the interaction between the physical, chemical, and biological conditions of the environment and the intrinsic properties of the particles themselves. The following sections summarize the major possible transformations of ZnO NPs.

1.2.2 Aggregation

Aggregation is one important criterion that should be taken into account in determining the mobility, transport, and bioavailability of NPs[60]. Aggregation is affected by the intrinsic properties of NPs and the extrinsic environmental variables. For example, as Ag NP size decreases, aggregation increases due to the increased surface free energy[61, 62]. Similarly, an increase in the concentration of NPs enhances aggregation[62] resultant from the increased probability of collisions between particles (although in soil, heteroaggregation processes are expected to dominate)[63, 64]. Extrinsic properties of the surrounding media also have an effect on the aggregation of NPs. For example, aggregation rate increases as the pH of the solution approaches the point of zero charge (PZC) - the point of zero charge of NPs (PZC) is the pH at which positive and negative surface charges are balanced resulting in an electrophoretic mobility of zero[65]- , which is reported to be 9.3[66, 67] for bare ZnO NPs , since electrostatic repulsion between particles is minimized. Extrinsic properties of the environment also affect nanoparticle behavior. Many studies reported that, consistent with classical Derjaguin, Landau, Verwey and

Ovebeek (DLVO) theory, increasing the ionic strength resulted in an increased aggregation of bare ZnO NPs[67-69]. The effect of electrolyte type and concentration on the stability of NPs in aqueous solutions has been investigated in many studies. As is well known from classical colloid science, the presence of polyvalent cations, such as Ca^{2+}, greatly enhances aggregation[68, 70].

1.2.3 Dissolution

Dissolution of nanoparticles as they enter the environment has been relatively well investigated; ZnO NPs dissolution eventually releases ionic forms of Zn[67, 71, 72]. Dissolution of Ag NPs can be size dependent regardless of the coating type and/or synthesis method [37]. This is attributed to the larger surface area and the increased lattice strain-induced surface energy associated with smaller Ag particles, which increases dissolution [58, 73]. Similarly, ZnO NP dissolution is more dependent on the primary size of the particles, where smaller NPs dissolve more quickly than larger particles[74]. It is also greatly affected by the transformations of the surface of nanoparticles to form more or less soluble minerals, such as sulfidation or phosphatation[75]. The pH of the media greatly affects the dissolution of metal oxide NPs. For metal oxides, in general, as the acidity increases, the number of protons attacking the surface of the NPs increase, leading to the polarization of the metal-hydroxyl bond, which eventually results in the detachment of the metal from the surface[76, 77]. Proton promoted dissolution of goethite nanorods was found to be an order of magnitude higher when pH was lowered from 2 to 1[78].

1.2.4 Coatings

Coatings are commonly used to impart stability and dispersivity to the NPs via steric

and/or electrostatic repulsion and can dramatically alter the PZC in the case of ionic coatings such as polyelectrolytes[79-81]. The influence of coatings on NP behavior has only begun to be explored. For example, in aqueous environments, sterically stabilized Ag NPs with the nonionic polymer polyvinylpyrrolidone (PVP) coating had greater colloidal stability than electrostatically stabilized Ag NPs with citrate coatings (CIT) under varied solution chemistry [57, 82]. Microbial communities may play a role in the persistence of these coatings. Kirschling et al., found that coatings could be bioavailable to bacteria[79], thus NPs could lose these coatings and become more prone to aggregation. We have also explored the role of coating molar mass and charge in determining the attachment of Ag and ZnO NPs to soils[83, 84]. We have also demonstrated that Ag NP coating influences the extent to which organic ligands in solution stimulate oxidative dissolution[85]. Inorganic coatings (also called shells), also play an important role in controlling the behavior of NPs. Our previous research has demonstrated that $Zn_3(PO_4)_2$ shells dramatically decrease the solubility of ZnO nanomaterials due to the insolubility of $Zn_3(PO_4)_2$ [86].

1.2.5 Interaction with Natural Organic Matter (NOM)

Zinc oxide NPs can have enhanced colloidal stability in the presence of NOM. by Natural organic matter acts as a stabilizer against aggregation by imparting negative charge to particles, [82]. Natural organic matter may adsorb to the surface of bare NPs[67] or replace the as-synthesized coating[87]. In addition to increasing colloidal stability of particles, we have also found that some NOM may enhance the dissolution of Ag NPs[85].

The concentration and characteristics of NOM present in an environment are among the key factors that influences the behavior and stability of NPs[88]. Previous studies reported that NOM could induce the aggregation of bare ZnO NPs at lower NOM

concentrations [67, 72, 83]. However, it is unlikely to be the case under relevant environmental conditions, where NOM concentrations are much greater than the predicted NP concentrations in terrestrial environments. Also, the presence of divalent cations such as Ca^{2+} at high concentrations can cause bridging between NOM molecules, leading decreasing the colloidal stability of the particles[89, 90].

1.2.6 Nanoparticle Transformation in Soil

The behavior of NPs is determined by an interplay between their intrinsic properties and the extrinsic properties of their surroundings. Soil pH is a major factor that affects the behavior of NPs in soils. In simple systems (e.g. in deionized (DI) water), the particles will be positively or negatively charged if the pH of the system is below and above the point of zero charge (PZC), respectively. Thus, in the soil environment, at a pH lower than PZC, the electrostatic attraction between the negatively charged soil components will be more dominant, whereas electrostatic repulsion would be rather dominant at a pH higher than the PZC. Several studies showed that increasing the soil pH decreased recovered Zn concentrations in pore water of soils spiked with bare ZnO NPs[91-93].

Increasing soil pH and organic matter (OM) concentration increased the bioavailability of bare ZnO NPs to *Folsomia candida* but not the Zn concentration in soil pore water[94]. Mobility, aggregation, and dissolution of NPs in soil solution, however, is far less straight forward, due to the complex nature of the soil system. Soil solution has a multitude of components, including clay mineral colloids, dissolved and particulate organic matter of different composition and concentration, Fe/Al oxohydroxides, organic and inorganic ligands, and cations of various concentration and valency[95]. Thus, to fully

understand the effects of soil properties on their partitioning and potential bioavailability for crop uptake, a comprehensive investigation should be performed considering all the interactions between these particles and their surroundings.

1.3 Research Objectives and Outline

The overarching objective of this research is to enhance Zn concentration in wheat grain using coated and uncoated ZnO NPs. Coating ZnO NPs imparted colloidal stability electrostatically by conferring a negative charge on the bare ZnO NPs through the formation of a shell made of poorly soluble Zn-phosphate (ZnO-core, $Zn_3(PO_4)_2$ shell, or core-shell NPs). Steric stabilization of ZnO NPs was achieved by using the nonionic polymer dextran (DEX-ZnO NPs), while the polyelectrolyte dextran sulfate stabilized ZnO NPs in an electrosteric manner by combining steric and electrostatic repulsive forces through negatively charged sulfate groups (DEX(SO_4)-ZnO NPs).

As a part of this project, we explored the application of ZnO nanomaterials with various inorganic and organic coatings both directly to soil and as a seed coating as a means of wheat fortification. We synthesized and characterized these NPs to give us insights about their expected behavior in simple and more complex systems (i.e. soil). Then we tested the performance of these NPs as seed coatings in a seed germination assay, followed by the investigation of the partitioning and dissolution of these NPs in the soil under different pH conditions. The outcome from both studies helped us select the ZnO NPs which would have better performance in moving forward with the greenhouse studies performed on wheat.

1.3.1 Research Hypotheses

1) At high pH (8), amendments with negatively charged ZnO NPs will result in higher total Zn concentrations in saturated paste extracts of soil than for neutral and positively charged ZnO NPs or $ZnSO_4$.
2) Dissolved Zn concentrations will be higher in saturated paste extracts of soil at lower pH (6) for all treatments (nano or ionic).
3) Increased total Zn in saturated paste extracts is more predictive of plant available Zn status than dissolved Zn for ZnO NP treatments.

Under conditions where Zn^{2+} would precipitate as hopeite or bind to negatively charged surface sites on minerals or DOM, the colloidal stability would be maximized for the negatively charged particles; core-shell structures and DEX(SO_4) ZnO NPS, while their attachment efficiency and heteroaggregation potential would be minimized. The result of these interactions would be that the total Zn concentrations in pore waters of soils amended with the core-shell structures would be maximized when pore water concentrations of Zn^{2+} would be minimized. Total Zn concentrations in the soil pore water for ZnO NPs includes Zn in both dissolved and particulate forms which would be more indicative of Zn bioavailability since plants have been shown to take up nanoparticulate metals[96-98]. Dissolution of Zn ions increases as soil pH decreases due to the decrease in sorption to soil and dissolution of Zn carbonates/hydroxides precipitates. When the soil pH is far below the PZC of ZnO NPs, it will induce dissolution of the NPs and subsequently result in higher dissolved Zn concentrations in soil pore water.

1.3.2 Dissertation Outline

Chapter 2 describes the synthesis of bare, core-shell, and polymer coated ZnO NPs. The evaluation of these particles performance as seed treatments in comparison to $ZnSO_4$ in a seed germination assay was also presented.

Chapter 3 presents the investigation of the effects of pH, ionic strength and organic matter content of on the aggregation and zeta potential (ζ) potential of the NPs in simple media. Chapter 3 also investigates the effect of pH on the attachment and dissolution of bare and coated ZnO NPs, in comparison to $ZnSO_4$ in soil.

Chapter 4 evaluates the capacity of ZnO NPs to enrich Zn levels in wheat grain, in comparison to $ZnSO_4$ in a greenhouse study. This chapter describes two major routes of exposing the seeds to the NPs, either through seed treatment, or via soil amendment in soils that were manipulated to have either alkaline or acidic pH.

Chapter 5 summaries the overall conclusions of this research, sheds light on the challenges encountered, and suggests future work that could be performed to further the development of ZnO nanofertilizers.

Chapter 2: Functionalized ZnO Nanoparticle Seed Treatments to Enhance Growth and Zn Content of Wheat (*Triticum aestivum*) Seedlings.

Zeinah Elhaj Baddar and Jason M. Unrine

2.1 Abstract

Seed coating with micronutrients, such as Zn, is considered an alternative to soil amendment with micronutrients, especially when the latter is of limited success. In this study, we synthesized and modified the surface of ZnO nanoparticles (NPs) to investigate their potential for enhancing the Zn nutrition in wheat (*Triticum aestivum*). We tested bare ZnO (bare ZnO), dextran coated (DEX), dextran sulfate (DEX(SO_4) coated ZnO nanoparticles, and $Zn_3(PO_4)_2$ shell-ZnO core NPs. Treating seeds with $ZnSO_4$ solution and deionized water served as an ionic and solvent control, respectively. Upon the termination of the assay, Zn concentrations in roots and shoots, biomass and lengths of roots and shoots, and seed percent germination were measured. We used non-linear regression to examine the relationship between Zn concentration in the exposure suspension/solution and seedling response. All ZnO nanoparticles were more effective than $ZnSO_4$ in increasing tissue Zn concentrations and seedling growth. Exposure to higher concentrations of $ZnSO_4$ decreased the growth and germination relative to controls and ZnO NPs. In contrast to the other treatments, bare and dextran coated ZnO NPs increased Zn concentrations in wheat without decreasing growth. While $ZnSO_4$ significantly inhibited seed germination, none of the ZnO NPs used in this study had a significant effect on seed germination.

Keywords: seed coating, Zn fortification, surface modified ZnO nanoparticles, wheat, seedlings growth, germination.

2.2 Introduction

Zinc deficiency has detrimental effects on plant health. Leaf necrosis and reduced leaf size [16], stunted overall plant growth, compromised yields, and decreased seed vigor[99], are examples of Zn deficiency effects on plants Zinc deficiency is also a widespread human health concern. It is often associated with growth impairment, neurological, cognitive, and immunological disorders [9, 100, 101]. Zinc deficiency may be related to low geogenic Zn concentrations in soil, or to poor Zn bioavailability [41]. Poor Zn bioavailability can occur when soil chemistry promotes the formation of insoluble Zn minerals, such as $Zn_3(PO_4).4H_2O$ (hopeite) and $ZnCO_3$, or strong binding to clay, iron oxyhydroxides or organic matter [102]. Alkaline soils, particularly those rich in phosphate, are prone to poor Zn bioavailability. In this case, addition of Zn salts, such as $ZnSO_4$, may have limited efficacy since the added Zn may have limited bioavailability.

Several previous studies have focused on ZnO nanoparticle (NP) toxicity, with different plant species, exposure media, and test conditions [103-118]. However, few studies have addressed potential use of nanoparticulate ZnO to enhance growth and Zn levels in plants. Germination, number of pods, and Zn levels of peanut (*Arachis hypogaea*) seeds treated with ZnO NPs were significantly enhanced compared to seeds treated with $ZnSO_4$ and chelated Zn forms [55]. Growth stimulation accompanied by increased Zn concentrations, in soybean (*Glycine max*) and sweet potato (*Ipomoea batatas*), have also been reported [103, 119]. Coating ZnO NPs onto the surface of macronutrient granules, such as urea or mono ammonium phosphate (MAP), achieved a slight advantage over bulk

ZnO in term of Zn bioavailability in calcareous alkaline soils [120]. Other studies found that bare ZnO NPs enhanced plants resistance to drought and diseases [121, 122,123].

Seed germination assay studies are widely used to test toxic effects of heavy metals and NPs on plants since they are fast and simple[124]. Some of these studies have showed that low concentrations of NPs had stimulatory effects on plant growth, even if higher concentrations were toxic [106-110]. Few such studies have primarily focused on the beneficial effects of ZnO NPs on plant health and growth[52, 106, 121-123, 125].

Although coating seeds with Zn salts has been a successful tool in enhancing Zn concentrations and yield in several crops such as wheat (*Triticum aestivum*)[126], barley (*Hordeum vulgare L.*)[51], and rice (*Oryza sativa L.)*[127], few studies investigated the use of ZnO NPs as a seed treatment to enhance Zn crop nutrition[52, 125].

Surface modification of NPs is commonly devised to impart colloidal stability to prevent their aggregation, helps control the size and shape of the NPs[128], and enhances their compatibility to the desired applications [128,129]. Several studies have investigated the effects of coating charge on NP bioavailability, uptake, translocation, and toxicity in plants [130, 131]. For example, two hydroponic studies performed on five different plants; rice, ryegrass (*Lolium perenne*), radish (*Raphanus Sativus*), pumpkin (*Cucurbita mixta*), and wheat reported that positively charged Au and CeO_2 adhered to the roots more strongly than neutral or negatively charged ones. Conversely, higher root to shoot translocation was reported for the neutral and negatively charged CeO_2 NPs and Au NPs [130, 131]. Also, our previous work indicated that negatively charged ZnO NPs (ZnO core-$Zn_3(PO_4)_2$ shell or core-shell NPs hereafter) may have enhanced Zn bioavailability to wheat seeds [132].

In this chapter, we present the results of a study where we explored the use of ZnO NPs with various surface coatings as seed treatments to enhance Zn nutrition in wheat. We investigated the effect of the coating charge and the mechanism of stabilizing the particles on their distribution in seedling tissue. The ZnO NPs used as the seed treatments had various surface chemistries including, negatively charged $Zn_3(PO_4)_2$ shell NPs which were electrostatically stabilized, neutral and negatively charged polymer coatings (dextran (DEX) and dextran sulfate (DEX(SO_4)), which were sterically and electrosterically stabilized, respectively), and positively charged bare ZnO NPs. These forms were compared to their ionic Zn counterparts dissociating from $ZnSO_4$.

A seed germination bioassay and analysis of tissues for Zn content were done. To the best of our knowledge, this is the first study that investigates the potential use of ZnO NPs with different modes of stabilization and different surface chemistries as seed treatments to enhance Zn nutritional value of wheat.

2.3 Materials and Methods

2.3.1 Synthesis and Surface Modification of ZnO Nanoparticles

We synthesized bare ZnO NPs using alkaline precipitation, following the procedure reported by Becheri et al [133]. A 0.2 M solution of $ZnCl_2$ was stirred in a 90 °C water bath. After ten minutes of stirring, a 5.0 M solution of NaOH was added in a drop-wise manner to the $ZnCl_2$ solution, causing a milky white precipitate to form. After cooling to room temperature, the white precipitate was centrifuged at 3220 X g until the supernatant was clear. The supernatant was decanted, and the pellet was re-suspended in 18 MΩ deionized (DI) water. This process was repeated four times.

To synthesize dextran (DEX) and dextran sulfate ($DEX(SO_4)$) coated ZnO NPs, the same synthesis protocol was followed with either 9-11 kDa dextran in a 1:6 dextran to Zn mass ratio, or 15 kDa dextran sulfate in a 1:4 dextran sulfate to Zn mass ratio, added to the reaction mixture prior to the addition of NaOH. Purification of the DEX and $DEX(SO_4)$ coated ZnO NPs was performed by dialysis using a 12-14 kDa molecular weight cutoff (MWCO) membrane to retain the smallest NPs (Spectra/Por ® 6 dialysis membrane). The dialysis water was changed once a day until the pH equilibrated to between 7 and 8, indicating that excess NaOH and NaCl had been removed. To synthesize core-shell NPs, we treated bare ZnO NPs with 1.58 mM HNa_2PO_4 at pH 8.0±0.2 to coat particles with a $Zn_3(PO_4)_2$ shell NPs following the protocol of Rathnayake et al [86]. We modified the protocol by increasing the concentrations of the reactants, while keeping the ratio of P to Zn the same and purifying by dialysis instead of centrifugation. After dialysis, the pH of the suspension was adjusted to 8.50±0.50 with 0.1M NaOH to increase the colloidal stability. All reagents were provided by Sigma Aldrich, St. Louis, MO, USA.

Three replicate batches of bare ZnO NPs, DEX and $DEX(SO_4)$ coated ZnO NPs, and core-shell NPs were synthesized to evaluate the reproducibility of the synthesis protocol. The yield of the bare ZnO NP synthesis was also evaluated by centrifuging the NP suspension and subsequent weighing, dissolution, and analysis of the dried pellet for Zn content by inductively coupled plasma mass spectrometry ICP-MS (Agilent 7500cx Santa Clara, CA, USA).

2.3.2 Nanoparticle Characterization

Dynamic light scattering (DLS) was used to measure the hydrodynamic diameter of the particles in deionized (DI) water. The results are reported as the intensity weighted mean (Z-average) hydrodynamic diameter. The electrophoretic mobility of the NPs was determined using phase analysis light scattering (PALS). Malvern Zetasizer Nano ZS (Malvern, United Kingdom) was used to perform DLS and PALS measurements. The zeta potential (ζ) was estimated from the electrophoretic mobility using the Smoluchowski's approximation. To measure the point of zero charge (PZC, the pH at which positive and negative surface charges are balanced, resulting in an electrophoretic mobility of zero)[65], we added increments of either 0.01M NaOH or HCl and measured the pH and zeta potentials corresponding to each of these increments. The values of PZC were estimated graphically by plotting pH values resulting from acid/base addition against the corresponding ζpotential. The Zn concentration used for the DLS and PALS measurements was 100 mg Zn/L in DI water. The pH of the DI water suspensions used for both measurements was between 7.15 and 7.8. The pH of the suspensions is higher than the normal pH of DI water (5.8) due to the buffering action of the ZnO NPs. Transmission electron microscopy (TEM) (Jeol 2010F, Tokyo, Japan) was used to measure the primary particle size of the NPs. Around 100 individual particles were measured from several different images to determine the average primary particle size using ImageJ software (http://rsb.info.nih.gov/ij/download.html). A powder X-ray diffraction (XRD) fractogram for the bare ZnO NPs was obtained using AXS D8 Discover diffractometer (Bruker, Madison, Wisconsin, USA) and compared to an authentic standard to verify that the product was ZnO.

We performed thermogravimetric analysis (TGA) (Discovery high resolution thermogravimetric analyzer, TA Instruments, New Castle, Delaware, USA) to measure the amount of coating on dextran and dextran sulfate coated NPs (Figure S 2.1). At a heating rate of 50°C per minute, around 8 mg lyophilized NPs were heated from 40 to 700°C. Mass losses were plotted against temperature. Coating amount was calculated by subtracting total mass losses in coated NPs from the total mass loss of bare ZnO NPs.

2.3.3 Seed Germination Assay

Winter wheat seeds (*Triticum aestivum*, cv. Pembroke, University of Kentucky, Lexington, KY) were surface sterilized with 8.25% NaOCl solution, washed three times and then soaked for 1 hour in DI water. Around 15 seeds were incubated in 3mL of treatment solution (bare ZnO, DEX, DEX(SO_4) and core-shell NPs, and $ZnSO_4$ and a DI water control) in a 15 mL centrifuge tube. Each treatment was replicated five times. The nominal concentrations used for each treatment were 100, 500, and 1000 mg Zn/L. ZnO NP suspensions were sonicated for 45 min at 100% amplitude using a cup horn sonicator (Qsonica, Newtown, Connecticut, USA).

For coating controls, we used TGA, *vide supra,* to quantify the amount of dextran and dextran sulfate in 1000 mg Zn/L suspensions of DEX-ZnO and DEX(SO_4)-ZnO NPs. For core-shell NPs coating control treatments, a 1000 mg Zn/L suspension of core-shell NPs was centrifuged for 30 min at 3220 g over a 3KDa Amicon filter (Millipore, Burlington, Massachusetts, USA). We used ion chromatography (Dionex ICS-3000, Sunnyvale, California, USA) to measure the amount of free phosphates (unattached to core-shell NPs) in the supernatant. The results of these analyses, using three replicates of Na_2HPO_4,

dextran, and dextran sulfate solutions, were 28.3, 42.5, and 32.4 mg/L, respectively, and are referred to hereafter as “coating controls”.

Seeds were treated with the solutions/suspensions for 24h on a shaker at room temperature. Seeds were removed the next day and were uniformly placed on DI-moistened filter paper in sterile 9 cm petri dishes. Seeds were not washed after incubation to maintain a coating of the test materials on the seed surface. Throughout this experiment, a petri dish is considered as a biological replicate. Five or three biological replicates per treatment, with 10 seeds each, were performed for Zn treatments and coating controls, respectively. The petri dishes were sealed with parafilm and incubated in the dark at 25°C. The germination assay was terminated once > 65% of the seeds in the DI water control treatments developed a radicle root of at least 20 mm length. After test termination, we measured percent germination, length of the radicle roots and shoots, and root and shoot dry biomass after lyophilization. Lyophilized roots and shoots were pooled from each plate and were digested together in metal-free 15 mL teflon vials using concentrated HNO_3. After cooling, digestates were diluted with DI water and Zn concentrations of the roots and shoots were measured using ICP-MS. Digestion blanks, duplicates, post digestion spiked samples, and standard reference material (SRM1515; apple leaves, National Institute of standards and Technology, Gaithersburg, MD, USA) were also included to validate the analyses. Relative percent difference between duplicates and spike and SRM1515 recoveries were 11.7± 5.5%, 96.0±7.2%, and 101.7±7.3%, respectively (mean ± one standard deviation).

2.3.4 Dissolution Experiments

Dissolution experiments were performed in DI water at 500 mg Zn/L for DEX-ZnO, DEX(SO_4)-ZnO, bare ZnO, and core-shell NPs. Nanoparticles were dispersed in 5mL DI water in 15 mL centrifuge tubes. Suspensions were sonicated for 45 min at 100% power. Each treatment was performed in triplicates. Two mL aliquots were transferred from each tube to 2.0 mL microcentrifuge tubes to measure dissolution. These tubes were left to equilibrate for 24 h, then centrifuged at 16,837 X g for 3 hours. One mL supernatant was transferred carefully from each tube to another 2.0 mL centrifuge tube. The supernatants were acidified to 0.158 M HNO_3 and analyzed for dissolved Zn using ICP-MS.

2.3.5 Statistical Analysis

Due to the variation in the actual Zn concentrations in exposure suspensions, we performed quadratic regression analysis to explain the relationship between actual Zn concentration in exposure solutions/suspensions and Zn accumulation and plant response. The relationship between Zn concentration in the exposure solution and germination was analyzed using linear regression. Although germination data followed a Poisson distribution, the variances were homogenous. Thus, we evaluated differences in germination between the control and $ZnSO_4$ treatments at each nominal Zn concentration using the pairwise t-test at α 0.05. We also used ANOVA followed by the Tukey HSD test to test significant differences among ZnO NPs in the dissolution experiment. ANOVA, followed by Dunnett's test, was used to test for significant differences between DI and coating control treatments on germination and seedlings growth. Statistical analysis was performed using JMP ®10.0.0. Sigmaplot 12.5 was used to generate the graphs.

2.4 Results

2.4.1 Particle Characterization

The primary particle size measured by TEM ranged from 20 to 30 nm and varied a little among treatments and among batches for the same treatment (Fig. 2.1). Primary particle sizes of three separate replicates from each NP are reported as mean ± one standard deviation (Table 2.1). Also, we averaged the primary particle sizes of the three replicates and reported the results as grand mean ± one standard deviation (Table 2.1). Primary particle sizes for bare, core shell, DEX-ZnO, and DEX(SO_4)-ZnO NPs were respectively: 24 ± 1, 27± 0.30, 18.0± 1.0, and 20±1.0, reported as grand means ± one standard deviation (Table 2.1).

The Z-average diameters (intensity-weighted) ranged from 304-755 nm, indicating some degree of aggregation in the suspensions (Table 2.2). The intensity weighted Z-average diameter is also heavily weighted towards larger particles [134]. Although the Z-average diameter is not a good representation of the physical distribution of particle sizes (by mass, volume, or number), it allows for a good comparison between treatments as it does not rely on the assumptions needed to convert the data to mass, volume, or number weighted distributions. All NPs, except the dextran coated ones, were aggregated to a similar degree, while DEX-ZnO NPs were significantly more aggregated. Since multiple peaks showed up when analyzing the hydrodynamic size, volume weighted averages are reported as well (Table 2.2).

The ζpotentials in DI water (Table 2.2), were positive for bare ZnO and DEX (+29.1 mV and +19.5 mV, respectively). Whereas, core-shell and DEX(SO_4)-ZnO NPs were negatively charged (-23.9 mV and -24.8 mV, respectively). The presence of a polymer on

the particle surface complicates the estimation of ζ potential from electrophoretic mobility data due to the uncertainty about the size of the electrical double layer[135,136]. However, the sign of the charge is certain, allowing for an accurate estimation of the PZC. Bare ZnO NPs had a PZC of 9.8, while DEX-ZnO NPs PZC was almost one pH unit lower (8.7). Core-shell and DEX(SO_4)-ZnO NPs had PZC values lower than 6.2 (Fig. S 2.2). Addition of a $Zn_3(PO_4)_2$ shell as well as coating ZnO NPs with dextran sulfate resulted in a significant decrease and a sign inversion in the zeta potential values in DI water (-20.7 and -24.8 mV, respectively) (Table 2.2) and (Fig S 2.2). This is because it decreased the PZC from 9.80 for bare ZnO NPs to less than 6.2 for DEX(SO_4) and core-shell particles. Our previous research indicated that treatment of ZnO with the HNa_2PO_4 solution at pH 8 results in an amorphous shell of $Zn_3(PO_4)_2$ on the surface of the particles[132]. The PZC value of $Zn_3(PO_4)_2.4H_2O$ is around 4.8[137]. We found that the PZC value of the core-shell particles was less than 6, accounting for the charge reversal observed in DI water as compared to bare ZnO NPs (Fig. S 2.2). A similar shift to a lower PZC value for the DEX(SO_4) particles was expected given that the pKa of dextran sulfate is approximately 2 [138] (Fig S 2.2).

There was a good agreement in XRD diffractograms between the bare ZnO NPs and the authentic zincite-structured ZnO standard (Fig S 2.3).

2.4.2 Zinc Concentration in Roots and Shoots

We found that the quadratic regression best fits the relationship between Zn concentrations in exposure solutions and root and shoot Zn concentration (Fig. 2.2, Table 2.3). We defined the concentration of Zn in the exposure solution corresponding to the maximum tissue Zn concentration, biomass, or root/shoot elongation on a regression line

as Zn optimum- (Zn_{opt}) for each parameter. Regression lines associated with Zn concentration achieved in roots and shoots of treated seeds in all treatments were statistically significant ($p<0.001$) at $\alpha=0.05$. Coating with DEX-ZnO NP resulted in the highest Zn concentration in the roots with 2143 µg/g at Zn_{opt} >1196 mg Zn/L, followed by the DEX(SO_4)-ZnO NPs treatment which delivered 1910 µg Zn/g roots at Zn_{opt} >936 mg Zn/L, while $ZnSO_4$ increased root Zn concentrations to 1871 µg/g at a Zn_{opt} of 725 mg/L. Bare ZnO and core-shell NPs had almost the same root Zn concentration with 1464 and 1454 µg Zn/g at Zn_{opt} values of 605 and 1017 mg Zn/L, respectively (Fig 2.2, Table 2.3).

Compared to root Zn concentrations, shoot Zn concentrations were almost an order of magnitude lower, with maximum tissue concentrations at much lower Zn_{opt} values (Fig 2.2, Table 2.3). Seeds treated with bare ZnO NPs achieved the highest mean shoot tissue Zn concentration (433 µg/g) at a lower Zn_{opt} value of 518 mg Zn/L. At a Zn_{opt} value of 894 mg/L, DEX-ZnO treatment resulted in 346 µg Zn/g of shoot tissue. Similarly, the DEX(SO_4)-ZnO NPs, $ZnSO_4$ and core-shell NPs resulted in 309, 302 and 266 µg Zn/g shoot dry mass at Zn_{opt} values of 674, 597 and 644 mg/L, respectively.

In general, all Zn treatments significantly enhanced tissue Zn concentrations in roots and shoots compared to controls. Dextran coated, DEX(SO_4)-ZnO NPs, and $ZnSO_4$ had maximum Zn concentrations in the roots that were 42, 38, and 37% higher than the controls, respectively. Bare and core-shell NPs were 29 times higher than the control in terms of root Zn concentration. Bare ZnO NP-treated seeds had the highest Zn concentration in their shoots, with 13 times greater than in the controls. Dextran coated and DEX(SO_4)-ZnO NPs respectively enhanced shoot Zn concentration by 10.5 and 9.4

times that of the control. Zn concentration in the shoots of $ZnSO_4$ and core-shell NPs were 9.1 and 8 times higher than the control.

Average shoot to root percentage of Zn concentration in the bare ZnO NP treatment was the highest at 30%, followed by the core-shell NP treatment with 18%. The rest of the treatments had ratios of about 16% (Fig. 2.2, Table 2.3).

With a nearly 67% increase in root biomass, the core-shell NP treatment achieved a significantly ($p<0.001$) higher maximum root biomass than the other treatments, at a Zn_{opt} of 500 mg Zn/L. Treating seeds with DEX-ZnO NPs significantly ($p<0.001$) increased root biomass (55% higher than control) compared to the other treatments at a Zn_{opt} of 428 mg/L. The maximum increase in root biomass achieved using $ZnSO_4$ did not exceed 24% at a Zn_{opt} of 238 mg Zn/L ($p<0.001$). Quadratic fits for the remaining treatments (bare and DEX(SO_4)-ZnO NPs) were not statistically significant at $\alpha=0.05$, indicating no stimulatory or inhibitory effect of Zn on biomass at any concentration (Fig. 2.3, Table 2.3).

At a Zn_{opt} of 314 mg/L, DEX-ZnO NPs significantly enhanced root elongation by 24% as compared to controls ($p=0.044$), while treating seeds with $ZnSO_4$ elongated the main root by 17% compared to the controls at a Zn_{opt} of 138 mg Zn/L ($p<0.001$). None of the corresponding quadratic fits associated with the rest of treatments were statistically significant at $\alpha=0.05$ (Fig. 2.3, Table 2.3).

Shoot biomass was 21% higher than control ($p=0.044$) in seeds treated with DEX-ZnO NPs at Zn_{opt} of 415 mg/L. While at 124 mg Zn/L, only a 2% increase in shoot biomass was achieved by $ZnSO_4$ treatment ($p= 0.003$). We found no significant effects of the rest of Zn treatments on shoot biomass (Fig. 2.4, Table 2.3).

Treating seeds with DEX-ZnO NPs significantly (*p=0.030*) increased shoot length compared to the rest of Zn treatments with a 27% increase in shoot length relative to the control at Zn_{opt} of 406 mg/L. Bare ZnO NP treatment had significantly (*p=0.033*) increased shoot length, with a 24% increase relative to the control at a lower Zn_{opt} of 242 mg/L. The core-shell NP treatment (*p=0.019*) was slightly lower than ZnO NPs with 23% increase in shoot elongation Zn_{opt} of 587 mg/L. Zinc sulfate treatments (*p=0.008*) had the smallest increase in shoot length by only 16% relative to controls at a Zn_{opt} of 297 mg/L. Treating seeds with DEX(SO_4)-ZnO NPs had no effect on shoots elongation (Fig 2.4, Table 2.3).

2.4.3 Germination

Percent germination was not related to Zn concentration for any treatment except for $ZnSO_4$, for which germination was negatively related to Zn exposure concentration at α = 0.05 (Fig. 2.5). For $ZnSO_4$, germination success was less than 50% at the highest exposure (nominal) concentration of 1000 mg Zn/L. Pairwise t-test between control and $ZnSO_4$ treated seeds showed that the latter significantly inhibited germination at a nominal Zn concentration of 500 mg Zn/L (Fig. S 2.4).

2.4.4 Effect of Coating Material on Growth and Germination

There was no significant effect of the coating material on either germination or root elongation (α=0.05; table S 2.1). Dextran sulfate, DEX, and $Zn_3(PO_4)_2$ coatings significantly increased (p<0.05) root and shoot biomasses by 59% and 55%, 23% and 22%, and 26% and 18%, respectively, compared to the DI control. Only DEX(SO_4) and DEX coatings caused significant increases (*p<0.05*) in shoot length by 62% and 11%, respectively, compared to the DI control.

Another way to examine the effect of Zn treatment on the seedling growth is by plotting Zn concentration in seedling tissue versus measured biomass. In seedling roots, the quadratic regression analysis for all Zn treatments (Fig. S 2.5 (A), Table S 2.2 (A)). Except for core-shell NPs, the rest of nanoparticulate ZnO treatments had almost an identical accumulation of biomass at root Zn concentrations lower than around 750 µg Zn/g biomass. Core-shell NPs tended to have a different behavior as the biomass accumulation was significantly higher than the rest of ZnO NP treatments which could explain the lower $Zn_{root\ max}$. Zinc sulfate treatment barely enhanced the root biomass relative to the control which could explain the relatively high $Zn_{root\ max}$ for this treatment. All ZnO NP treatments accumulated higher relative root biomass when compared to $ZnSO_4$ over the entire range of Zn root concentration (Fig. S 2.5(A), Table S 2.2). For relative shoot biomass, the quadratic regression analysis was not statistically significant for all but $ZnSO_4$ treatment, perhaps due to the spread of the data points. Therefore, no inferences could be drawn from such relationships (Fig. S 2.5(B), Table S 2.2).

2.4.5 Dissolution of ZnO Nanoparticles

Dissolution data showed that, at a nominal Zn concentration of 500 mg/L, dissolved Zn concentrations for DEX(SO_4), DEX, bare ZnO, and core-shell NPs were 22.6, 19.0, 4.8, and 4.0 mg Zn/L, respectively (Fig. 2.6).

2.4.6 Critical Zn Levels

Since Zn_{opt} values that we previously defined for each treatment vary depending on the regression variable (e.g. biomass, elongation) and the shape of the quadratic regression, optimizing the Zn concentration in the coating solution to achieve maximum Zn levels and plant growth simultaneously must be done carefully. Thus, it is important

to define a Zn concentration in the exposure solution beyond which adverse effects occur for any of the endpoints[139]. We hereafter define this concentration as the critical Zn exposure concentration for toxicity or Zn_{crt}. The graphical relationship between Zn_{crt} and Zn_{opt} is also shown (Fig. 2.7).

Since toxic effects occur at higher concentrations than deficiency effects, Zn_{crt} will always be higher than Zn_{opt} for a given endpoint. Finding the points of a quadratic equation (exposure Zn concentration) when the response equals 1 gives two solutions; 0 (Zn concentration for control treatments) and a positive number which represents Zn_{crt} (Fig. 2.7). We determined the values of Zn_{crt} for each treatment and at each physiological endpoint (Table 2.4). We also determined corresponding Zn_{opt} values for comparison (Table 2.4). Data derived from statistically significant regressions for each treatment showed that Zn_{crt} was at least twice as great as Zn_{opt} for all the Zn treatments and at each physiological endpoint, except for germination (Table 2.4). Germination did not follow this rule, because there was no response for any treatment except $ZnSO_4$. This treatment had a linear decrease in germination success with increasing exposure concentrations. In cases where the Zn_{opt} or the Zn_{crt} values exceeded the maximum concentration that was tested, we indicate that it is greater than the maximum tested concentration.

For the core-shell NP treatment, regardless of the physiological endpoint, Zn_{crt} values were all similar and slightly exceeded 1000 mg/L, which represents the maximum nominal Zn concentration used in this study. ZnO NPs coated with dextran followed the same pattern, though Zn_{crt} values were equal to or slightly lower than the maximum tested Zn concentration (1000 mg Zn/L). Given that none of the regression lines associated with DEX(SO_4)-ZnO NPs treatments were statistically significant, no

stimulatory or inhibitory effects on seedlings growth could be deduced. Thus, there are no Zn_{crt} and Zn_{opt} values. Bare ZnO NP treatment followed a similar pattern, except for shoot elongation, which had a Zn_{crt} of 750 mg Zn/L.

Values of Zn_{crt} for $ZnSO_4$ treatments were much lower than these for the rest of the ZnO NPs. Root growth (biomass and elongation) and shoot elongation all had Zn_{crt} values that were almost half the values of Zn_{crt} for core-shell and DEX-ZnO NPs treatments. Shoot biomass was the most sensitive endpoint for the $ZnSO_4$ treatment, where Zn_{crt} = 280 mg Zn/L. This was 72% lower than Zn_{crt} for DEX-ZnO NPs (Table 2.4).

2.5 Discussion

In this study, we demonstrated that treating seeds with ZnO NPs, at nontoxic concentrations, could improve plant growth and Zn concentrations in wheat seedlings. All Zn treatments, regardless of Zn form, significantly enhanced tissue Zn concentrations in roots and shoots, compared to the control. We also found that the nanoparticulate treatments were more effective than $ZnSO_4$ in enhancing root and shoot Zn concentrations and growth. Germination was not affected by any of the Zn nanoparticle treatments, whereas $ZnSO_4$ significantly inhibited germination even at the lowest tested concentration of 100 mg Zn/L. Furthermore, surface chemistry of the ZnO NPs in this study led to different patterns in tissue targeting and growth stimulation. To our knowledge, no previous studies have investigated the use of different surface coatings on ZnO nanomaterials to alter their surface chemistry and performance characteristics so as to enhance Zn tissue concentrations in plants.

While ZnO NP seed treatments have not been well studied from a nutritional standpoint, a few studies have examined ZnO toxicity and Zn accumulation. De la Rosa et al reported that alfalfa, tomato (*Solanum lycopersicum*), and cucumber (*Cucumis sativus)* accumulated 4700, 2100, and 1600 µg Zn per g of seedling, respectively, when seeds were treated with ZnO nanoparticle concentrations of 1285 mg Zn/L. On the other hand, seedlings of these plants accumulated less Zn (3500, 1100, and 800 µg Zn/g in alfalfa, tomato, and cucumber, respectively) in their tissue when seeds were treated with $ZnSO_4$ at 250 mg Zn/L[110]. Likewise, Chinese cabbage seeds that were treated with 30 nm bare ZnO NPs at 64 mg Zn /L had about 23 times higher Zn concentration in the seedlings compared to $ZnSO_4$ at 2 mg Zn /L[140]. In the present study, we found that Zn_{max} in the roots and shoots of wheat seedlings in bare ZnO NP treated seeds surpassed Zn_{max} values for $ZnSO_4$ at Zn_{opt}, indicating that ZnO NPs are more efficient at delivering Zn to plant tissues than $ZnSO_4$.

Enhanced delivery of Zn to the seeds by ZnO NPs relative to $ZnSO_4$, appears to operate through a particle-specific mechanism. We found that at equal Zn concentrations, some NPs deliver more Zn to plants than $ZnSO_4$, despite much lower dissolved Zn concentrations in the NP treatments. For example, the data showed that in 500 mg Zn/L ZnO exposure solutions, which is close to Zn_{opt} values for shoot concentrations, dissolved Zn concentrations were low (4-22 mg Zn/L). The $ZnSO_4$ treatment had a similar Zn_{opt} for shoot concentrations (597 mg/L). However, the mean shoot tissue concentrations were higher for the bare ZnO NP than for the $ZnSO_4$ treatment (433 µg/g vs 302 µg/g) at the Zn_{opt} values. Previous studies have reported similar findings where accumulation of Zn from NP treatments was higher than would be predicted based on the

dissolved Zn concentration[110, 140]. As further evidence of different mechanisms of uptake for particles versus dissolved ions, data from Zhang et al suggest that the seed coat of maize (*Zea mays)* is a greater barrier to absorption of Zn from ZnO than $ZnSO_4$. Even in this case, however, bare ZnO NPs caused a significantly higher amount of Zn in the roots compared to $ZnSO_4$, even with an intact seed coat[108]. Taken together, these findings suggest a nano-specific Zn delivery mechanism that requires further investigation.

Similarly, ZnO NPs were more effective at increasing biomass, suggesting that absorbed Zn from ZnO NPs can supply biologically available Zn. The present data showed that shoot and root biomass were increased by 21% to 67%, relative to the control, at Zn_{opt} values that ranged from 415 to 500 mg Zn/L. Several previous studies focusing on the toxicity of ZnO NPs reported growth stimulation. Root and shoot biomass of mung bean (*Vigna radiata*) from seeds treated with bare ZnO NPs at 16 mg Zn /L were increased by 41% and 76% , respectively, relative to the control [109]. Bare ZnO NP treated gram (*Cicer arietinum*) seeds developed root and shoot biomass that was, respectively, 37% and 27% higher than the controls at an exposure concentration of 20 mg Zn/L as well [109]. Exposure to bare ZnO NPs at 642 mg Zn/L enhanced tomato (*Lycopersicon esculentum*) seedling biomass by 36% compared to the control[110].

Root and shoot elongation have been shown to be increased by exposure to ZnO NPs in several studies. Our study showed that root and shoot elongation was significantly enhanced when seeds were treated with ZnO NPs at non-toxic Zn concentrations. In one previous study that did focus on using seed treatments to enhance crop Zn nutrition, Subbaiah et al found that bare ZnO NPs enhanced the growth of maize. The study showed that ZnO NPs at 1204 mg Zn /L significantly enhanced root and shoot elongation

by 40% and 35%, respectively, when compared to the control, and by 60% and 55%, respectively, when compared to $ZnSO_4$ at a concentration of 1606 mg Zn/L[52]. In another study, at 8.03 mg Zn/L, radicle length of maize was significantly increased by 20% compared to the control [108]. Cucumber seeds treated with bare ZnO NPs at Zn concentrations of around 160 and 320 mg Zn/L developed radicles that were 1.9 and 2.7 times as long as these of non-treated seeds, respectively[110]. In sweet sorghum (*Sorghum bicolor*), germination success, and root and shoot elongation, were all improved relative to the untreated seeds at 100 mg Zn/L added in the form of bare ZnO NPs [125]. In soybean seeds treated with bare ZnO NPs at 405 mg Zn/L, root elongation was 30% greater than that of the control [107]. In comparison, we found that most of our reported Zn_{opt} values for wheat seedling root and shoot elongation were between 200 and 600 mg Zn/L with corresponding increases in root and shoot elongation of around 23-40% relative to the control.

In general, all tested coating materials significantly enhanced seedling growth (biomass and elongation) which could indicate that there was an interaction effect of ZnO NPs and the coating material on seedling growth which requires further investigation.

Regardless of Zn concentration, ZnO NPs did not affect wheat germination success. Most previous germination studies have reported similar results on soybean[107], cucumber[108, 141], radish and rape[118], lettuce[114, 141], maize and rice[116], and zucchini (*Cucurbita pepo*)[56]. A few studies have shown adverse effects of ZnO NPs on germination. For example, Jain et al found that at a concentration 803 mg Zn /L, bare ZnO NP inhibited the germination of wheat, tomatoes, and pearl millet (*Pennisetum glaucum L.*)[106]. Subbaiah et al and Zhang et al reported a significant increase in maize

germination compared to the control at 1204 and 8.03 mg Zn/L, respectively [52, 108]. These contradictory findings could be attributed to test conditions or to the specific cultivars used.

In general, previous research has shown that ZnO NP seed treatments induced less toxicity than $ZnSO_4$ seed treatments [52, 107, 108, 125, 140, 142]. We also found that $ZnSO_4$ inhibited germination and caused adverse effects on growth at Zn concentrations much lower than applied as ZnO NPs. The ZnO NP treated plants tended to have both higher Zn concentrations and higher biomass at a given exposure concentration. In our study, germination was significantly inhibited at a nominal Zn concentration of 500 mg Zn /L applied as $ZnSO_4$, as compared to the control. None of the ZnO NP treatments had an effect on germination. The maximum nominal Zn concentration in ZnO NPs treatment used in this study was 1000 mg Zn/L which was probably not high enough to induce inhibitory effects on seed germination with ZnO NPs. In sweet sorghum, $ZnSO_4$ treatments inhibited seed germination and root and shoot elongation at 25 mg Zn/L[125]. In maize, at 1000 mg Zn/L in bare ZnO NPs suspension, germination was not affected even with the seed coat removed. Lin and Xing found a significant negative effect of bare ZnO NPs on seed germination[118]; but this particular study involved a 2h exposure period followed by keeping the seeds on a filter paper soaked with 5 mL of the exposure suspension at 1606 mg Zn/L over the course of the germination study, which probably resulted in much higher Zn uptake.

This is the first study showing that changing the surface chemistry of the particles by applying different coatings changed their performance in terms of plant growth and tissue Zn targeting. A few previous studies have focused on the effect of charge on the

partitioning of NPs in plant tissue [130, 131], but this was for whole plants in hydroponic culture, not for seed treatments. These studies were also focused on toxicity of non-nutrient materials. Surface charge and the type of ligand on the coatings affected the translocation of gold nanoparticles in tomato and rice [143]. Li et al found that although cysteine and thioglycolic acid ligands were negatively charged and had similar size, cysteine coated Ag NPs were more efficiently translocated to rice and tomato shoots compared to thioglycolic coated Ag NPs, which were not different from the positively charged cysteamine coated particles [143]. Positive charge on CeO_2 nanoparticles[130] and Au nanoparticles [131, 143] preferentially increased the Au and Ce root tissue concentrations compared to neutral and negatively charged coatings in wheat, ryegrass, tomatoes and rice. Conversely, neutral and negatively charged CeO_2 and Au NPs were more effectively translocated to the shoots and leaves [130, 131]. In this study, DEX(SO_4) caused much higher root Zn in as compared to core-shell NPs, although both nanoparticles had a negative charge. This could be attributed to the combined effect of the higher dissolution and the stabilizing mechanism used. DEX(SO_4) NPs are polymer coated, which may have enhanced their adherence to the roots/seed coat, compared to the core-shell NPs, through hydrophobic interaction [144]. Similarly, DEX-ZnO NPs exhibited a higher dissolution and were likely adsorbed more efficiently on to the roots/seed coat through hydrophobic interaction especially when compared to core-shell NPs. Bare ZnO NPs accumulated the highest Zn concentration in the shoots, compared to the rest of ZnO NPs, even though they had a positive zeta potential. Previous studies have shown that positively charged particles preferentially accumulate in roots [130, 131, 143]. This observation requires further study to determine the mechanisms involved.

The decision over which Zn concentration and particle coating should be used depends on the objective. For example, if the objective is to get maximum enrichment of shoot tissue Zn without negatively affecting the plant growth, then for the DEX-ZnO treatment, selecting the value of Zn_{opt} for tissue Zn concentration (894 mg/L) is the best, since it is less than all Zn_{crt} values for root and shoot biomass (1031, 985 mg Zn/L, respectively) and elongation (910, 968 mg Zn/L, respectively). For the $ZnSO_4$ treatment, all but shoot elongation had Zn_{crt} lower than Zn_{opt} for shoot tissue Zn concentration (597 mg Zn/L). The most sensitive endpoint was shoot biomass which had a Zn_{crt} of 280 mg Zn/L. Hence, Zn concentration in the exposure solution should not exceed that value. Since ZnO NPs tended to target different seedling parts, bare ZnO NPs for example, would be a better choice for enhancing shoot Zn nutrition compared to the rest of the treatments, while DEX and DEX(SO_4) ZnO NPs would be superior to the rest of the NPs to enhance root Zn concentration.

The results of this study collectively showed that engineered ZnO nanomaterials performed better than $ZnSO_4$ as a seed treatment, since higher biomass, root and shoot elongation and tissue Zn concentrations, depending on treatment, could be achieved without causing adverse effects. Within the ZnO NP treatments, surface chemistry affected the distribution of Zn within the plant. Surface chemistry also affected enhancement of elongation and biomass within the plants. Thus, it is possible to tune the surface chemistry to optimize the performance of the ZnO NPs depending on the desired effects

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Tables:

Table 2.1 Primary particle sizes of three replicates of each type of nanoparticles (NPs) as measured by transmission electron microscopy. Stdev = one standard deviation

Nanoparticle	**Replicate(1) Avg.(nm)± Stdev.**	**Replicate(2) Avg.(nm)± Stdev.**	**Replicate(3) Avg.(nm)± Stdev.**	**Grand mean (nm)± Stdev.**
Core-shell	27 ± 11	28 ± 9	27 ± 11	27 ± 0.3
Bare ZnO	23 ± 10	25 ± 9	23 ± 9	24 ± 1
DEX-ZnO	18 ± 10	17 ± 6	19 ± 7	18 ± 1
DEX(SO_4)-ZnO	22 ± 11	19 ± 6	19 ± 7	20 ± 1

Table 2.2 Hydrodynamic size, zeta potential, pH at which zeta potential values were measured, and point of zero charge (PZC) for ZnO NPs. Results are reported as the average of three independent replicates of each ZnO NPs ± one standard deviation

Nanoparticles*	**Hydrodynamic diameter † (nm)**	**Hydrodynamic diameter †† (nm)**	**Zeta potential (mV)**	**pH**	**PZC**
Bare ZnO	314 ± 33	623 ± 139	29.1± 0.6	7.15	9.8
DEX-ZnO	755 ± 192	974± 542	19.5± 1.1	7.80	8.7
DEX(SO_4)-ZnO	304 ± 36	574 ± 83	-24.8± 0.4	7.40	<6.2
Core-shell	531.5 ± 44.6	586.8 ± 205.3	-23.9± 2.3	7.61	<6.2

† Intensity weighted z-average †† Volume weighted z-average.

Table 2.3 Effect of Zn treatment on root and shoot lengths, dry biomass, and Zn concentration. *p*-values in bold means regression was statistically significant at α=0.05.

Plant response	Regression parameter	Core-shell NPs	DEX-ZnO NPs	DEX(SO_4)-ZnO NPs	Bare ZnO	$ZnSO_4$	Control*
Zn concentration in roots (µg/g)	r^2	0.91	0.84	0.81	0.94	0.89	NA
	p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	NA
	Zn_{opt}(mg/L)	1017	>1196	>936	605	725	NA
	$Zn_{roots\ max}$ (µg/g)	1454	2143	1910	1464	1871	50.6±4.8
Zn concentration in shoots (µg/g)	r^2	0.91	0.78	0.87	0.84	0.80	NA
	p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	NA
	Zn_{opt}(mg/L)	644	894	674	518	597	NA
	$Zn_{shoots\ max}$ (µg/g)	266	346	309	433	302	33.2±8.1
Shoots to Roots Zn (%)		18	15	11	30	16	66
Relative root biomass	r^2	0.76	0.66	0.14	0.024	0.73	NA
	p-value	**<0.001**	**<0.001**	0.3772	0.8744	**<0.001**	NA
	Zn_{opt}(mg/L)	500	428	NS	NS	238	NA
	$Root_{max\ biomass}$	1.67	1.55	NS	NS	1.24	NA
Relative root length	r^2	0.19	0.38	0.47	0.27	0.69	NA
	p-value	0.2627	**0.044**	**0.016**	0.131	**<0.001**	NA
	Zn_{opt}(mg/L)	NS	314	450	NS	138	NA
	$Roots_{max\ length}$	NS	1.24	1.48	NS	1.17	NA
Relative Shoot biomass	r^2	0.20	0.38	0.038	0.064	0.58	NA
	p-value	0.2330	**0.044**	0.7761	0.6492	**0.003**	NA
	Zn_{opt}(mg/L)	NS	415	NS	NS	124	NA
	$Shoot_{max\ biomass}$	NS	1.21	NS	NS	1.02	NA
Relative shoot length	r^2	0.46	0.42	0.078	0.41	0.53	NA
	p-value	0.0188*	0.0294*	0.5859	0.0329*	0.0076*	NA
	Zn_{opt}(mg/L)	587	406	NS	242	297	NA
	$Shoot_{max\ length}$	1.23	1.27	NS	1.24	1.16	NA

* Zn tissue concentrations and root and shoot biomass and elongation of control treatment are reported as mean ± one standard deviation. Zn_{opt} is the concentration of Zn in the exposure solution resulting in the maximum corresponding Zn concentration in tissue, $Zn_{root\ max}$, $Zn_{shoot\ max}$, are maximum Zn concentration in roots and shoots calculated as the y-axis value corresponding with Zn_{opt} at the maximum of the quadratic fit. Root/shoot max biomass, max length are the corresponding biomasses and lengths of the main roots and shoots of the seedlings at Zn concentration in the exposure solution resulting in a maximum response. NA: not available. NS: non-significant regression.

Table 2.4 Critical and optimum Zn concentrations (Zn_{crt} and Zn_{opt}, respectively) in exposure solutions for Zn treatments at different physiological endpoints and for root and shoot Zn content. Zn_{opt} values corresponding with maximum Zn concentration in roots and shoots for each Zn treatment are listed. NS: not statistically significant

	Core-shell NPs		**DEX-ZnO NPs**		**DEX(SO_4)-ZnO NPs**		**Bare ZnO**		**$ZnSO_4$**	
	Zn_{crt}	**Zn_{opt}**	**Zn_{crt}**	**Zn_{opt}**	**Zn_{crt}**	**Zn_{opt}**	**Zn_{crt}**	**Zn_{opt}**	**Zn_{crt}**	**Zn_{opt}**
	mg/L									
Relative root biomass	>1075	500	1031	428	NS	NS	NS	NS	580	238
Relative root elongation	NS	NS	910	314	847	450	NS	NS	510	138
Relative shoot biomass	NS	NS	985	415	NS	NS	NS	NS	280	124
Relative shoot elongation	>1075	587	968	406	NS	NS	750	242	634	297
Root tissue Zn concentration	NS	1017	NS	>1535	NS	>1910	NS	605	NS	725
Shoot tissue Zn concentration	NS	644	NS	894	NS	674	NS	518	NS	597

Figures

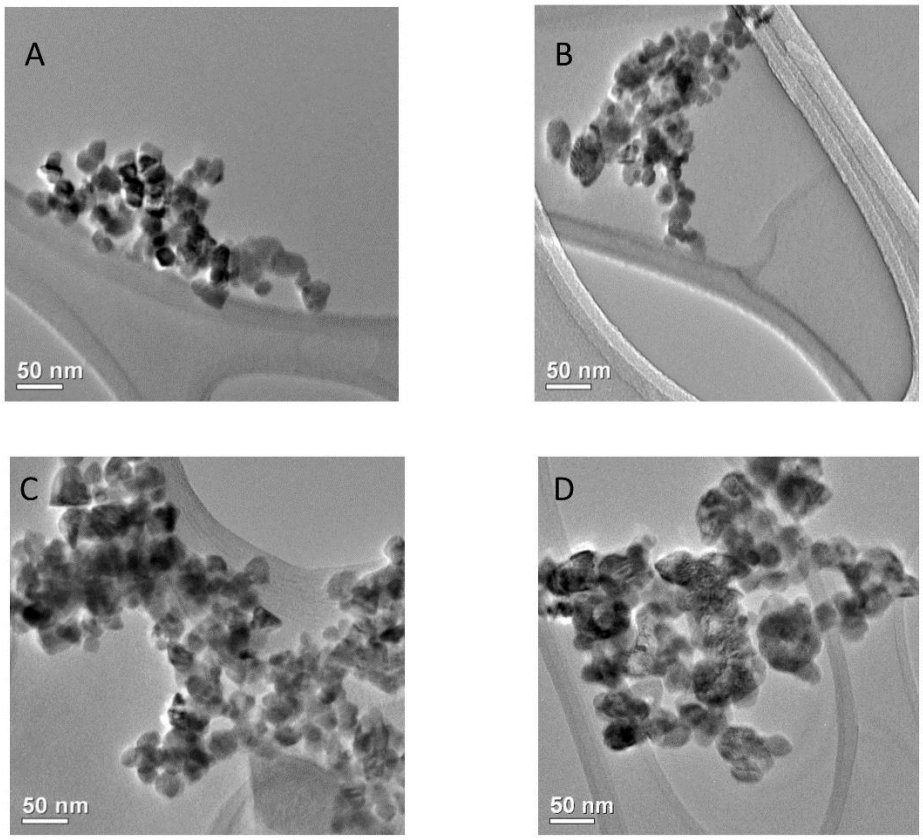


Figure 2.1 TEM images of bare ZnO NPs (A), dextran coated (DEX -ZnO NPs) (B), dextran sulfate coated (DEX (SO_4)-ZnO NPs) (C), ZnO-$Zn_3(PO_4)_2$ core-shell NPs (D). Scale bar is 50 nm

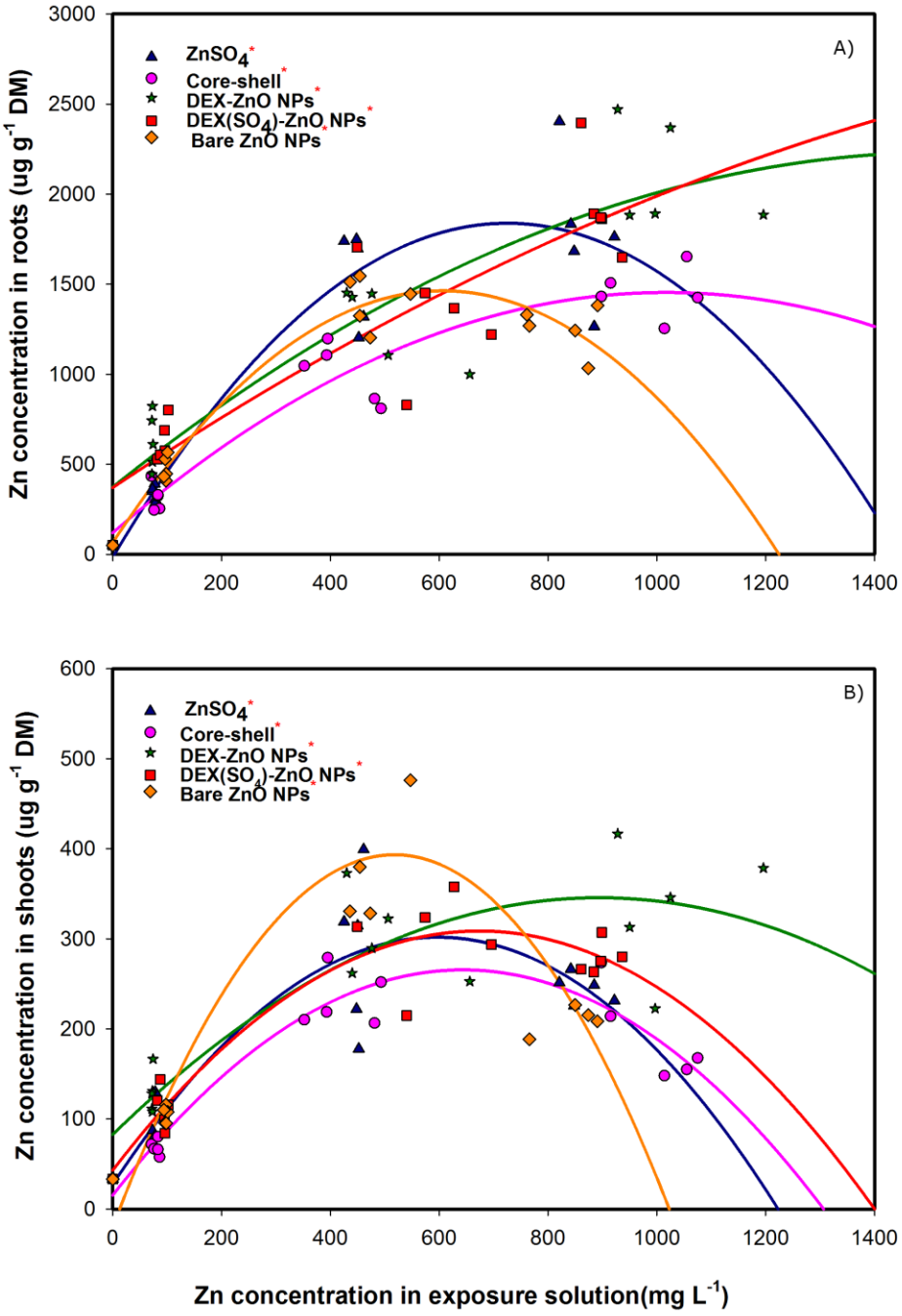


Figure 2.2 Quadratic regression for Zn concentration in root tissue on dry mass basis (A), in shoot tissue on dry mass basis (B) versus total Zn concentration in exposure solution in mg/L.

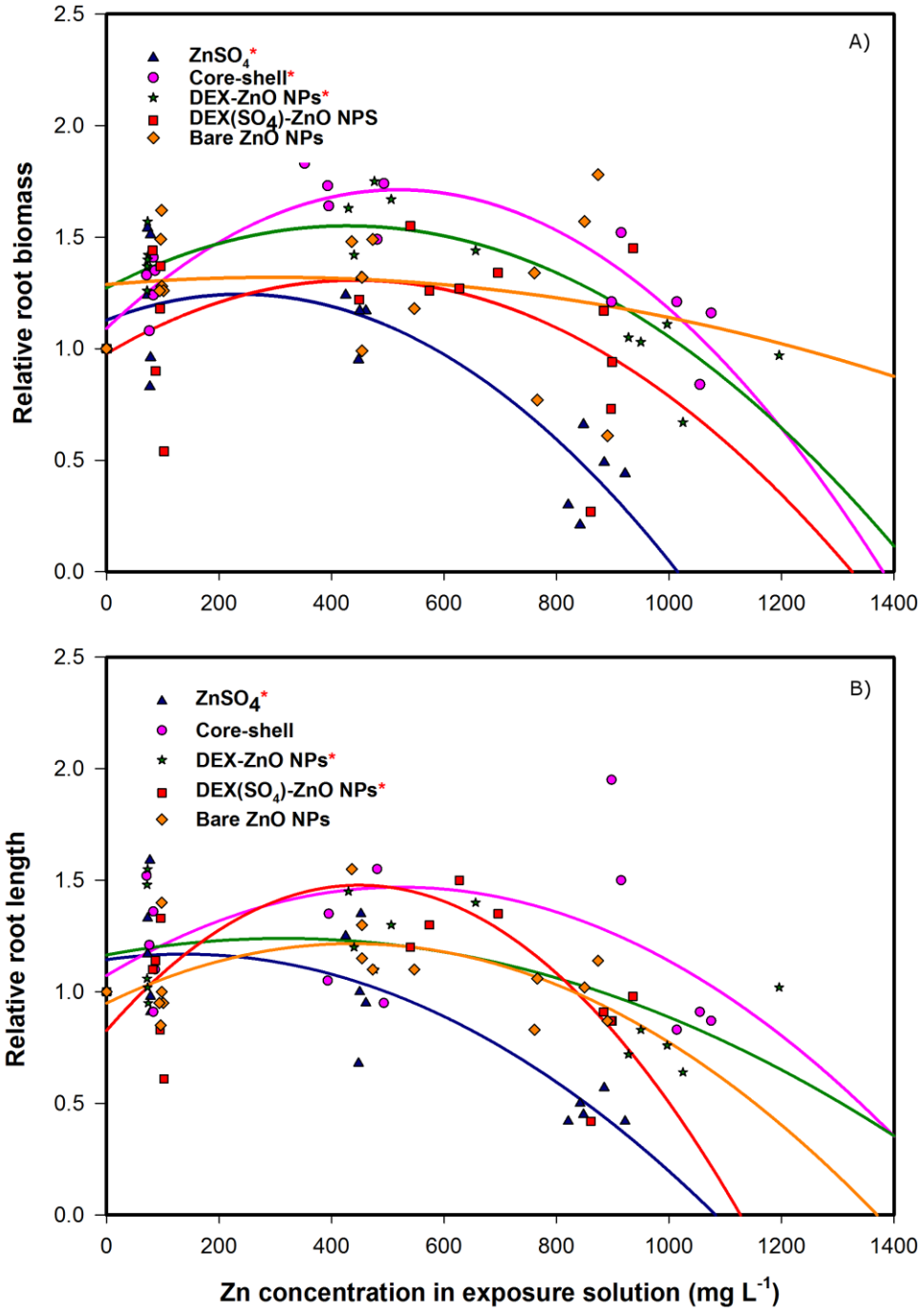


Figure 2.3 Quadratic regression for root biomasses (A), and root elongation (B) versus total Zn concentration in exposure solution in mg/L.

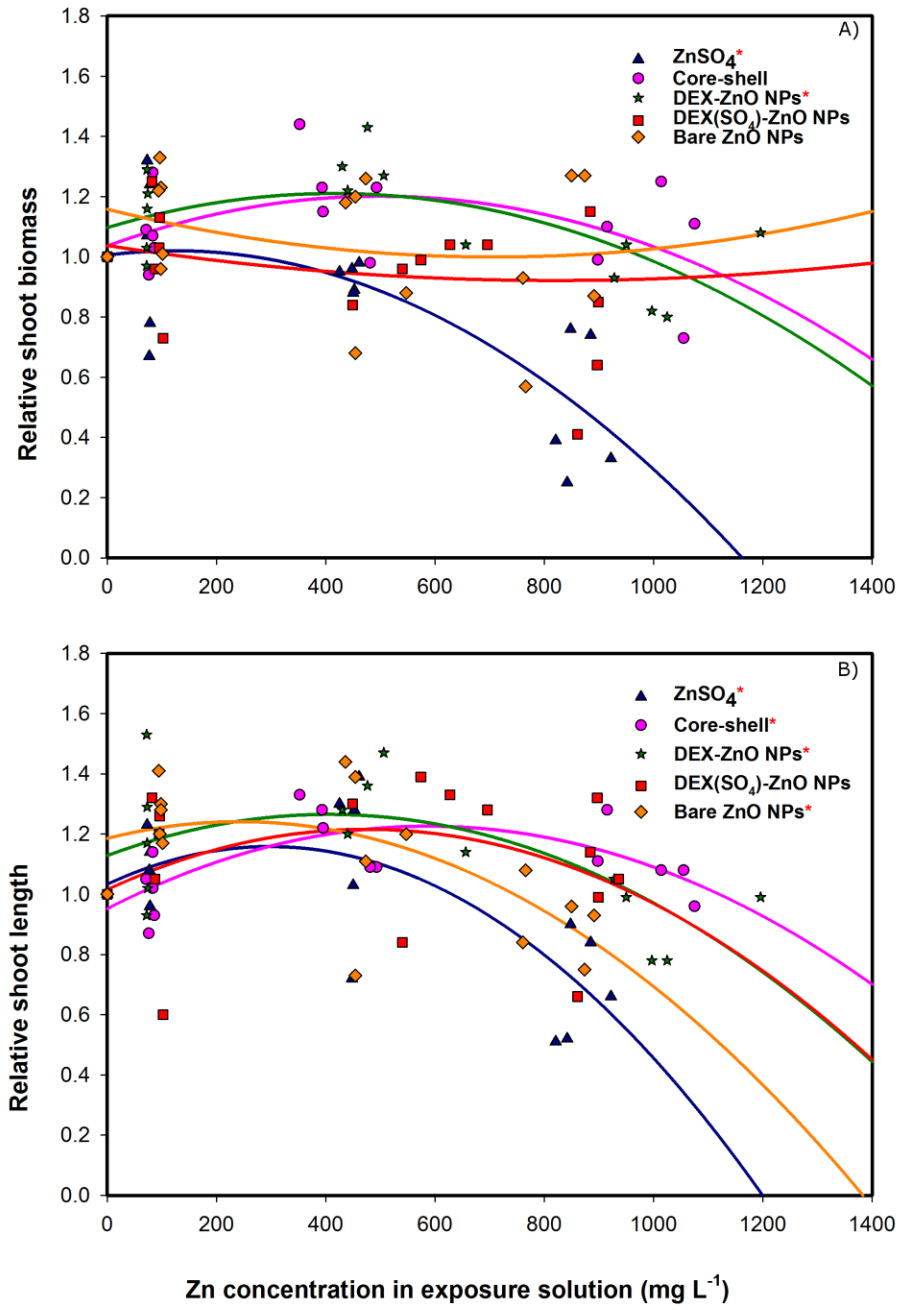


Figure 2.4 Quadratic regression for shoot biomasses (A), and shoot elongation (B) versus total Zn concentration in exposure solution in mg/L.

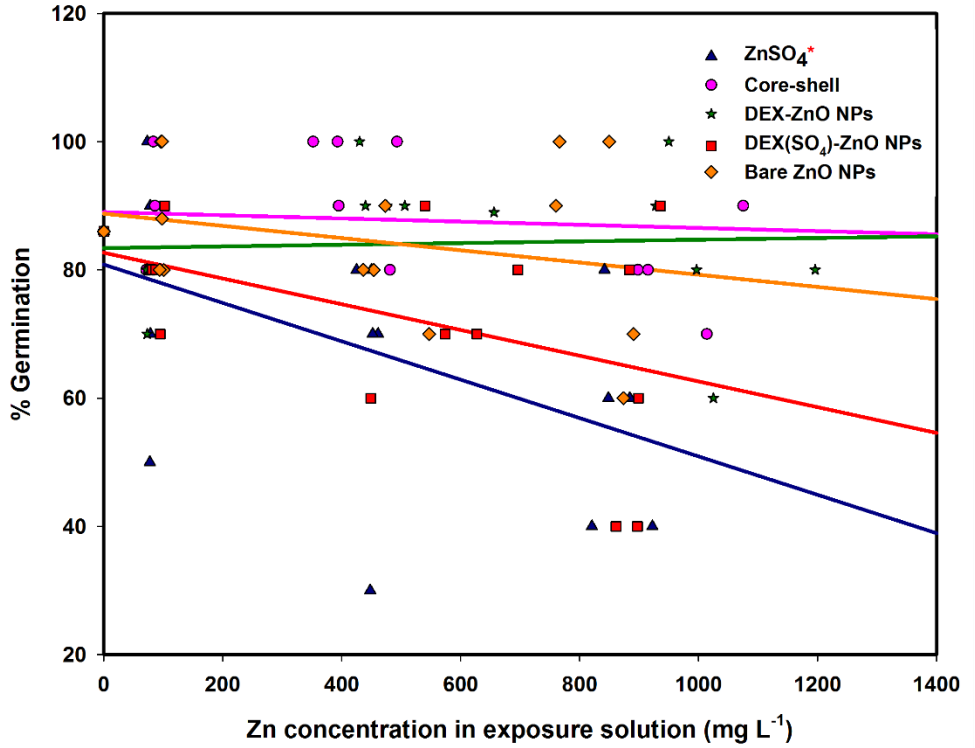


Figure 2.5 Linear regression for % germination versus total Zn concentration in exposure solution in mg/L.

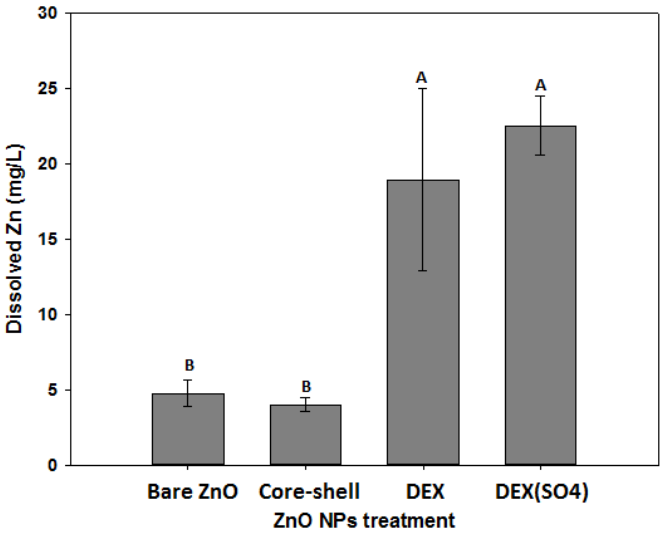


Figure 2.6 Dissolution of ZnO NPs in mg Zn/L in deionized (DI) water at a nominal Zn concentration of 500 mg Zn/L. Treatments connected with different letters are not significantly different at α=0.05.

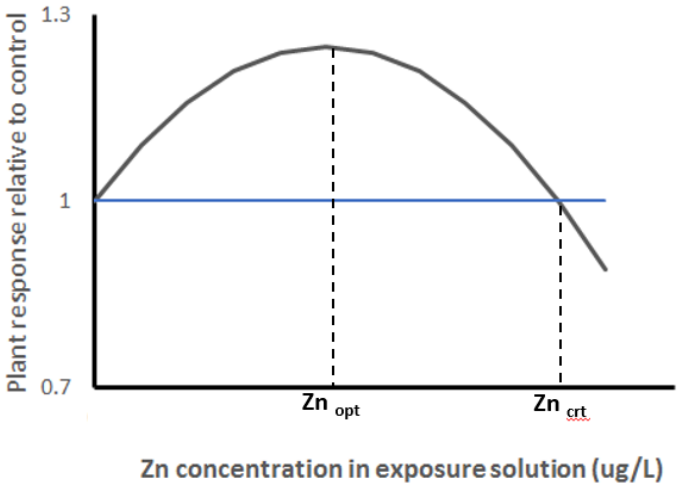


Figure 2.7 A graphical illustration of the relationship between optimum Zn (Zn_{opt}) and critical Zn concentration (Zn_{crt}) on a generic quadratic regression showing the effect of Zn concentration in the exposure solution on plant response relative to the control.

Supplementary information

Functionalized ZnO nanoparticles as seed coatings to enhance growth and Zn content of wheat (*Triticum aestivum*) seedlings.

Tables:

Table S 2.1 Effect of coating material on % germination, root and shoot lengths and dry biomass*

Coating type	Germination (%)	Radicle Root length (mm)	Root biomass (mg)	Shoot length (mm)	Shoot biomass (mg)
DI water	84±11[a]	20.0±8.3 [a]	19.7±5.5 [a]	36.8±6.8 [a]	32.5±7.1 [b]
DEX(SO_4)	93±6 [a]	24.3±1.5 [a]	31.3±1.7 [b]	59.7±5.1 [b]	50.5±1.4 [a]
Dextran	96±6 [a]	25.0±7.2 [a]	24.2±2.2 [b]	40.8±5.0[b]	39.7±2.6 [b]
$Zn_3(PO_4)_2$	96±6 [a]	22.7±6.8 [a]	24.9±8.4 [b]	43.3±9.1 [a]	38.2±6.9 [b]

*Results are reported as mean ± one standard deviation (n=5 for deionized (DI) water, n=3 for the coatings). ANOVA followed by Dunnett's test were used to test for significant differences between the coating controls and DI control. Coating controls connected with different letters are not statistically significant from DI control at α=0.05.

Table S2.2: Quadratic regression parameters for root and shoot Zn concentration versus relative root and shoot biomass.

Treatment	Equation	r^2	*p* -value
$ZnSO_4$	Relative **root** biomass = (-4E-07*(Zn in **roots**) ^2) + (0.0006*Zn in **roots**) + 1	0.39	0.032*
	Relative **shoot** biomass = (4E-06*(Zn in **shoots**) ^2) - (0.0018*Zn in **shoots**) + 1	0.16	0.028*
Core-shell	Relative **root** biomass = (-9E-07*(Zn in **roots**) ^2) + (0.0015*Zn in **roots**) + 1	0.62	<0.001*
	Relative **shoot** biomass = (-2E-06*(Zn in **shoots**) ^2) + (0.001*Zn in **shoots**) + 1	0.05	0.066
Bare ZnO	Relative **root** biomass = (-7E-07*(Zn in **roots**) ^2) + (0.0011*Zn in **roots**) + 1	0.13	0.007*
	Relative **shoot** biomass = (-3E-06*(Zn in **shoots**) ^2) + (0.0013*Zn in **shoots**) + 1	0.17	0.22
DEX	Relative **root** biomass = (-4E-07*(Zn in **roots**) ^2) + (0.0009*Zn in **roots**) + 1	0.62	<0.001*
	Relative **shoot** biomass = (-3E-06*(Zn in **shoots**) ^2) + (0.0012*Zn in **shoots**) + 1	0.04	0.12
DEX(SO_4)	Relative **root** biomass = (-5E-07*(Zn in **roots**) ^2) + (0.001*Zn in **roots**) + 1	0.81	0.022*
	Relative **shoot** biomass = (9E-08*(Zn in **shoots**) ^2) - (0.0002*Zn in **shoots**) + 1	0.02	0.73

*Regression is statistically significant at α=0.05.

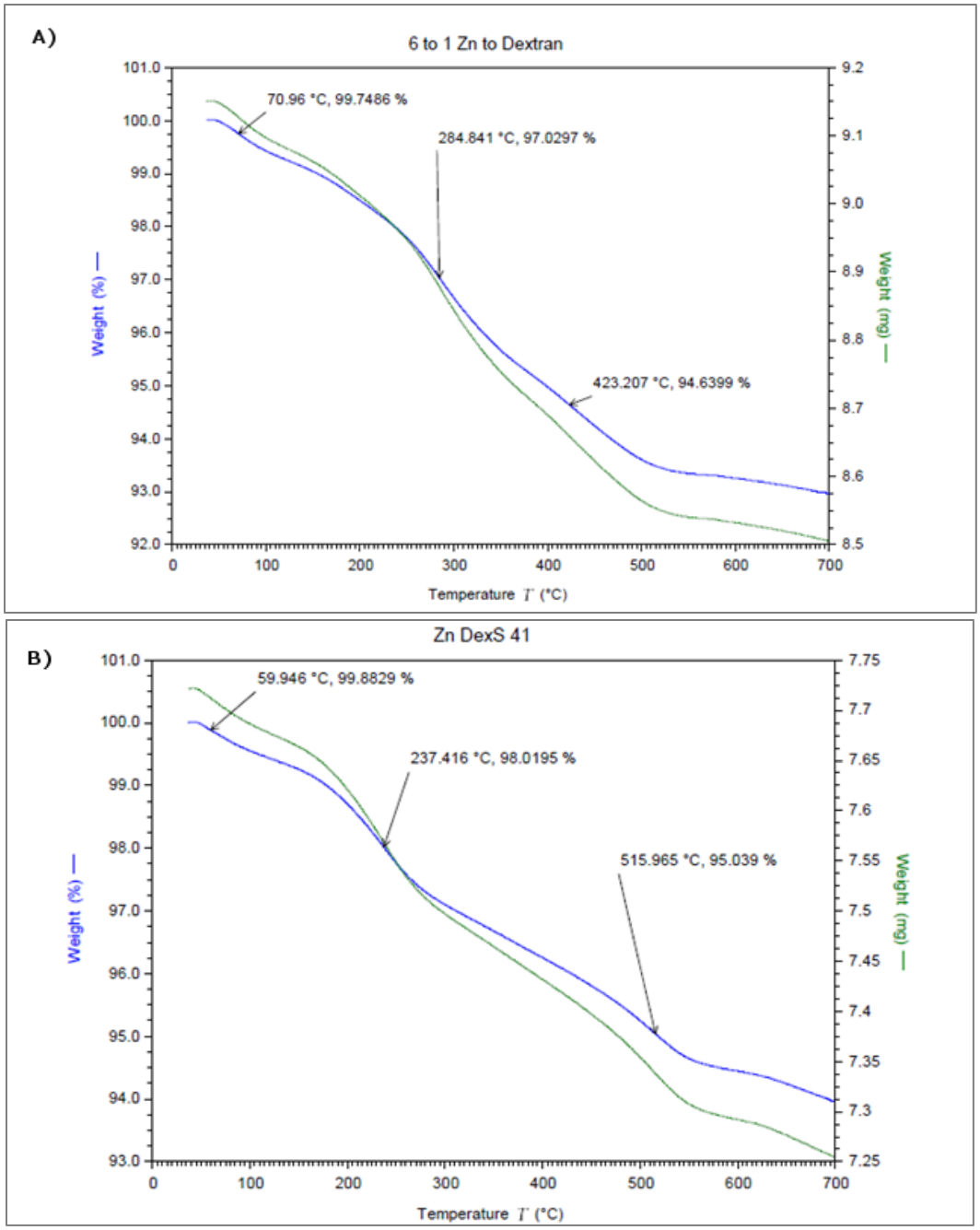


Figure S 2.1 Thermogravimetric analysis (TGA) graphs for dextran coated (DEX-ZnO) NPs(A) and dextran sulfate coated DEX(SO_4)-ZnO NPs (B)

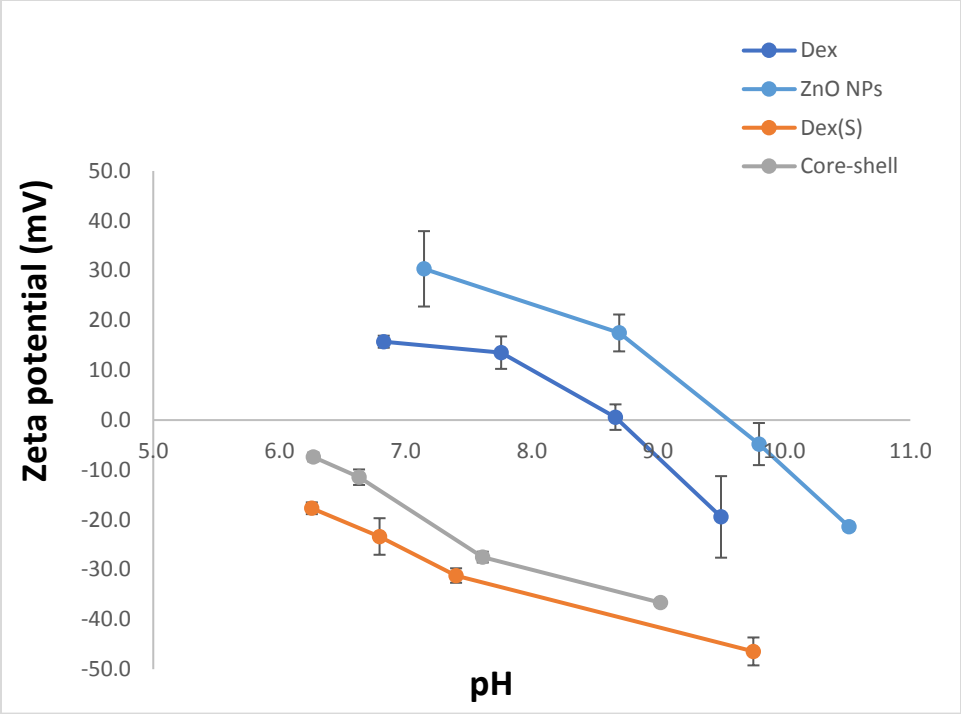


Figure S 2.2. Electrophoretic mobility of bare ZnO NPs, dextran coated ZnO NPs (DEX-ZnO), $Zn_3(PO_4)_2$-ZnO NPs (core-shell), and dextran sulfate coated ZnO NPs (DEX(SO_4)-ZnO NPs as a function of pH in deionized (DI) water. Zn concentration was 100 mg/L for all tested suspensions

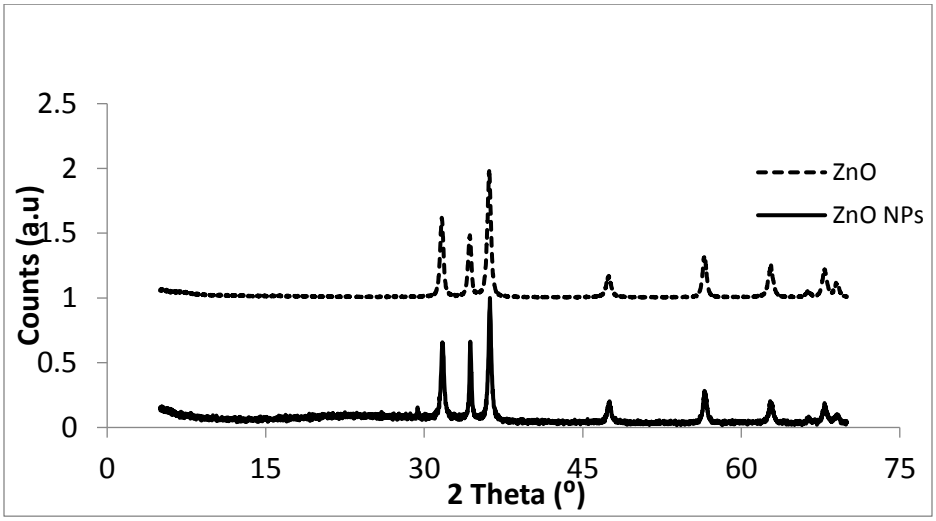


Figure S 2.3.XRD diffractogram of bare ZnO NPs and bulk ZnO

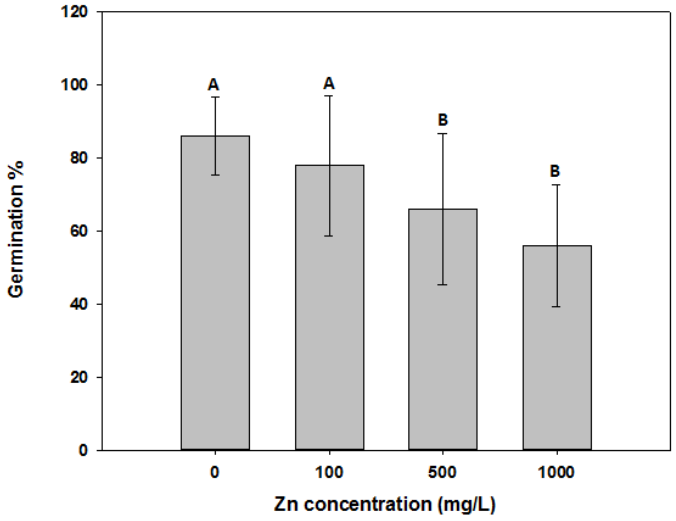
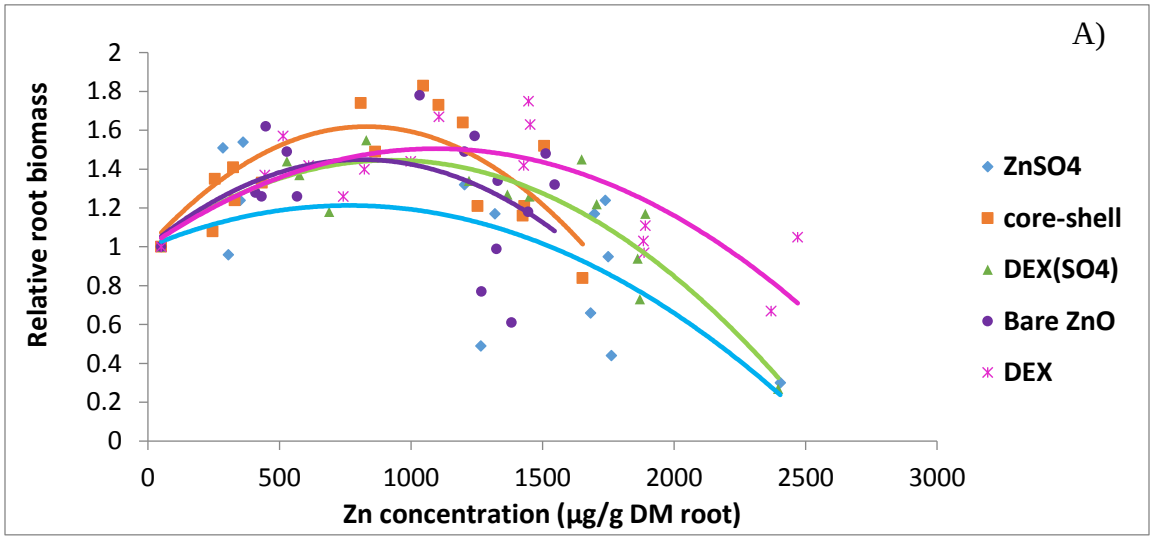


Figure S 2.4 Effect of Zn^{2+} ions concentration in exposure solution in (mg Zn/L) on germination success %. Each concentration was separately compared to 0 mg Zn/L (deionized (DI) water control) using two-sided t-test. Zn treatments connected with different letters are significantly different from the control at α=0.05



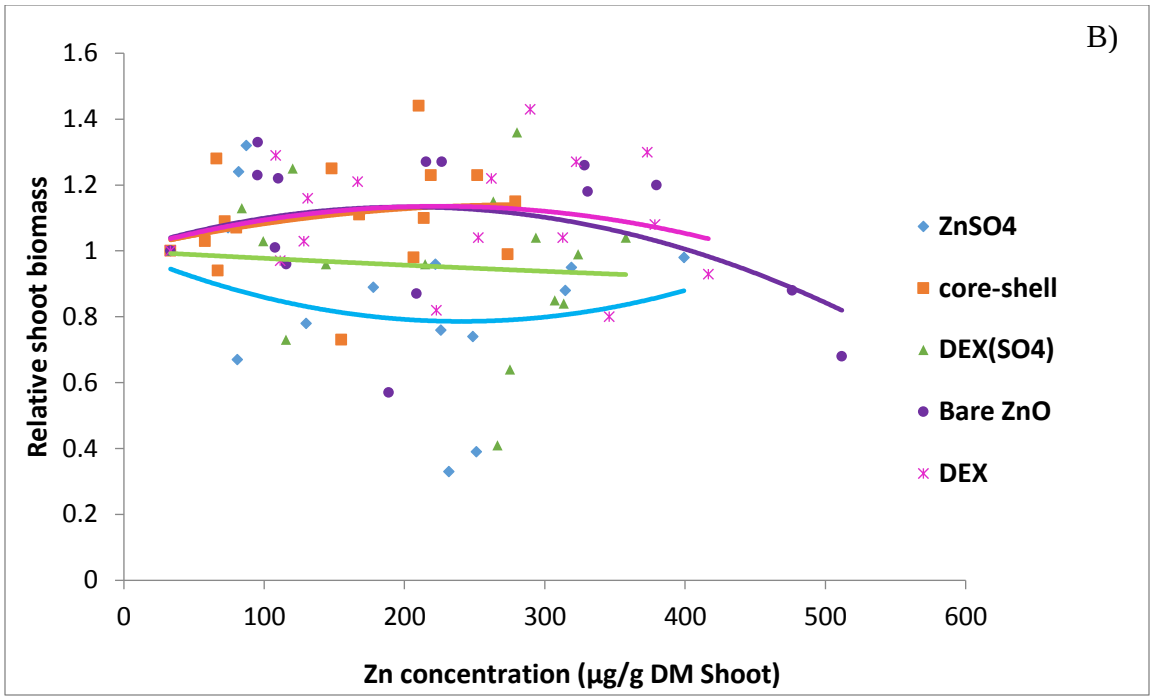


Figure S 2.5 Effect of Zn concentration in seedling in (µg Zn/g dry matter DM) on seedling growth. (A) Effect on root biomass, (B) Effect on shoot biomass. Quadratic regression was used to fit the data.

Chapter 3: Surface Coating Effects on the Sorption and Dissolution of ZnO Nanoparticles in Soil.

Zeinah Elhaj Baddar, Chris J. Matocha, and Jason M. Unrine

3.1 Abstract

Soil pH and dissolved organic matter (DOM) content are among the most important factors affecting the bioavailability of Zn and the binding and dissolution of ZnO nanoparticles (NPs). We investigated the effect of NP surface chemistry and DOM on the behavior of ZnO NPs and $ZnSO_4$ in soil solution at pH 6 and 8. To this end, we synthesized electrostatically stabilized (bare positively charged ZnO, and negatively charged ($Zn_3(PO_4)_2$ core-shell NPs), and sterically and electrosterically stabilized (neutral dextran (DEX), and negatively charged dextran sulfate (DEX(SO_4))-ZnO NPs, respectively. We hypothesized that negatively charged ZnO NPs will have higher total Zn concentrations as opposed to neutral and positively charged ZnO in soil pore water at higher pH, with higher dissolution of the NPs at lower pH. We found that all soils amended with ZnO NPs had significantly higher total Zn concentrations in saturated paste extracts compared to $ZnSO_4$, at both pH 6 and 8. At pH 8, core-shell and DEX-ZnO NP amendments had significantly higher total Zn concentration than $ZnSO_4$. To further investigate the unexpected behavior of the neutral DEX-ZnO NPs, we performed sorption isotherm experiments which showed that DEX-ZnO NPs had the highest affinity for DOM of all ZnO NPs, which likely enhanced their colloidal stability and partitioning in soil pore water, especially at pH 8. In simple aqueous solution with increasing ionic strength, negatively charged core-shell and DEX(SO_4) ZnO NPs were the most stable

against aggregation. When DOM was introduced in to the system, the as-synthesized surface chemistry of the particles was altered, and all NPs became negatively charged. No differences in total or dissolved Zn concentrations in soil extracts were observed among the different NP types while $ZnSO_4$ amended soils had the highest dissolved Zn among all treatments.

3.2 Introduction

There is an increasing interest in the use of nanoparticles (NPs) for delivery of agrochemicals such as micronutrients and pesticides[39]. One application under investigation has been the use of ZnO NPs as micronutrient fertilizers. A few studies have reported significant increases in yields and tissue Zn concentrations in peanuts (*Arachis hypogaea*)[55], maize (*Zea mays*)[52], and sorghum (*Sorghum bicolor*)[125] when bare ZnO NPs were added as soil amendments. Even when applied at a concentration that was ten times lower than that of $ZnSO_4$, foliar application of chitosan coated ZnO NPs at 40 mg/L enhanced wheat (*Triticum aestivum*) grain Zn concentration by 30% as compared to a 50% increase achieved with $ZnSO_4$ at 400 mg/L[54].

In general, total metal concentration is a poor indicator of Zn biooavailability[145]. Zinc bioavailability for plant uptake is limited by high soil pH, low geogenic Zn levels and high contents of phosphates, clay, natural organic matter (NOM), and carbonates[102]. Therefore, using soluble Zn salts as fertilizers is often of limited success under these conditions. Likewise, soil properties may affect the bioavailability, fate, and behavior of ZnO NPs. Thus, the partitioning of nanoparticles to the soil pore water is an important determinant of the mobility and bioavailability of these nanoparticles for plant uptake.

Soil pH has a tremendous effect on the behavior of Zn ions. Higher soil pH is often associated with restricted bioavailability of nutrients, including Zn. The increase in soil pH is accompanied by the deprotonation of hydroxyl groups present on soil components such as clay aluminosilicates, Al/Fe oxohydroxides, or organic ligands, leading to the retention of Zn ions[22, 23, 146]. Moreover, high soil pH induces Zn ion precipitation as poorly soluble Zn minerals (e.g. Zn carbonates, phosphates, and hydroxides)[22]. On the other hand, lower soil pH enhances the bioavailability of Zn by solubilizing Zn complexes, in addition to the desorption from the now more protonated exchange sites on soil colloid surfaces.

Nanoparticle behavior is also dramatically affected by soil pH If the pH in a medium approaches the point of zero charge PZC (the pH at which positive and negative surface charges are balanced, resulting in an electrophoretic mobility of zero[65]), the particles will have a greater tendency to aggregate[72]. On the other hand, at a pH higher than the PZC, particle surfaces will be more negatively charged, conferring a higher NP colloidal stability in the soil solution as they will most likely be repelled from negatively charged colloids in the soil, thus enhancing their partitioning to the soil solution. For bare ZnO NPs the PZC is about 9.3, giving them a net positive charge at most likely soil pH values. Solubility of ZnO is also strongly pH dependent. Solubility in water begins to increase below a pH of 7.3. Previous work has shown that low soil pH enhances the dissolution of ZnO NPs and results in an increase in Zn ion concentration[92-94, 147-150].

Ionic strength also affects the NP colloidal stability. When electrolyte concentration increases, the electric double layer is compressed due to charge screening, which reduces the separation distances between particles and allows attractive forces to dominate,

inducing aggregation. One study reported that the propensity of Ag NPs to aggregate in an electrolyte solution was found to be dependent on their surface chemistry, specifically organic coatings, according to the following order: bare Ag NPs > Ag NPs sterically stabilized with polyvinylpyrrolidone (PVP)> Ag NPs electrosterically stabilized with gum arabic (GA)[144].

Modification of NP surface chemistry by adding coatings is often implemented to impart colloidal stability and minimize aggregation[128]. The initial surface chemistry of the as-synthesized particles can be dramatically altered in complex environmental media due to the loss of coating and/or replacement with natural organic material[58, 151]. Dissolved organic matter can overcoat or replace original coatings on NP surfaces[67, 87]. Due to the low pKa values of carboxylate functional groups, humic acids (HA) tend to have a negative charge under environmentally relevant conditions. Thus, whether DOM replaces or overcoats existing coatings, a negative charge will be imparted to these NPs, potentially enhancing their colloidal stability in soil solution. On the other hand, DOM could also induce NP dissolution due to the ligands exchange which occurs on the surface of these NPs[74].

This study investigated the effect of ZnO NP surface chemistry on their partitioning in soil pore water. According to the classical Derjaugin–Landau–Verwey–Overbeek (DLVO) theory, the interplay between attractive (Van der Waals) and repulsive (electrostatic) forces determines the aggregation status of colloids[152]. Coatings can increase particle stability by increasing electrostatic or steric repulsion. Electrostatic stabilization involves imparting a charge to the particle surface to enhance their electrostatic repulsion. Steric stabilization is caused by osmotic constraints due to the

conformation of macromolecules on two particle surfaces as they come into close proximity , thus increasing particle repulsion[135]. Electrosteric stabilization combines both effects to further enhance particle stability against aggregation. In order to enhance NP resistance against aggregation, we stabilized bare ZnO NPs sterically through adding a nonionic coating (dextran), electrosterically through adding a polyelectrolyte (dextran sulfate) coating, and electrostatically by forming a shell of $Zn_3(PO_4)_2$ on a core of ZnO NPs. We will refer to these particles as: DEX, DEX(SO_4), and core-shell ZnO NPs hereafter. Core-shell and DEX(SO_4) ZnO NPs are negatively charged, therefore, we hypothesized that this would likely enhance their partitioning to the soil solution in comparison with the positively charged bare ZnO and the neutral DEX-ZnO NPs, especially under alkaline conditions. We expected DEX-ZnO NPs to initially bind to soil particles, as it has been shown that particles coated with neutral polymers have a high affinity for surfaces which are not coated with a like polymer[153]. We also expected that acidic soil pH will induce the dissolution of ZnO NPs regardless of their surface chemistry.

3.3 Materials and Methods

3.3.1 Zinc Oxide Nanoparticles

A detailed description of synthesis protocols and characterization of ZnO NPs can be found in our previous work[154]. In brief, alkaline precipitation in water was used to produce bare ZnO NPs. The aging of these NPs in phosphate solution under certain conditions leads to the formation of a core made of ZnO NPs that is covered by a shell of amorphous $Zn_3(PO_4)_2$[86]. The addition of nonionic (dextran) and polyelectrolyte (dextran

(SO_4)) of 9-15 kDa at 1:6 and 1:4 coating to Zn mass ratio during the synthesis resulted in the formation of DEX and DEX(SO_4) ZnO NPs, respectively.

Transmission electron microscopy (TEM), x-ray diffraction (XRD), dynamic light scattering (DLS), phase analysis light scattering (PALS), and thermogravimetric analysis (TGA) were used to determine, the primary particle size and shape, chemical form, hydrodynamic diameter, electrophoretic mobility, and the mass of coating on the particles, respectively. We also determined the PZC of these NPs by measuring the electrophoretic mobility of 100 mg Zn/L ZnO NPs suspensions at different pH values upon titrating with either HCl or NaOH.

3.3.2 Stability of ZnO Nanoparticles as a Function of Ionic Strength

To test the effect of ionic strength on the stability of ZnO NPs, suspensions of bare and coated particles were prepared at 500 mg Zn/L in DI using cup horn sonication (Qsonica, Newtown, Connecticut, USA) at 100% amplitude for 45 minutes. Then 0.26 mL of each NP suspension was aliquoted in a 2 mL microcentrifuge tube, where 1.04 mL of 0, 1, 10, and 100 mM NaCl solutions were added to achieve a final concentration of 100 mg Zn/L. Hydrodynamic diameters and electrophoretic mobilities were measured using Zetasizer.

3.3.3 Effect of pH and DOC on Zeta (ζ) Potential and Dissolution of ZnO Nanoparticles in Solution

We performed saturated paste extractions[155] from an unamended Sadler soil at both pH 6 and pH 8. The collected extracts were centrifuged for 4 hours at 16,837 x g (using a particle density of 2.67 g/cm^3 for soil particles to obtain a size cut off of 35 nm diameter according to Stoke's law). The supernatants were aliquoted and referred to as particle free

soil solution (PFSS) hereafter. To buffer the soil solution pH values, which decreased due to equilibration with the atmosphere, we added 2-(N-morpholino) ethanesulfonic acid (MES) and tris (hydroxymethyl) aminomethane (TRIS) buffers at a final concentration of 1 mM to achieve pH values of 6 and 8, respectively.

Zinc oxide NP suspensions were prepared at a nominal concentration of 250 mg/L Zn in DI water. Cup horn sonication for 45 minutes at 100% amplitude was used to disperse the NPs. In a 2 mL microcentrifuge tube, 0.12 mL of each ZnO NP suspension and DI water (Zn free control) were added to 1.15 mL PFSS at either pH 6 or pH 8 to achieve a final concentration of 25 mg Zn/L. Three replicates of each treatment were prepared. Sample pH values were measured prior to and after 24 h mixing at room temperature on a sample rotator that was set at maximum speed. Electrophoretic mobilities were measured after 24 h using phase analysis light scattering (PALS; zetasizer nanoZS, Malvern Instruments, Malvern, United Kingdom). Particle ζ potential was estimated from electrophoretic mobilities using the Smoluchowski's approximation. These samples were then centrifuged for 3 hours at 16,837 X g, then a 0.5 mL aliquot of the supernatant was acidified to 0.16 M HNO_3 to measure the dissolved Zn in the PFSS using ICP-MS.

To address the effect of dissolved organic matter on the ζ potential of ZnO NPs, we added PPHA at a concentration of either 25 or 100 mg C/L to a 25 mg Zn/L suspension of each ZnO NP treatment in either PFSS, DI water, or MHRW. Samples were left on a tube rotator as mentioned above. Sample pH was measured in all suspensions at each C concentration level (in DI water and MHRW), and in the PFSS at both pH levels. Particle ζ potential and dissolution were determined as described above.

3.3.4 Dissolved Organic Carbon Sorption Experiments

We performed batch experiments to discern the sorption of DOC on to the surface of the different ZnO NPs. Pahokee peat humic acid (PPHA) (International Humic Substances Society, IHSS, 1S103H) was used as the model DOC source. We dissolved 10 mg of PPHA in 100 mL DI water. The pH of the solution was brought up to 9 using 0.1M NaOH to facilitate dissolution. The solution was left to stir overnight at room temperature (22°C) and then filtered with a 0.2 μm nylon filter, and stored at 4°C. The DOC concentration in PPHA stock solution was 43 mg C/L as determined using a carbon analyzer.

Zinc oxide NP suspensions at 1000 mg Zn /L were sonicated for 45 minutes at 100% amplitude. Moderately hard reconstituted water (MHRW) was prepared according to EPA method 600/4-90/027F[156]. Briefly, in 1L DI water, the following salts were added to achieve the following measured concentrations, in g/L: 0.067, 0.123, 0.096, and 0.004 of $CaSO_4.2H_2O$, $MgSO_4.7H_2O$, $NaHCO_3$, and KCl, respectively. The pH of MHRW was adjusted to 8 throughout the sorption experiments to match the pH of PFSS which was 8. Batch experiments were carried out at room temperature (~ 22°C) in 15 mL metal free centrifuge tubes where 2 mL of MHRW was added to 0.8 mL ZnO NPs at a Zn concentration of 1000 mg Zn/L (final Zn concentration was 100 mg Zn/L). Serial dilution of PPHA stock solution was done as the volume was brought up to 8 mL using DI water. All suspensions were prepared in triplicate and incubated for 24 h on a sample rotator set at full speed to establish equilibrium. We previously determined that equilibrium was obtained in 24 hours in a separate experiment (Fig. S 3.1). The suspensions were then centrifuged for 3 h at 16,837 x g to obtain non-sorbed DOC.

To determine free dissolved organic carbon concentration, 75 μL were withdrawn from the supernatants and aliquoted into a 96 well plate, and a microplate reader was used to measure the absorbance at 254 nm[157, 158]. We determined the molar extinction coefficient at 254 nm using the DOC concentration of the stock solution measured using a carbon analyzer ((TOC-V_{CPN} total carbon analyzer, Shimadzu Corporation, Columbia, MD, USA).

The plots of the free DOC concentration (C_e), in mg C/L, against sorbed DOC (q_e) in mg/kg best fit a Freundlich isotherm model, which is described by the following formula:

$$q_e = k_f C_e^{(1/n)} \qquad (1)$$

whereq_e is the amount of PPHA (mg C) adsorbed per unit mass of ZnO NP (g) at equilibrium, C_e is the concentration of free PPHA (mg C/L) at equilibrium, n is the linearity parameter, and k_f is the Freundlich coefficient which describes the binding affinity of PPHA to the surface of the particles.

The linearization approach was used to determine the Freundlich isotherm equation parameters, for each treatment where both (C_e) and (q_e) were log-transformed. Then, log(C_e) values were plotted against log(q_e) values. Linear regression was used to fit the data points. Slope and intercept in each regression represented (1/n) and k_f, respectively. These parameters were then used to plot the data points according to the Freundlich model.

3.3.5 Soil Characterization

Surface Sadler silt loam (fine-silty, mixed, mesic Fragiudalf) surface soil was obtained from The University of Kentucky Research and Education Center at Princeton (KY, USA). The soil was air dried, ground, and sieved (<2mm). Chemical and physical

characterization of the soil included the determination of pH in a 1:1 ratio of soil to 18MΩ DI water or 1M KCl[159], particle size distribution (texture) by hydrometer[160], and total organic C and N by Dumas combustion (1112 Series NC soil analyzer, Thermo Electronic Corporation, Waltham, MA, USA)[161]. A factor of 1.724 was multiplied by the TOC value to convert soil TOC into organic matter content[161]. Acid extractable major cations and trace metals were determined following EPA method 3052[162]. We placed 0.25g soil and 10 mL concentrated nitric acid in Teflon bombs. Closed vessel microwave digestion (MARS Express microwave reaction system (CEM, Matthews, NC) was performed and the digestates were further diluted before measuring major cation and trace metal concentrations. Major cation concentrations were measured using inductively coupled plasma-optical emission spectrometry (ICP-OES, Vista Pro Simultaneous ICP-OES, Varian, Palo Alto, CA, USA). Trace metal concentrations were measured using Inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500cx Santa Clara, CA, USA). Major anions were extracted from the soil with water according to the method described by Judy et al. [163]. Analysis of recovered anions from soil samples was performed using ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA). Colorimetric methods; molybdate blue-stannous chloride[161] and indophenol blue[164],were used to determine total phosphorous and ammonium concentrations, respectively, in soil. Soil water holding capacity (WHC) was determined using pressure plate extractor (Soil Moisture Equipment Corp., Santa Barbra, CA, USA)[165]. A Mehlich III extraction was used to estimate the bioavailable Zn fraction in soil[166].

3.3.6 Soil Spiking

In a 150 mL capacity disposable plastic beaker, 50 grams of air-dried soil were thoroughly mixed by a wooden stick with either 40 or 54 mg of $MgCO_3$ or MgO to increase soil pH to 6 and 8, respectively. The carbonates and oxide of Mg were chosen because, due to their relative acid neutralizing capacities, we could add similar amounts of Mg to each treatment. We chose to use $MgCO_3$ and MgO instead of Na_2CO_3 and NaOH because Na^+ acts as a dispersing agent and disrupts the soil structure, dispersing a large quantity of soil colloids. Given that we were investigating colloidal stability of ZnO NPs, we didn't want to cause conditions that would artificially increase colloid dispersal. We also avoided using $CaCO_3$ or CaO due to the tendency for Ca^{2+} to cause aggregation of colloids. Magnesium ions cause less aggregation than Ca^{2+} especially in the presence of dissolved organic matter[167]. To achieve 30% WHC, 5 mL of DI water were added to each soil sample. After thorough mixing, the beakers were weighed and covered with parafilm perforated by a few holes to allow air exchange.

Soil samples were left to equilibrate to the desired pH for 7d in an incubator at 15°C. At the end of the incubation period, masses were checked, and DI water was added as needed to compensate for evaporation. Zinc oxide NP suspensions of 1000 mg Zn/L were prepared by adding a known mass of NP powder to 5 mL DI water in a 15 mL centrifuge tube. The suspensions were sonicated using a cup horn sonicator at 100% amplitude for 45 minutes. The suspensions were then added to the soil samples and mixed thoroughly with a wooden stick, which also raised the moisture content to 60% WHC. The beakers were covered with parafilm with several holes to allow air exchange and were kept in the incubator at the same temperature for two more weeks. The masses

of beakers were recorded, and DI water was added as needed to replace water lost during the incubation period, once every week. The experiment was terminated after 14 d of incubation.

3.3.7 Saturated Paste Extraction

In order to minimize dissolution and colloidal dispersion artifacts from using large ratios of water to soil, while still obtaining sufficient soil water for analysis, we prepared saturated pastes for extraction of total and dissolved Zn using standard methods[161]. Hyperbaric filtration (Fann instrument company, Houston, TX, USA) was used to extract soil solution from the saturated paste. We used Ahlstrom 10 μm pore size filters. Saturated pastes were transferred to the filter unit and 600 kPa pressure was applied using air. The soil solution was collected and kept at 4°C. Recovery of Zn NPs or Zn ions through the filters ranged from 99-104%. Greater than 90% of 1 μm polystyrene/latex beads passed through the filters.

3.3.8 Total Zn in Spiked Soil and Total and Dissolved Zn in Saturated Paste Extracts

Total Zn concentrations were determined in soils prior to and after extracting the soil solution. Around 2.0 g of soil was dried to constant weight in the oven at 105 °C. Dried soil was ground, and 0.25 g were digested with concentrated HNO_3 as mentioned above using EPA method 3052[162].

Saturated paste extracts were vortexed for 30 seconds and 1mL was transferred to a 15 mL tube. Then, 0.75 mL of concentrated HNO_3 was added to each tube and an open vessel microwave digestion was performed according to EPA method 3005 A[168]. The Zn concentration in these samples is defined as total Zn. Another 2 mL fraction of the

extracted soil solution was centrifuged at 16,837 X g for 3 hours to eliminate particles of >7 nm diameter as calculated using Stoke's law. A one mL aliquot of supernatant was subsequently acidified to 0.16 M HNO_3. We defined this Zn fraction as the dissolved Zn. Inductively coupled plasma-mass spectrometer was used to measure Zn concentration in both fractions.

3.3.9 Statistical Analysis

Hydrodynamic size and the electrophoretic mobility data in the electrolyte solutions followed ANOVA assumptions of normality and variance homogeneity. Therefore, we performed ANOVA, followed by Dunnett's test to examine the effect of electrolyte concentration on the hydrodynamic size and the electrophoretic mobility for each ZnO NP treatment separately. Electrophoretic mobility and dissolution data in PFSS, DI and MHRW followed the ANOVA assumptions and were analyzed in a similar manner, where, the Tukey HSD multiple comparisons test was performed between different ZnO coatings at each PFSS soil pH or DOM concentration. Evaluation of total and dissolved Zn concentration in saturated paste extracts was performed with a randomized complete block design, where at each pH level each Zn treatment was replicated three times within each experimental block. There were two experimental blocks performed on different days. We used Proc GLM to test for significant main effects and 2 -way interactions at $\alpha = 0.05$. Multiple comparisons between treatments within statistically significant interaction or main effects were performed using Tukey's HSD adjustment (SAS 9.4, SAS Institute, Cary, NC).

3.4 Results

3.4.1 Zinc Oxide Nanoparticle Characterization

Detailed characterization of the ZnO NPs can be found in our previous work[154]. Transmission electron micrographs (Fig. 3.1) showed that the particles were nearly spherical. The primary particle sizes were 24 ± 1, 27 ± 0.3, 18 ± 1, and 20 ± 1 nm (mean ± one standard deviation) for bare, core-shell, DEX, and DEX(SO_4) ZnO NPs, respectively. Intensity weighted hydrodynamic diameters were: 314± 32.8, 532 ± 44.6, 755± 191.8, and 304 ± 36.2 for bare, core-shell, DEX, and DEX(SO_4) - ZnO NPs, respectively. Smoluchowski's approximation was used to calculate ζ potential values from electrophoretic mobilities measured in DI water, which were positive for the bare and DEX-ZnO NPs (29.1± 0.6 and 19.5± 1.1 mV) and negative for DEX(SO_4) and core-shell ZnO NPs (-24.8± 0.4 and -23.9± 2.3 mV). Point of zero charge values were determined graphically by plotting zeta potential values across a range of pH values (Fig. S3.2). Bare ZnO and DEX-ZnO NPs had higher PZC than DEX(SO_4) and core-shell ZnO NPs, where the former two had PZC values of 9.8 and 8.7, respectively, and the latter two had PZC values less than 6.2.

3.4.2 Effect of Ionic Strength on Hydrodynamic Diameter and Zeta (ζ) Potential

The increase in the intensity weighted (z-average) hydrodynamic diameter of ZnO NPs in response to the increase in the electrolyte concentration indicates significant aggregation, especially at 100 mM NaCl (Fig. 3.2A). In contrast to the coated particles, bare ZnO NPs started aggregating at the lowest NaCl concentration of 1mMwhere the mean intensity weighted hydrodynamic diameters doubled (from 408 ± 83 nm to 816 ± 72 nm (mean ± one standard deviation)). DEX-ZnO NPs were aggregated to some

degree even at 0 mM NaCl, at 809 ± 189 nm (mean ± one standard deviation). Negatively charged ZnO NPs (core-shell and DEX(SO_4)) were more resistant to aggregation. At 1 and 10 mM NaCl, negatively charged particle diameters was around 40% and 25% lower than that found with the bare and neutral particles (ZnO and DEX). At the highest electrolyte concentration of 100 mM, all NPs exhibited an increase in aggregation (Fig. 3.2A).

The ζ potential values for core-shell and DEX(SO_4)- ZnO NPs remained negative as electrolyte concentration increased (Fig. 3.2B). Whereas for DEX and bare ZnO NPs, ζ potential remained mostly positive (except for DEX at 100 mM NaCl) and significantly decreased especially when the electrolyte concentration increased to 10 mM. ζ potential values were 0.28 and 0.50 lower for the bare and DEX-ZnO NPs, respectively, in comparison to ζ potential values in DI water. Likewise, at 100 mM NaCl, bare and DEX ZnO NPs had ζ potential values were respectively 4 and 20 times lower than those measured in DI water. For core-shell and DEX(SO_4)-ZnO NPs, increasing the concentration to 1 and 10 mM produced similar ζ potential values which were 1.32 times lower than ζ potential in the absence of the electrolyte. On the other hand, increasing the concentration to 100 mM increased the ζ potential of the core-shell NPs which was not significantly different from the DI treatment (Fig. 3.2B). Zinc oxide NPs coated with dextran sulfate DEX(SO_4) followed a similar pattern, albeit more pronounced changes in ζ potential values can be observed. Compared to DI water treatment, ζ potential values for DEX(SO_4)-ZnO NPs were 1.6, 1.9, and 1.3 lower at 1, 10, and 100 mM NaCl, respectively (Fig. 3.2B). The pH of all treatments ranged from 7 to 8, so the pH effect on ζ potential was minimal.

3.4.3 Zeta (ζ) Potential of Particles in Simulated Soil Solutions

Increasing DOC concentration in DI tended to lower ζ potential values for all ZnO NPs (Fig. 3.3A). At 0 mg C/L, only DEX-ZnO NPs exhibited a positive ζ potential (24.1 ± 3.3 mV) (mean ± one standard deviation), while other ZnO NPs had negative ζ potentials and were not significantly different from one another. When DOC concentration increased to 25 mg C/L, bare ZnO NPs had significantly lower ζ potential of -50.7 ± 1.34 mV, when compared to the other ZnO NPs, which were not significantly different from one another. The ζ potential for DEX-ZnO NPs at 100 mg C/L was significantly higher ($p = 0.016$) than the rest of treatments (-45.5 ± 0.78 mV).

Likewise, in MHRW, increasing DOC concentration lowered zeta potential of ZnO NPs (Fig. 3.3B). There were no significant differences among the NPs at 0 or 25 mg C/L. However, at 100 mg C/L, DEX-ZnO NPs had the highest ζ potential (-37.2 ± 0.7, mV) of all treatments ($p < 0.05$), followed by DEX(SO_4), bare ZnO, and core-shell NPs with ζ potentials of (-41.9 ± 0.7, mV), (-44.8 ± 1.1, mV), and (-45.7 ± 1.7, mV), respectively (mean ± one standard deviation). All ZnO NPs were negatively charged in PFSS, regardless of soil pH (Fig 3.3C). In PFSS at pH6, DEX and DEX(SO_4) ZnO NPs were significantly ($p = 0.005$) different from one another (-13.99 ± 0.15) vs (-16.67 ± 1.20) mV. Bare ZnO and core shell NPs had similar ζ potential and were not significantly different from DEX or DEX(SO_4) ZnO NPs. At pH8, DEX and bare ZnO NPs had significantly ($p = 0.014$) different ζ potential values of (-12.20 ± 0.26) and (-13.90 ± 0.72) mV, respectively. Core-shell and DEX(SO_4)-ZnO NPs were not significantly different from each other or from the other two NPs. The increase in carbon

concentration in MHRW and DI was accompanied by an increase in the pH values of all ZnO NP suspensions (Table S 3.1).

3.4.4 Dissolution in Simulated Soil Solutions

Particle free soil solution pH had a tremendous effect on the dissolution of ZnO NPs in the buffered, extracted soil water (Fig. 3.4A). Dissolution at pH 6 was higher than at pH8 for all ZnO NP treatments. The nominal total Zn concentration in each treatment was 25 mg/L. At pH 6, dissolution of core-shell NPs was the lowest with a dissolved Zn concentration of 15.2 ± 0.2 mg Zn/L. Bare ZnO dissolution (18.6 ± 0.8 mg Zn/L) was not significantly different from either core-shell NPs or DEX-ZnO NPs (21.9 ± 1.8 mg Zn/L). Dissolution of DEX(SO_4)-ZnO NPs was the highest (26.2 ± 3.1 mg Zn/L), which was not significantly different from DEX-ZnO NP. The same trend carried on at pH 8. Core-shell NPs had the lowest dissolution (2.9 ± 0.1 mg Zn/L), followed by bare ZnO, DEX and DEX(SO_4)-ZnO NPs with dissolution of (3.6 ± 0.3 mg Zn/L), (4.0 ± 0.1 mg Zn/L), and (4.4 ± 0.2 mg Zn/L), respectively (Fig. 3.4A).

The DOC concentration also had a big effect on ZnO NP dissolution. Dissolution of DEX(SO_4)-ZnO NPs in DI water (5.6 ± 0.1 mg Zn/L) was about twice as great as that for bare ZnO NPs (2.9 ± 0.2 mg Zn/L) (Fig. 3.4B). The dissolution of DEX(SO_4)-ZnO, DEX-ZnO, and core-shell NPs was the same. Introducing DOC to the ZnO NP suspensions in DI water generally increased the dissolution of the NPs. At 25 mg/L DOC (1:1 C to Zn mass ratio), DEX(SO_4)-ZnO NPs had the highest dissolution of all ZnO NPs (9.0 ± 0.4 mg Zn/L). The other ZnO NPs had lower dissolution of around 7.2 mg Zn/L. Increasing C to Zn ratio 4 times almost doubled the dissolution, from around 6-7 mg

Zn/L at 25 mg/L DOC to about 16 mg Zn/L. However, no significant differences were found between ZnO NP treatments (Fig. 3.4B).

In MHRW at 0 mg C/L, core-shell and bare ZnO NPs tended to have lower dissolution, 2.0 and 2.2 mg Zn/L, whereas DEX and DEX(SO_4)-ZnO NPs both had a dissolution of 2.4 mg Zn/L (Fig. 3.4C). Like DI water, increasing DOC concentration in MHRW significantly increased dissolution; at 25 and 100 mg C/L, dissolution of ZnO NPs was two and five to six times greater than dissolution in the absence of DOC. When DOC concentration was 25 mg C/L, bare ZnO NPs had the lowest dissolution of all ZnO NPs (3.7 ± 0.1 mg Zn/L; $p < 0.05$). Core-shell NP dissolution (4.3 ± 0.5 mg Zn/L) was significantly lower than that of DEX(SO_4)-ZnO (5.0 ± 0.1 mg Zn/L). The latter was not significantly different from DEX-ZnO NP dissolution (4.7 ± 0.1 mg Zn/L). Dissolution was similar (10.3-12.5 mg C/L) among all ZnO NPs at a DOC concentration of 100 mg C/L (Fig. 3.4C). All NP suspensions experienced a carbon concentration dependent increase in pH (Table S 3.1)

3.4.5 Natural Organic Matter Sorption

The Freundlich model was fitted to the sorption isotherm of dissolved organic matter to ZnO NPs (Fig. 3.5). All r^2 values suggested that Freundlich isotherm model fitted the data well (Table S 3.2). The Freundlich constant (k_f) value was similar for most ZnO NPs (0.052-0.054; Table S 3.2) indicating that similar amounts of PPHA were sorbed at low concentrations. The exception was the core-shell NPs (k_f = 0.041), which sorbed less at low concentrations. The treatments differed in 1/n values (Table S 3.2), which indicated a difference in the decline in binding as the PPHA concentration increased. Zinc oxide NPs coated with dextran had the highest 1/n value as compared to the rest of

the particles with 1/n = 0.577, giving it a more linear sorption isotherm and greater sorption of PPHA at higher concentrations.

On the other hand, DEX-(SO_4)-ZnO had the lowest value (1/n = 0.345) among all treatments, and core-shell and bare ZnO NPs had intermediate 1/n values of 0.515 and 0.433, respectively (Table S 3.2, Fig. 3.5). The net result was higher sorption of PPHA for DEX-ZnO and bare ZnO NPs as compared to the other treatments at higher PPHA concentrations (> 4 mg/L).

3.4.6 Soil and Soil Solution Characterization

Major physiochemical properties of Sadler silt loam are listed (Table 3.1). Acid leachable, exchangeable, and Mehlich III extractable metals can be found in Table S 3.3. Major cations and anions, DOC, and ionic strength (IS) for the extracted soil solution for Zn unamended Sadler soil at pH 6 and pH 8 are also listed (Table S 3.4).

3.4.7 Total Zn Concentration in Soil

Acid leachable Zn recovery of total Zn from the SRM (NIST 2710a, Montana Soil I) was 92.7 ± 2.3% (n=4). The recovery of soil total Zn after saturated paste extraction, as compared to the nominal spiking concentration for $ZnSO_4$, bare, core-shell, DEX- and DEX(SO_4)-ZnO NPs at pH 6 was: 93.1 ± 1.0 %, 92.1 ± 8.5, 94.5 ± 3.0, 87.6 ± 9.8, and 107.2 ± 9.4 %, respectively. Whereas at pH 8, recovered soil Zn was 84.3 ± 4.4, 91.9 ± 7.3, 91.5 ± 13.5, 100.3 ± 11.1, and 85.9 ± 6.9 % for $ZnSO_4$, bare, core-shell, DEX- and DEX(SO_4)-ZnO NPs, respectively. Data presented as (mean ± one standard deviation).

3.4.8 Total and Dissolved Zn Concentration in Soil and Saturated Paste Extracts

For total Zn in soil pore water (Fig. 3.6A), main effects (pH and treatment) were statistically significant (p <0.001, and 0.004, respectively). The treatment by pH

interaction was not significant. We performed multiple comparisons between different Zn treatments within each pH level independently. In contrast to $ZnSO_4$, all ZnO NP treatments had significantly increased Zn concentration in the soil solution as compared to the nonamended soil, at both pH 6 and pH 8. When compared to $ZnSO_4$ treated soils at pH 6 (165.1 ± 71.5 µg Zn/L), total Zn concentrations were increased significantly by factors of 3, 2.6, and 2.4 when soils were spiked with core-shell (486.1 ± 95.1 µg Zn/L), DEX-ZnO (422.2 ± 191.4 µg Zn/L), and bare (401.6 ± 161.2 µg Zn/L) NPs, respectively. Dextran sulfate coated ZnO NP (376.1 ± 157.4 µg Zn/L) treatments were not significantly different from $ZnSO_4$ treated soil at pH 6. At pH 8, core-shell (583.9 ± 199 µg Zn/L) and DEX-ZnO (576.9 ± 218.3 µg Zn/L) NP treated soils had twice as much total Zn concentration as $ZnSO_4$ at pH 8 (277.3 ± 125.4 µg Zn/L) ($p = 0.05$). Total Zn concentrations for DEX(SO_4) (471.9 ± 37.7µZn/L) and bare (478.6 ± 149.6 µg Zn/L) ZnO NP treatments were higher but not significantly different from $ZnSO_4$ at pH 8. None of the nanoparticle ZnO treatments were significantly different from one another in terms of total Zn in soil solution at either pH value (Fig. 3.6A).

We also looked at the effects of the ζ potential of ZnO NPs in PFSS on the total Zn concentration in the saturated paste extracts at both pH 6 and 8 (Fig. S 3.3). We found that, regardless of soil pH, linear regression between particle ζ potential in PFSS, and total Zn concentration in soil solution was not statistically significant at $\alpha=0.05$.

For dissolved Zn (Fig. 3.6B), the interaction between Zn treatment and pH was statistically significant ($p<0.001$). The dissolved Zn concentration for soil spiked with $ZnSO_4$ at pH 6 was 21 times (108.3 ± 67.3 µg Zn/L) higher than that of the nonamended soil (5.2 ± 3.0 µg Zn/L) ($p < 0.001$). Also, $ZnSO_4$ treated soil at pH 6 had significantly

higher (7-9 times) dissolved Zn in soil solution as compared to the rest of Zn treatments at pH 6 and about 5.5 times higher than all Zn treatments at pH 8. For pH 8 soil, except for the bare ZnO NPs, all Zn treatments (nano and ionic) were not significantly different from one another. The bare ZnO NP treatment had significantly higher dissolved Zn than the core-shell treatment. Dissolved Zn concentration in soil solution was 40 % higher for ZnO NPs at pH 8 (22.1± 5.6 µg Zn/L) compared to pH 6 (15.8 ± 5.9 µg Zn/L). At pH 8, DEX-ZnO (21.0 ± 4.7 µg Zn/L) and bare ZnO NPs treatments had significantly (3 times) higher dissolved Zn in soil solution, as compared to the control (7.3 ± 4.4 µg Zn/L).

3.5 Discussion

In the present study, we aimed to evaluate the effect of surface coatings on the behavior of ZnO NPs in soil at two different pH levels (moderately acidic and alkaline). Our hypothesis stated that, in comparison to positively charged and neutral particles (bare and DEX-ZnO NPs), negatively charged ZnO NPs (core shell-and DEX(SO_4)-ZnO NPs) would have significantly higher partitioning to the soil solution, resulting in an increase in the total Zn concentration in a saturated paste extract. This would be due to the electrostatic repulsion between the negatively charged natural colloids in the soil solution and the negative charge on these NPs, especially at higher soil pH.

The behavior of ZnO NPs in simple aqueous media was mainly dictated by the surface chemistry of the NPs. Negatively charged ZnO NPs (core shell and DEX(SO_4)-ZnO NPs) were more stable against aggregation compared to the neutral DEX-ZnO and the positively charged bare ZnO NPs, especially at the highest concentration of the electrolyte (100 mM), which is comparable to the ionic strength reported in the saturated paste extracts of the Sadler silt loam.

Introducing NOM into the media had a profound effect on the behavior of ZnO NPs and tended to negate the effects of the surface coatings applied during NP synthesis. Although at pH 6 and 8, bare and DEX-ZnO NPs should be positively charged given that their PZC is around 9, all NPs became negatively charged in DI, MHRW, and PFSS, likely due to coating replacement or overcoating with NOM. This behavior is consistent with reports for other kinds of NPs, including bare ZnO NPs[74, 88], gum arabic (GA) coated Ag NPs[85], and bare CuO NPs[169].

In the absence of NOM in DI water and PFSS, core-shell and ZnO NPs exhibited lower dissolution than DEX and DEX(SO_4)-ZnO NPs. This could be due to the low solubility of the $Zn_3(PO_4)_2$ ($Ksp = 9 \times 10^{-33}$) present in the shell structure. There were differences among ZnO NPs in their dissolution at the lower carbon concentration (25 mg C/L). However, at 100 mg C/L, which is equivalent to a 1: 4 Zn to DOC mass ratio, all the differences among the ZnO NPs diminished and they all produced similar dissolved Zn concentrations.

The reported concentration dependent increase in pH values of the suspensions as dissolved organic carbon concentration increased is a result of the enhanced dissolution of all ZnO NPs regardless of their as-synthesized coatings. Dissolution of ZnO NPs is well known to raise the pH of the solution due to the consumption of hydrogen ions during the reaction [67, 170].

The sorption isotherm experiments clearly showed that the neutral coating-dextran had the highest binding to NOM at higher NOM concentrations, perhaps due to hydrogen bonding, whereas the negatively charged NPs (DEX(SO_4)- ZnO and core-shell) both had

lower binding to the NOM, likely due to the electrostatic repulsion between the coatings and the negatively charged functional groups on the NOM, such as carboxylates.

Most of spiked Zn remained within the soil solid phase (~90%), indicating high retention of Zn to the soil regardless of the Zn form. A relatively small fraction of Zn was partitioned to soil solution as determined by saturated paste extraction. Likewise, retention of >80 % of PVP-Ag NPs [171], multi-walled carbon nanotubes (MWCNT)[172], and CIT-ZnO NPs[173] has been reported.

Total Zn concentration data in the saturated paste extracts showed no differences among NP treatments, even at the higher pH values. This relates to the observation made in aqueous solutions that sorption of NOM conferred a net negative charge to all the NPs, regardless of initial surface chemistry. Our hypothesis still holds true in the sense that negatively charged NPs partitioned more Zn to the soil solution at higher soil pH than at lower soil pH. However, the initial charge of the particles was not as important. Our results are in agreement with Whitley et al, 2013, who found that the prolonged aging of electrostatically stabilized CIT-Ag NPs versus sterically stabilized PVP-Ag NPs yielded the same total Ag concentration in sandy loam soil solution, despite the initial higher partitioning of total Ag from CIT-Ag NPs[84]. This was likely due to replacement or over coating of the pristine coatings with NOM, although the exchange or overcoating was faster for CIT coating due to its lower molecular weight as compared to the PVP used in this study[84].

The dissolution pattern in saturated paste extracts was different from that in PFSS. There were no differences in dissolution of ZnO NPs at the two pH levels, 6 and 8. One possible explanation for this discrepancy could be the combined effect of NOM and

divalent cations such as Ca^{2+}, which could have facilitated the bridging and subsequent heteroaggregation with clay colloids[174], which may have lowered the surface area and thus the dissolution[67]. It is also possible that the soil simply acted as a buffer, removing dissolved Zn ions from solution as they were generated.

Although several studies have been performed to test the effect of soil properties on the concentration of ZnO NPs, versus Zn ions, in soil solution[91-94], the methods applied for the extraction of NPs from the soil removed a large proportion of Zn in the nanoparticle form that would have formed heteroaggregates[149, 175]. Read et al.[147] found differences in soil Zn concentration at soil pH 5.9 and 7.2 only when the spiking concentration exceeded 500 mg Zn/kg soil, whereas soil Zn concentration was not significantly different at the lower concentrations such as the ones we used in the present study.

Overall, compared to $ZnSO_4$, DEX and core-shell ZnO NPs were able to achieve significantly higher total soil Zn concentrations, especially at pH 8, but not higher dissolved Zn concentrations. This result suggests that nanoscale fertilizers could be more effective in providing plants with Zn, especially under conditions where conventional fertilizers are of limited efficacy. This suggestion relies on the assumption that Zn from ZnO NPs in suspension is bioavailable to plants. Based on our previous research, we believe that this is likely the case[154, 163]. The efficacy of such amendments could be greatly improved by selecting coatings with a high affinity for soil organic matter and could eventually prove to be a successful means of providing the Zn required for plant growth.

Tables

Table 3.1 Major physiochemical properties of Sadler soil at unadjusted pH (native pH) and the two adjusted pH levels; 6 and 8.

Soil	pH		Particle size distribution			Texture class	OM %	Total N %	CEC cmol/kg
	DDI	1M KCl	Sand %	Silt %	Clay %				
Native pH	5.54	3.93	9	70	21	Silt loam	1.29	0.13	9.5
pH6	6.19	5.33	NM	NM	NM	NM	NM	NM	NM
pH8	7.4	6.66	NM	NM	NM	NM	NM	NM	NM

NM: Not measured, DDI: Distilled deionized water, OM: Organic matter, CEC: Cation exchange capacity.

Figures

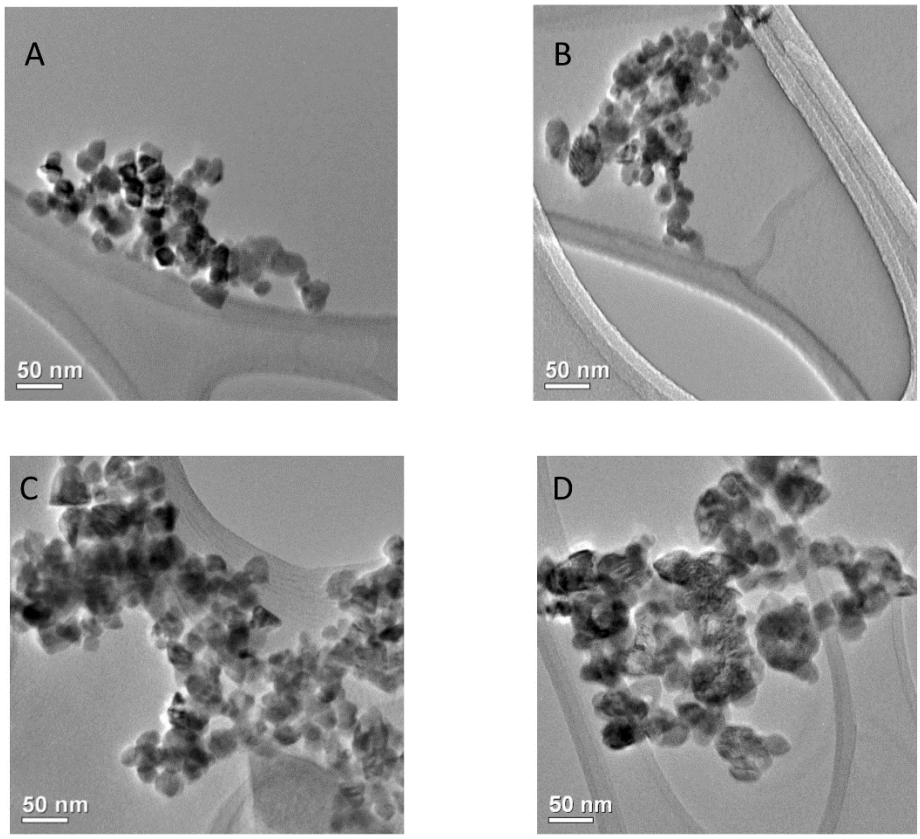


Figure 3.1 TEM images of bare ZnO NPs (A), dextran coated (DEX -ZnO NPs) (B), dextran sulfate coated (DEX (SO_4)-ZnO NPs) (C), ZnO-$Zn_3(PO_4)_2$ core-shell NPs (D). Scale bar is 50 nm.

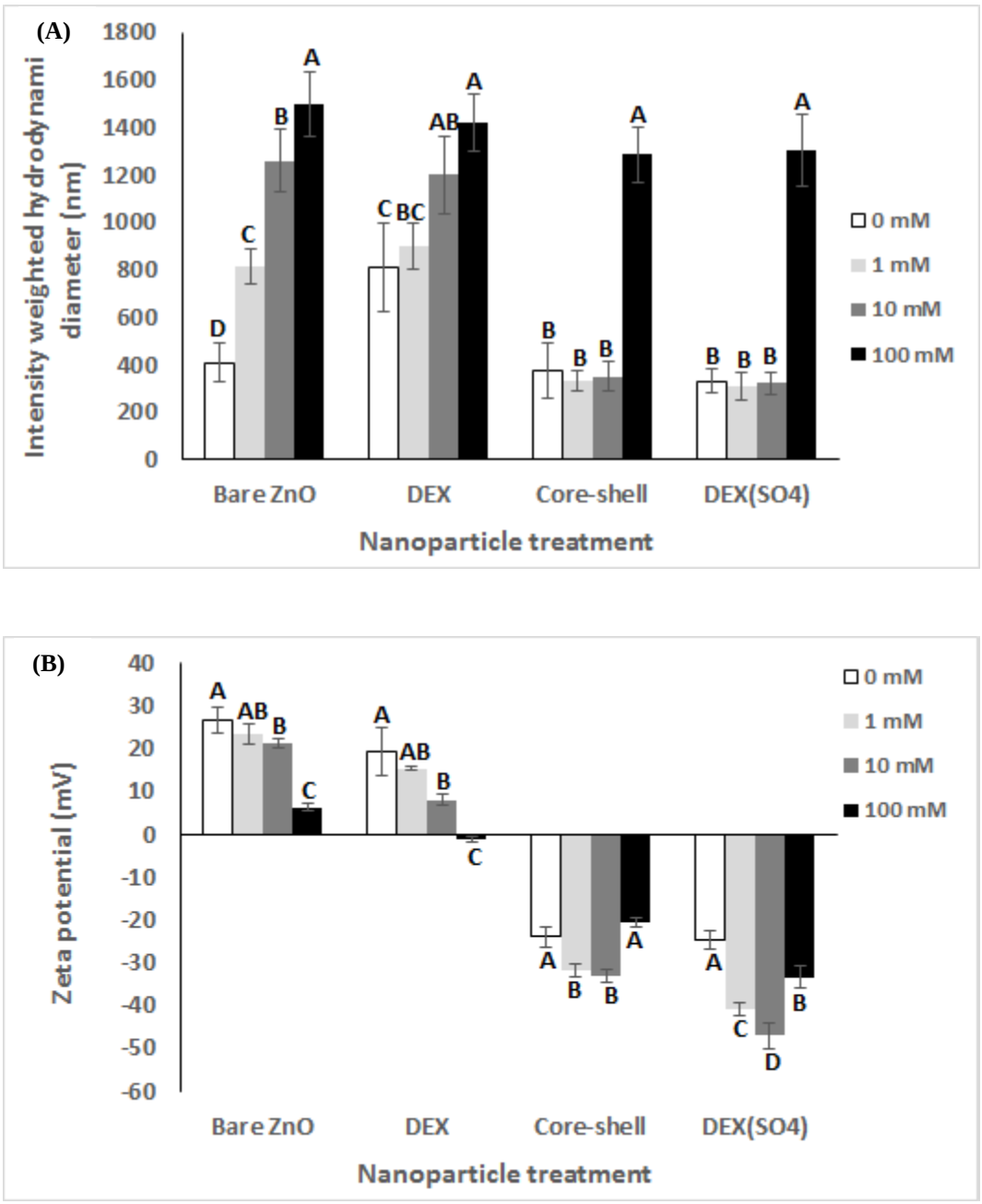
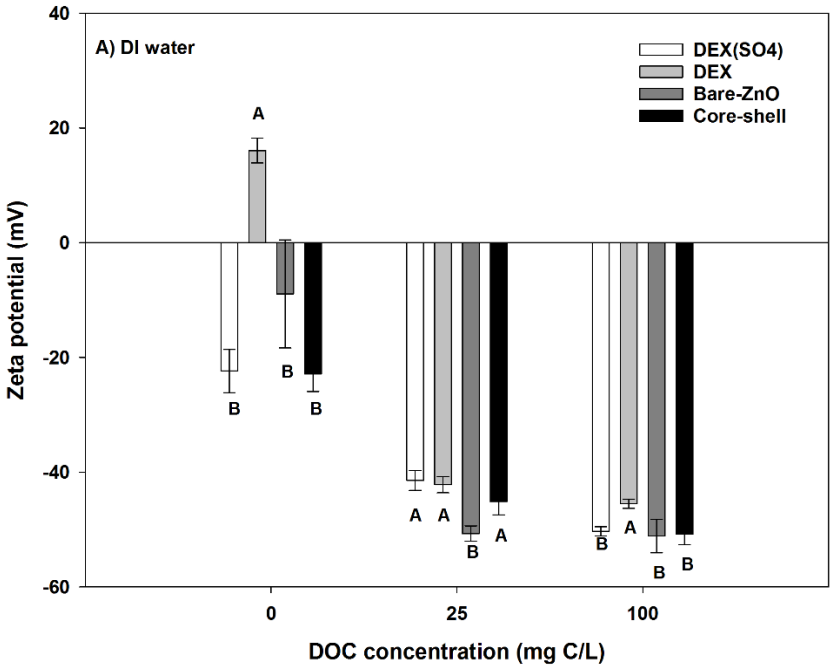
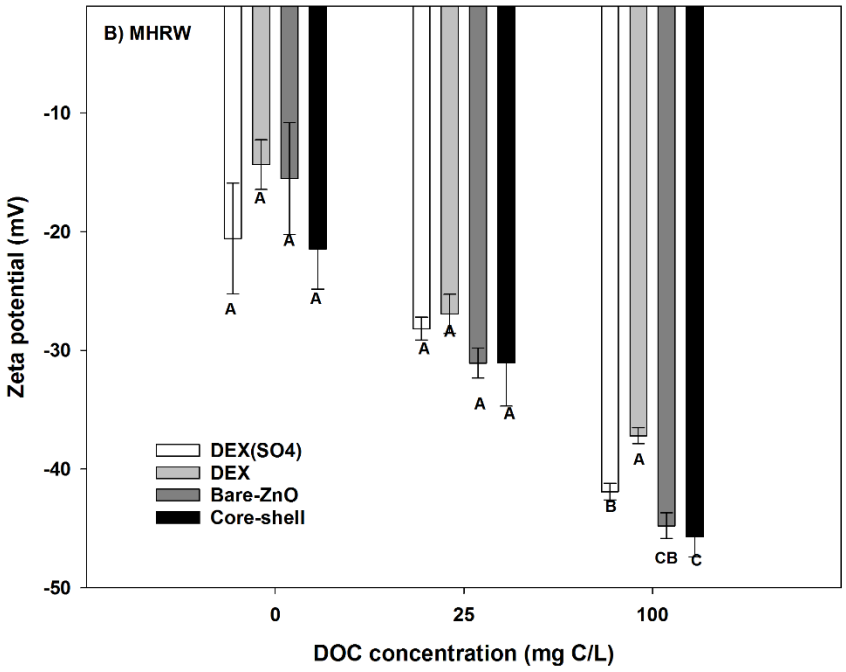


Figure 3.2 Effect of NaCl concentration on the hydrodynamic size (A) and zeta (ζ) potential (B) of ZnO NPs. Treatments connected by different letters within the same ZnO NP treatment are significantly different at α=0.05.


A) DI water
DEX(SO4)
DEX
Bare-ZnO
Core-shell
Zeta potential (mV)
40
20
0
-20
-40
-60
A
B
B
B
A
A
B
A
B
A
B
B
0
25
100
DOC concentration (mg C/L)


B) MHRW
Zeta potential (mV)
-10
-20
-30
-40
-50
A
A
A
A
A
A
A
A
B
A
CB
C
DEX(SO4)
DEX
Bare-ZnO
Core-shell
0
25
100
DOC concentration (mg C/L)

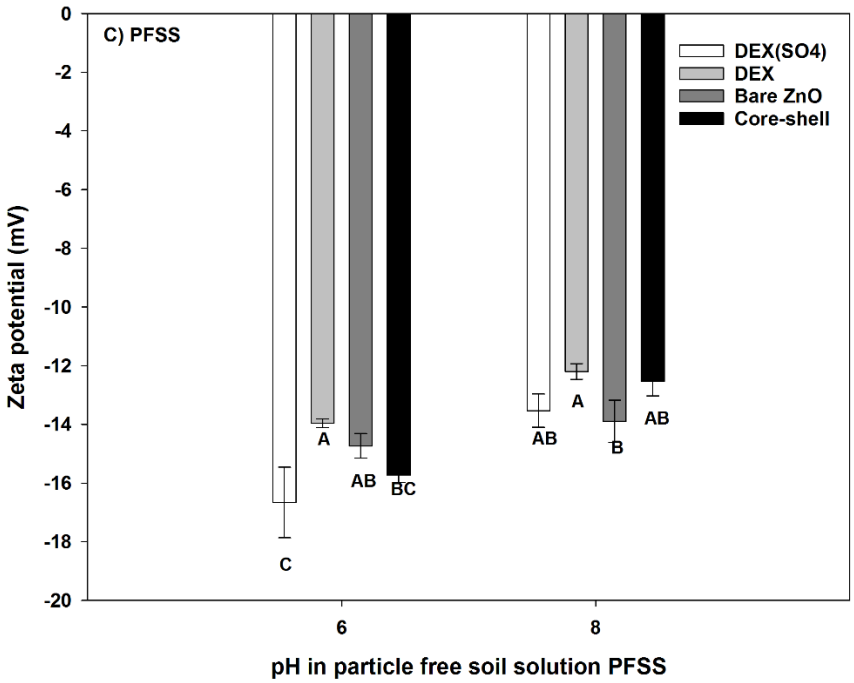
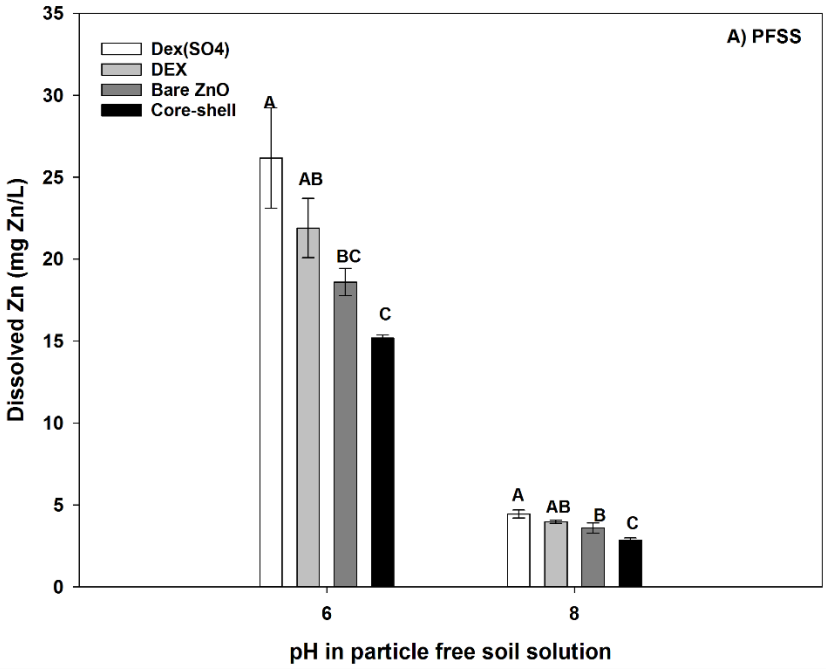
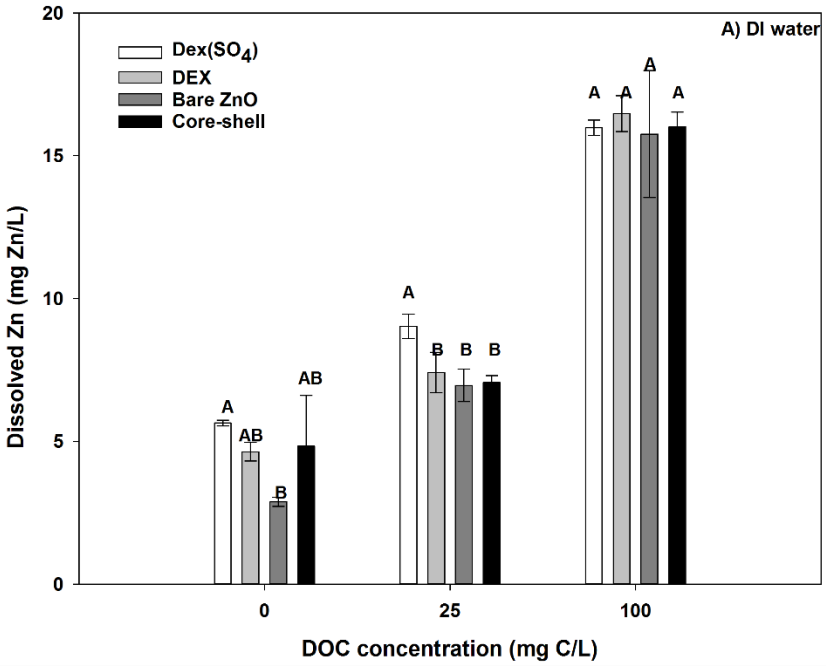


Figure 3.3 Zeta (ζ) potential of 25mg/L Zn- ZnO NPs suspended in Pahokee peat humic acid -PPHA at 0, 25, and 100 mg C/L in deionized (DI) water (A), moderately hard reconstituted water (MHRW) (B), and in particle free soil solution (PFSS) (C). Treatments connected by different letters at each PPHA or pH level are significantly different at α=0.05


A) PFSS
Dex(SO4)
DEX
Bare ZnO
Core-shell
Dissolved Zn (mg Zn/L)
35
30
25
20
15
10
5
0
A
AB
BC
C
A
AB
B
C
6
8
pH in particle free soil solution


A) DI water
Dex(SO4)
DEX
Bare ZnO
Core-shell
Dissolved Zn (mg Zn/L)
20
15
10
5
0
A
AB
B
AB
A
B
B
B
A
A
A
A
0
25
100
DOC concentration (mg C/L)

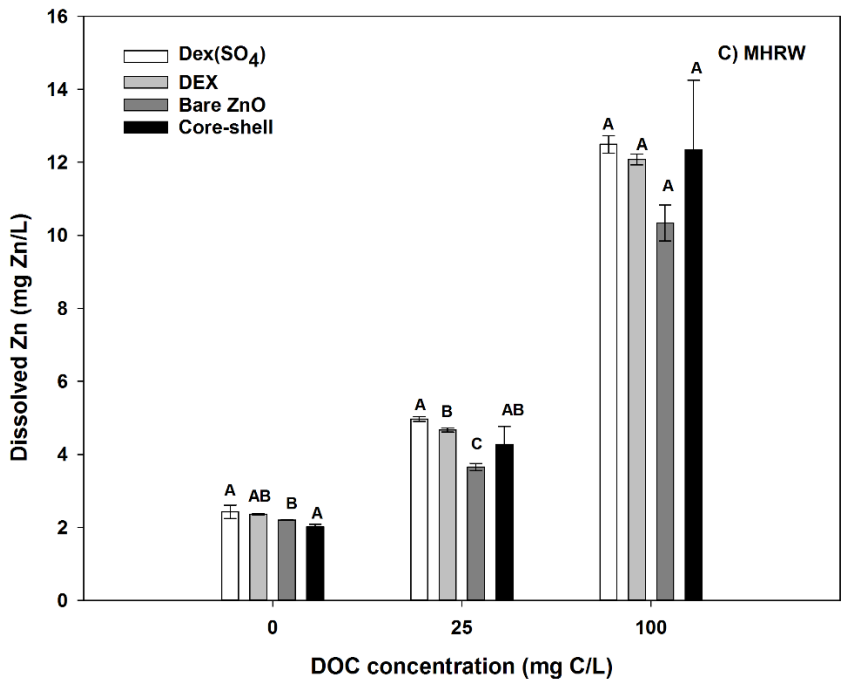


Figure 3.4 Dissolution of 25mg/L Zn- ZnO NPs in particle free soil solution (PFSS) at pH 6 and 8 (A), in Pahokee peat humic acid (PPHA) solutions at 0, 25, and 100 mg C/L in deionized (DI) water (B), and in moderately hard reconstituted (MHRW) (C). Treatments connected by different letters at each PPHA or pH level are significantly different at α=0.05.

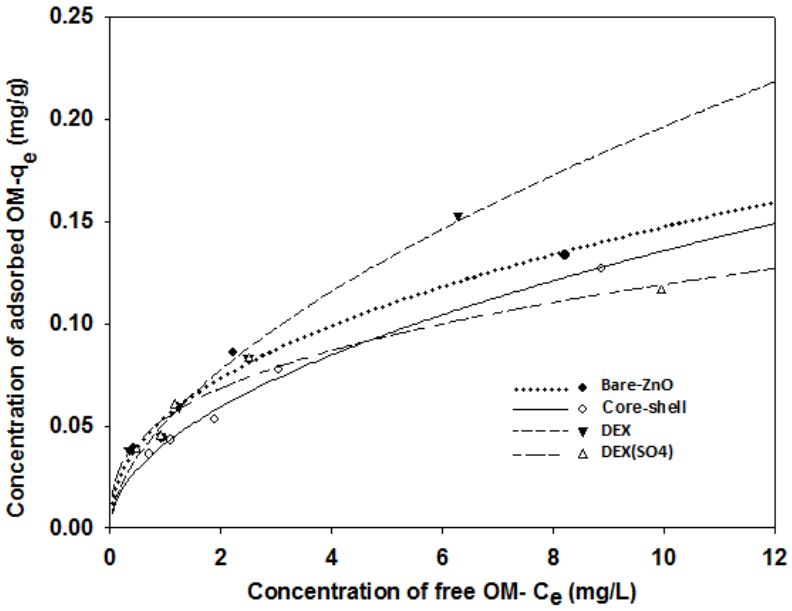


Figure 3.5 Freundlich sorption isotherm model fitted to dissolved organic carbon in sorption batch experiments.

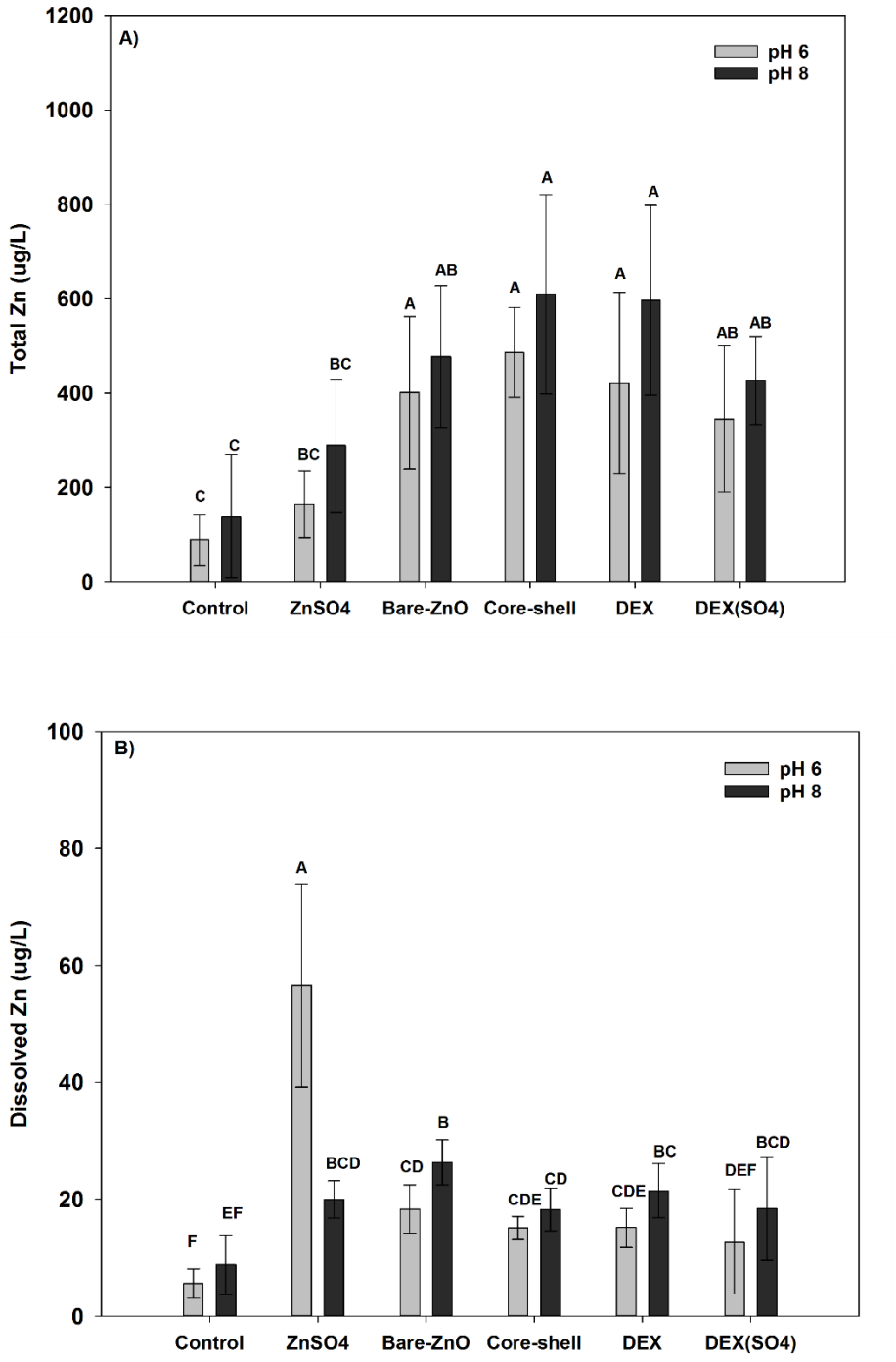


Figure 3.6 Total (A) and dissolved (B) Zn concentration in saturated paste extracts. In panel (A), treatments within the same pH level with different letters are significantly different at α=0.05. In panel (B) treatments at both pH levels with different letters are significantly different at α=0.05. Error bars represent ± one standard deviation.

Supplementary material

Tables

Table S 3.1 pH of 25 mg Zn/L as ZnO NPs in particle free soil solution (PFSS), deionized (DI) water and moderately hard reconstituted water (MHRW) at 0, 25, and 100 mg C/L

Medium	PPHA (mg C/L)	Bare ZnO	Core-shell	DEX	DEX-(SO_4)
			pH		
DI	0	6.67 ± 0.01	7.38 ± 0.06	7.43 ± 0.15	7.33 ± 0.05
DI	25	8.43 ± 0.06	7.67 ± 0.21	8.30 ± 0.10	7.74 ± 0.06
DI	100	9.10 ± 0.06	8.52 ± 0.12	8.92 ± 0.14	8.89 ± 0.03
MHRW	0	6.65 ± 0.06	6.93 ± 0.06	6.93 ± 0.05	6.72 ± 0.15
MHRW	25	7.18 ± 0.06	7.18 ± 0.04	7.30 ± 0.01	7.07 ± 0.01
MHRW	100	8.08 ± 0.18	8.06 ± 0.12	8.01 ± 0.10	8.02 ± 0.13
PFSS pH6	-	6.23 ± 0.04	6.12 ± 0.12	6.48 ± 0.08	6.42 ± 0.07
PFSS pH8	-	8.00 ± 0.05	7.84 ± 0.06	7.97 ± 0.09	7.90 ± 0.12

Table S 3.2 Sorption isotherm parameters for ZnO NPs and dissolved organic carbon (DOC) sorption studies

ZnO NPs treatment	r^2	*P* at α=0.05	K ($mg^{(1-(1/n))}.g^{-1}.L^{(1/n)}$)	n	1/n
Bare-ZnO	0.971	0.0021	0.054	2.31	0.433
Core-shell	0.993	0.0003	0.041	1.94	0.515
DEX	0.981	0.0011	0.052	1.73	0.577
DEX(SO_4)	0.960	0.0035	0.054	2.90	0.345

Table S 3.3 Selected chemical properties of the Sadler surface soil

	Na	Ca	Mg	K	Al	Fe	Zn	P
				(mg/kg)				
Acid leachable	NA	776	2,223	763	18,	NA	32	287
Exchangeable	23	766	173	35	37	NM	NM	NM
Mehlich III	NA	841	136	47	907	147	0.3	2.5

NM: Not measured

Table S 3.4: Soil solution chemical properties at pH 6 and 8.

Soil pH	Cations (mg/L)						Anions (mg/L)			IS	DOC
	Na	K	Ca	Mg	Al	Fe	F	Cl	SO_4	(M)	(mg C/L)
6	403	508	659	817	4,842	3,870	3	11	27.8	0.9	125
8	350	430	907	1,894	4,364	3,543	7	20	36	1.2	237

Figures

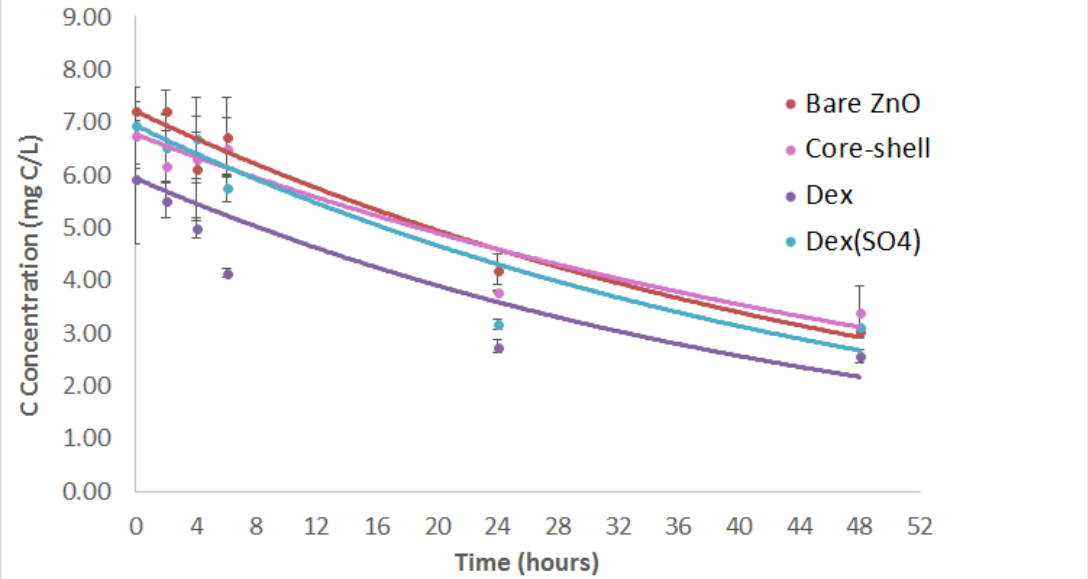


Figure S 3.1 Equilibrium time reached after 24 h incubation period in batch sorption isotherms experiments, each point is the average of three independent replicates. Error bars represent ± one standard deviation.

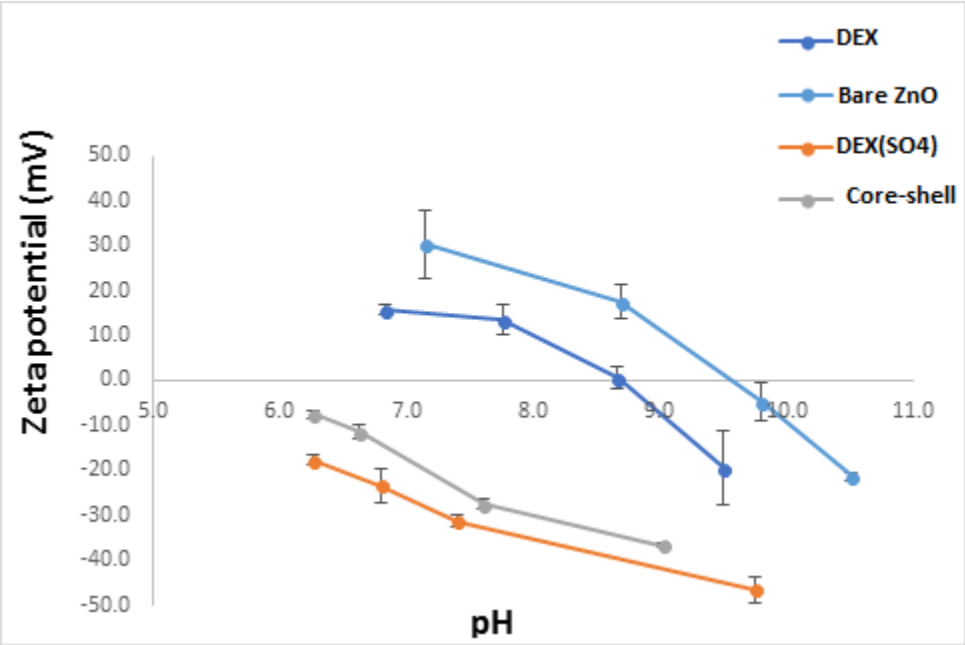
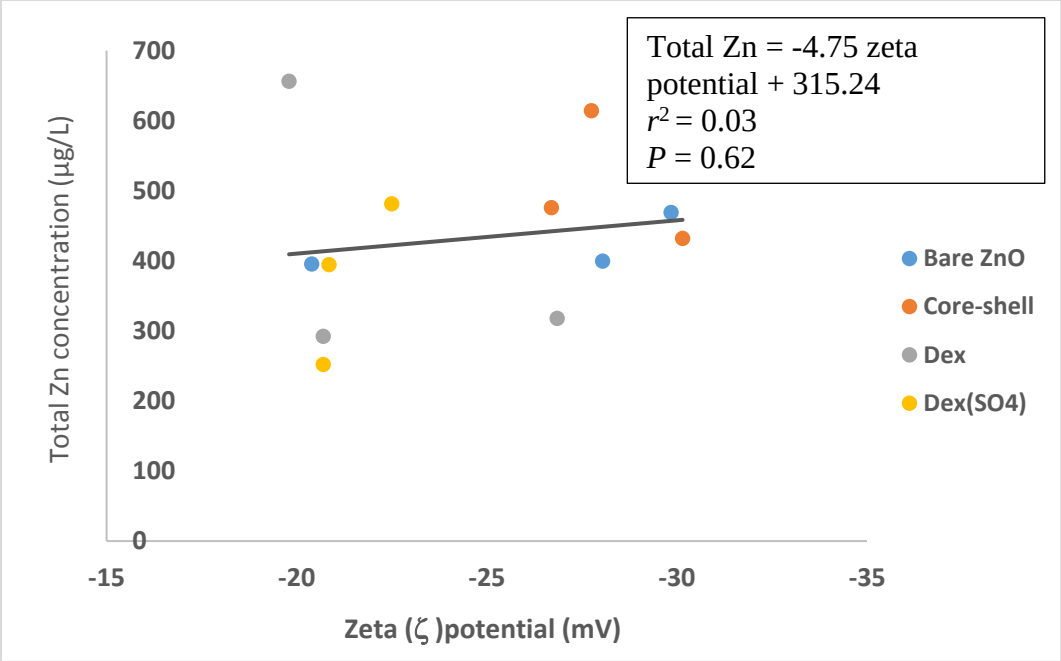


Figure S 3.2 Electrophoretic mobility of bare ZnO, dextran coated, (DEX-ZnO), ZnO-$Zn_3(PO_4)_2$ (core-shell), and dextran sulfate coated (DEX(SO_4)-ZnO NPs as a function of pH in deionized (DI) water. Zn concentration was 100 mg/L for all the tested suspensions.

A)



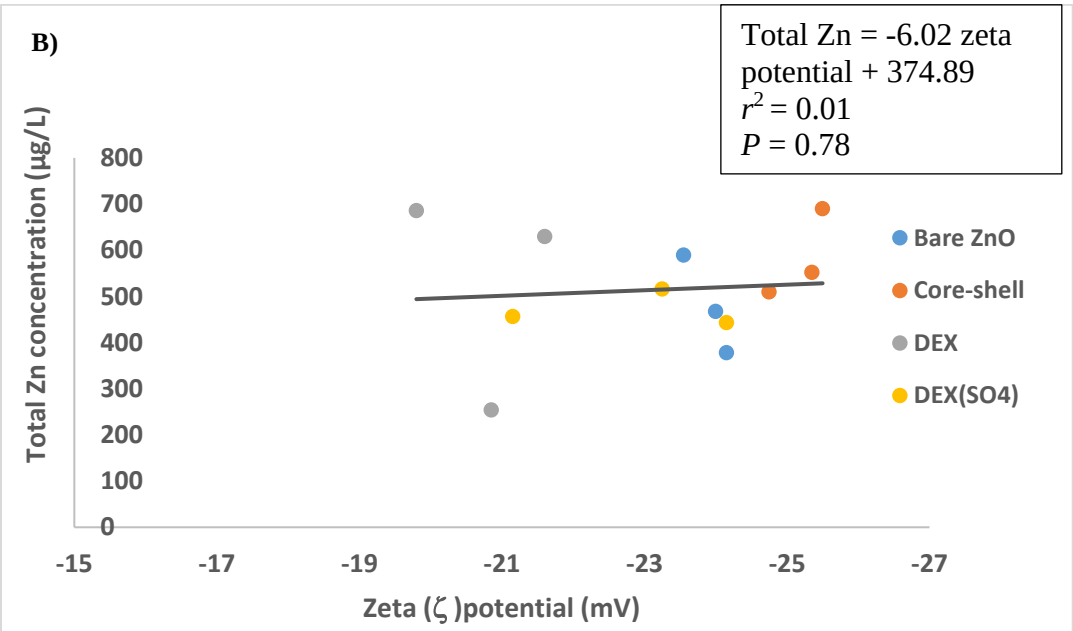


Figure S 3.3 Linear regression between zeta potential and total Zn in soil solution at pH 6 (A), and pH 8 (B). Inserts: regression equation, r^2, and significance of the model (p) at α=0.05

Acknowledgements:

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Chapter 4: Effects of Soil pH and Coatings on the Efficacy of Polymer coated ZnO Nanoparticulate fertilizers in Wheat (*Triticum aestivum*)"

Zeinah Elhaj Baddar and Jason M. Unrine

4.1 Abstract

Zinc deficiency in human is widespread. A diet heavily dependent on cereals often leads to Zn malnutrition. Zinc deficiency in plants could be detrimental to crop yield and nutritional value. Higher soil pH (>7) significantly lowers the bioavailability of Zn to crops. The objective of this study was to use ZnO nanoparticles (NPs) as seed treatments (experiment (A)) and as soil amendments (experiment (B)) to enhance Zn concentrations in wheat grain. In experiment (A), seeds were treated with dextran coated (DEX-ZnO) and bare ZnO NP suspensions, in addition to $ZnSO_4$, at 500 mg Zn /L. Seeds were then sown in Sadler silt loam soil until physiological maturity. In experiment B, soil pH was adjusted to 6 and 8, then soils were spiked with 15 mg Zn/kg soil in the form of DEX-ZnO and bare ZnO NPs, as well as $ZnSO_4$. The plants were grown in the spiked soil until physiological maturity. Zinc concentration and dry biomass of stems, leaves, and grain, as well as plant height, were all measured for the plants in both experiments. Results from experiment A showed that seeds treated with bare ZnO NPs resulted in significantly higher Zn concentration. In Experiment B, grain Zn concentration was the same across all Zn treatments regardless of soil pH. Soil pH had a significant effect on Zn accumulation and biomass of leaves and stems, where pH 6 resulted in higher accumulation and slightly stimulated growth as compared to pH 8. None of the Zn treatments were significantly different from one another in terms of tissue Zn concentrations, regardless the plant part. In both experiments, plant height differences were more pronounced

during the vegetative growth stage (vernalization period), but these differences tended to diminish as soon as the plants moved to the reproductive stage.

4.2 Introduction

Zinc deficiency has drastic effects on plant and human health. Crops suffering from Zn deficiency have lower yields and poor Zn nutritional value. Zinc deficiency in human is widespread, occurring in about a third of the global population [5]. The majority of people in developing countries have limited access to food sources, such as meat, which are rich in Zn [102]. Their diet is dependent mostly on cereals, which do not provide adequate Zn intake.

In some cases, Zn deficiency in crops is caused by low geogenic levels of Zn. However, soil physiochemical properties have an immense effect on Zn bioavailability. Total Zn concentrations in soil do not necessarily reflect Zn availability for plant uptake[21, 176]. Higher pH, and high contents of organic matter, clay, Al or Fe oxide-hydroxides, phosphate, and carbonate significantly decrease bioavailable Zn[102, 177, 178]. Soil pH is an important property that affects the biogeochemical cycling of macro and micronutrients. As soil pH increases, the quantity of negative charge on exchange sites of natural colloids (clay minerals, organic matter (OM), and Fe/Al oxides) increases, leading to the sorption of metals, including Zn ions[178]. Also, higher soil pH results in the precipitation of low solubility forms of Zn such as Zn carbonates and hydroxides[179].

Previous studies have shown that using $ZnSO_4$ as a fertilizer is less efficient in calcareous/alkaline soils. Peirzenski et al found that liming soil lowered soybean (*Glycine max*) tissue Zn concentration by half [180]. Likewise, Zn concentration in Swiss

chard tissue at soil pH of 5.3 was 313 µg Zn/g while after liming the soil to pH 7.4, Zn concentration dropped to 60 µg/g [181].

Nanotechnology has recently been applied to the delivery of pesticides and nutrients and there has been an interest in using ZnO nanoparticles, rather than $ZnSO_4$, as Zn fertilizers [46,47, 120]. Nanomaterials (materials having at least one dimension less than 100 nm[42]) have unique properties compared to their bulk counterparts and have become more widely incorporated in numerous commercialized products. Zinc oxide nanoparticles (NPs) are classed among the metal oxide nanomaterials, and are commonly used in semiconductors[182]. They are also widely used in sunscreen due to their ability to block harmful UV radiation[183]. A few studies have investigated the use of ZnO NPs as fertilizers and have shown positive effects on yields and Zn nutritional values in crops [52, 54, 55, 119, 125]. The majority of such studies have focused on amending soil with ZnO NPs as the method of application[52, 54, 55, 119, 120, 184]. Foliar application is also widely used and sometimes proved a more efficient means of delivering Zn to plant edible tissues, although it is likely that soluble Zn salts are more effective than ZnO NPs when applied to leaves[185]. Previous work showed that Zn nutrition[141], resistance to stress[127] and crop yield[126, 127] could all be enhanced by soaking seeds in soluble Zn salt solutions. Likewise, coating seeds with ZnO NPs enhanced yield, Zn nutrition, and mitigated salinity stress in lupin (*Lupinus termis*) [122].

Surface coatings are often added to enhance the colloidal stability and compatibility of NPs for specific applications[128, 129]. Our previous work showed that dextran coated ZnO NPs (DEX-ZnO) and ZnO-$Zn_3(PO_4)_2$ core-shell NPs gave significantly higher total Zn concentrations in soil solution as compared to $ZnSO_4$, especially at high soil pH[83] where

the bioavailability of most metals/nutrients, including Zn, becomes limited[177]. Also, in seed germination studies we showed that bare ZnO , DEX-ZnO NPs, and core-shell NPs showed promising results in enhancing tissue Zn concentration and growth in wheat seedlings[132, 154]. Bare ZnO and DEX-ZnO NPs also tended to produce higher total Zn concentrations in soil. This was likely due to their affinity for dissolved organic matter (DOM) in soil solution, which confers a net negative charge to the NPs, causing them to be repelled by negatively charged soil surfaces.

In this study, we investigated the potential use of bare and dextran coated ZnO NPs as nanofertilizers to enhance wheat (*Triticum aestivum*) yield and tissue Zn concentration in comparison to $ZnSO_4$ (commonly used fertilizer Zn form). To this end, we performed two separate experiments. The first experiment involved coating seeds with ZnO NPs prior to growing them in a natural soil. In the second experiment, we examined the soil pH effect on Zn uptake and yield of wheat after spiking the soil with Zn treatments (ZnO NPs and $ZnSO_4$).

4.3 Materials and Methods

4.3.1 Synthesis and Characterization of ZnO Nanoparticles

The synthesis of bare and dextran coated ZnO NPs (DEX-ZnO) is mentioned elsewhere[154]. In brief, bare ZnO NPs were prepared following alkaline precipitation as mentioned in a previous work[133]. Dextran (9-11 kDa, from Sigma-Aldrich, St. Louis, MO, USA) was added during the synthesis in a 1:6 dextran to Zn mass ratio.

Primary particle size was determined using transmission electron microscopy (TEM). Dynamic light scattering (DLS) and phase analysis light scattering (PALS) were used to measure the hydrodynamic size and the electrophoretic mobility, respectively. The Zn

concentration in nanoparticle suspension was measured using inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7500cx Santa Clara, CA, USA).

4.3.2 Treating Seeds with ZnO Nanoparticles

A suspension of 500 mg Zn/L was prepared with bare and DEX-ZnO NPs. The suspensions were sonicated for 45 min at 100% amplitude using a cup horn sonicator (Qsonica, Newtown, Connecticut, USA). Twelve mL of 500 mg Zn/L ZnO NPs suspensions, or 500 mg Zn/L as $ZnSO_4$ solution to serve as an ionic Zn control, in addition to 18 MΩ deionized (DI) water used as a solvent control, were prepared in separate 50 mL centrifuge tubes. Seeds (soft red winter wheat, cv. Pembroke) were soaked in the treatment solutions overnight on an end over end sample rotator at 22°C.

4.3.3 Soil Preparation

Sadler silt loam (fine-silty, mixed, mesic Fragiudalf) soil was obtained from the University of Kentucky Research and Education center at Princeton, KY(USA). The soil was air dried, ground, and sieved (<2mm). Soil characterization is described elsewhere[83]. Briefly, soil pH in DI at a 1:1 mass ratio was 5.54, cation exchange capacity (CEC) was 9.5 $cmol_c/kg$, and organic matter content (OM) was 1.29%.

Phosphorous, nitrogen, and potassium were added to all soils according to fertilizer recommendation for growing wheat in Sadler soil[186] as follows, diammonium phosphates $(NH_4)_2HPO_4$ (102.3 mg/kg soil), KCl (55.4 mg/kg soil), and two additions of 64.3 mg/kg soil of ammonium nitrate (at the Feekes 3 and 5 wheat growth stages).

4.3.4 Experiment (A) Treated Seed Greenhouse Assay

Seeds coated with the ZnO NPs, $ZnSO_4$ at 500 mg Zn/L, and DI were sown in plug trays containing Sadler soil at its native pH. Water was added to reach the soil field

capacity. The trays were kept at 4°C for eight weeks to induce vernalization. The rest of the soil was kept under the same condition. Seedlings were then transplanted to 1 kg potted soil. Each treatment was comprised of nine pots, and each pot contained three seedlings from the same treatment. Plant height was measured once a week until heading (Feekes stage 10).

4.3.5 Experiment (B) Spiked Soil Greenhouse Assay

In the second greenhouse experiment, which involved seed exposure to ZnO NPs via the soil, soil pH was adjusted to 6 and 8 using $MgCO_3$ and MgO, respectively and left to equilibrate for 2 weeks. For an explanation of why $MgCO_3$ and MgO were used to adjust soil pH, see Elhaj Baddar, et al. [83]. Soil at both pH levels was spiked with DI water or with a Zn suspension /solution to provide 15 mg Zn/kg soil in the form of bare ZnO NPs, DEX-ZnO NPs, or $ZnSO_4$. We selected this concentration to be similar to the highest concentration at which soils would normally be amended with Zn (typical range in Zn application rates is 2-15 mg/kg)[102]. Seeds were left to imbibe water overnight on an end over end sample rotator in DI water at room temperature. The next day, seeds were sown in a portion of the spiked soil that was added to plug trays. The trays were left at 4°°C to vernalize. The rest of the soil was incubated at 4°C until transplant day. After 8 weeks of vernalization, three seedlings from each treatment were transplanted into pots containing 1 kg of soil. There were eleven pots per Zn treatment per soil pH. Plant height was measured every week until heading (Feekes 10).

4.3.6 Harvesting Plants and Acid Digestion

After maturity, aboveground plant tissue was harvested in three separate parts; grain, leaves, and stems. Harvested tissues were gently washed with DI water and oven dried at

100 °C. Dry biomass was measured, and acid digestion was performed as follows: plant tissues were digested at room temperature overnight in 10 mL concentrated nitric acid in 50 mL polypropylene centrifuge tubes. Then, open vessel digestion was performed for 20 minutes at 100 °C. Samples were cooled to room temperature, then 2.5 mL H_2O_2 was added to each tube and the heating step was repeated. Plant digestates were left to cool down to room temperature and brought up to 50 mL using DI water. Digestion blanks, standard reference material samples (SRM1515, apple leaves, National Institute of Standards and Technology, Gaithersburg, MD, USA), duplicates and spikes were included to check the quality of the analyses. Zinc recovery from the SRM, spike recovery, and relative percent difference between duplicates were 94.4 ± 1.2%, 98.7 ± 3.9 %, and 0.52 ± 4.3 %, respectively.

4.3.7 Statistical Analysis

Tissue Zn and biomass accumulation data were analyzed in a similar way. Data were first checked for ANOVA assumptions of normality and homoscedasticity using Shapiro-Wilk and Levine tests, respectively. Whenever ANOVA assumptions were met, ANOVA was performed, and the Tukey-HSD multiple comparison test were performed on each plant part, separately.

The Proc-GLM procedure was used to determine the main and interaction effects (pH and Zn treatment), and multiple comparison tests (Tukey-HSD) were performed across all treatments, regardless of pH, whenever the interaction term was significant.

For plant height, we performed repeated measures ANOVA using the Proc- GLM procedure with the Repeated option to take into account autocorrelation in the effect of treatment on the height measurements. Tukey-HSD multiple comparison tests were used

to compare the treatments for height differences at each time point. All statistical analyses were performed using SAS 9.4 (SAS institute, Cary, NC).

It is worth mentioning that we considered a single pot as the experimental unit. Therefore, we pooled tissue Zn concentrations, biomass, and height data from three plants in each pot and treated that as the average plant response in both experiments.

4.4 Results

4.4.1 Zinc Oxide Nanoparticle Characterization

A detailed characterization of the nanoparticles was reported previously [154]. Briefly, TEM analysis showed that bare and DEX-ZnO NPs had primary particle sizes of 24 ± 1 and 18 ± 1 nm, respectively. Bare ZnO NPs had an intensity weighted hydrodynamic diameter (z-avg) of 314.4 ± 32.8 nm in DI water whereas DEX-ZnO NPs had a z-avg of 755.2 ± 191.8 nm. Smoluchowski's approximation was used to convert electrophoretic mobility values to zeta potential. Bare and DEX-ZnO NPs had ζ potentials of 29.1± 0.6 mV and 19.5± 1.1 mV in DI water (pH 5.8), respectively. Powder XRD confirmed that the particles were zincite structured ZnO[154].

4.4.2 Experiment (A) Treated Seed Greenhouse Assay

4.4.2.1 Zinc Concentration

Zinc concentration in grain of bare ZnO NPs treatments was significantly (p = 0.01) higher than the control treatments by 33% (Fig. 4.1). Grain Zn concentration in DEX-ZnO NPs and $ZnSO_4$ treatments were neither statistically significant from each other nor from bare ZnO NPs or the control treatments. Leaf tissue Zn concentrations were similar for all treatments (30-35 μg Zn/g) (Fig. 4.1). All Zn treatments (ions and nano) caused

an average 41% higher ($p < 0.001$) Zn concentration in wheat stems, as compared to the control treatment (Fig. 4.1).

4.4.2.2 Biomass

Grain biomass in the Zn treatments (nano and ions) were not significantly different from one another or from the control (Fig. 4.2). Regardless of Zn form (nano or ionic), plants from seeds treated with Zn tended to have, on average, 20% lower leaf biomass as compared to the control ($p < 0.001$) (Fig. 4.2). Zinc treatments had no significant effect on stem biomass.

4.4.2.3 Plant Height

During the vegetative growth period, plants in the control treatment were taller than the rest of the Zn treatments during the vernalization period (55, 40, and 32% taller than DEX-ZnO, $ZnSO_4$, and bare ZnO NP treatments, respectively). However, differences in plant height diminished during the post-transplant period (reproductive growth period), where control treatments produced plants that were not significantly taller than plants treated with the bare ZnO NP or $ZnSO_4$ treatments after 6 weeks (Fig. 4.3). Nine-week-old plants treated with bare ZnO NP were not significantly different from untreated plants. DEX-ZnO NP treated plants were significantly shorter than the plants in the control treatment. However, at week 11, there were no significant differences among treatments. The same pattern carried on two weeks later (week 13).

4.4.3 Experiment (B) Spiked Soil Greenhouse Assay

4.4.3.1 Zinc Concentration

There was a significant effect of Zn treatment and pH interaction on Zn concentrations in grain ($p < 0.001$, Fig 4.4A). All plants grown in Zn amended soils-except for plants

grown on soil treated with bare ZnO at pH 8 which was not statistically significant from the control at the same pH- had similar Zn concentrations in the grain which was, on average, 93 µg Zn/g grain, around 33% more Zn compared to the control. There was a significant effect of Zn treatment and pH interaction (p <0.001) on Zn concentrations in leaves. Zinc concentrations in the leaves were about twice as high in the pH 6 treatments (averaged 136 µg Zn/g dry biomass) as compared to the pH 8 (averaged 68 µg Zn/g dry biomass) for all Zn treated soils, whereas Zn concentration in control leaves was the same regardless of soil pH (30 µg Zn/g dry biomass). There was also a significant effect of Zn treatment on Zn concentrations in the leaves. Leaf Zn concentrations at pH 6 and pH 8 was 4.4 and 2.0 times greater than the control, respectively (Fig. 4.4B). However, there were no differences in leaf Zn concentration across all Zn treatments regardless of Zn form within the same soil pH. A similar trend was observed for Zn concentrations in stems. There was a significant interaction between Zn treatment and soil pH ($p<0.001$). At pH 6, all Zn treatments had significantly higher Zn concentration in the stems than the control but were not significantly different from each other. Stem tissue Zn concentrations for Zn treatments at pH 6 averaged 100 µg Zn/g, which was 6.3 times higher than the control. At pH 8, except for DEX treated soils, Zn treatments were not significantly different from each other and averaged 49 µg Zn/g which was 2.2 times higher than the control. Zn concentrations in stems of plants grown in soils treated with Zn at pH6 were twice as much as compared to plants grown in Zn treated soils at pH 8 (Fig. 4.4C).

4.4.3.2 Biomass

There was a significant (p = 0.013) interaction between Zn treatment and soil pH. At pH 6, while not significantly different from $ZnSO_4$, bare ZnO NP treatment produced grain of 40 and 32% higher biomass compared to the grain produced from the control and DEX-ZnO NP treatments, respectively (Fig. 4.5A). No significant differences in grain biomass were detected among the different Zn treatments (nano or ionic) nor when compared to the control at pH 8 (Fig. 4.5A). There was no significant interaction between Zn treatment and soil pH on leaf biomass (Fig. 4.5B). None of the Zn treatments were significantly different from one another or from the control at soil pH 6. While not significantly different from one another, DEX-ZnO NP and $ZnSO_4$ treated soils at pH 8 produced plants of biomass higher than those of the control treatment by 51 and 30 %, respectively. Biomass of leaves from plants grown on soil treated with DEX-ZnO NPs was 28% higher than of those grown on soil treated with bare ZnO NPs at pH 8. There were no differences in leaf biomass between $ZnSO_4$ and bare ZnO NPs (Fig. 4.5B). For stem biomass, only main effects (pH and Zn treatment) were statistically significant (p = 0.001) at $\alpha = 0.05$ (Fig. 4.5C). Stem biomass for control and DEX-ZnO NP treatments were not significantly different from one another at pH 6. Likewise, bare ZnO NP and $ZnSO_4$ treatments produced plants with similar stem biomass (0.154 g average dry mass) at pH 6, while both treatments resulted in plants with stem biomass that were 23 and 16% higher than the control and DEX-ZnO NP treatments, respectively (Fig. 4.5C). At pH 8, all Zn treatments produced plants of similar stem biomass, whereas only bare ZnO NP treatment resulted in plants with stem biomass that was 17% higher than those of the control treatment (Fig. 4.5C).

4.4.3.4 Plant Height

Repeated measures ANOVA showed a significant treatment effect on plant height. We performed multiple comparisons at selected important time points in plant growth stage; one week after sowing, when vernalization ended, when stem elongation started and at flag leaf emergence (Fig. 4.6). One week after sowing, bare ZnO NP-treated plants, at both pH 6 and 8, were the tallest plants, compared to the rest of the treatments. ($p = 0.05$). Plants grown in bare ZnO amended soils at pH 6 were 28, 26, 23, 22, 21, and 15% taller than plants grown in soils amended with $ZnSO_4$ at pH 6, control at pH 8, control at pH 6, DEX-ZnO NP at pH 6, DEX-ZnO NP at pH 8, and $ZnSO_4$ at pH 8, respectively. (Fig. 4.6). By the end of the vernalization period (week 7), all other treatments had plants as tall as the ones grown in the bare ZnO NP pH 6 treatment, except for control plants at pH 8 and $ZnSO_4$ at pH 6. The gap between the height of plants grown in soils amended with bare ZnO NP at pH 6 and $ZnSO_4$ at the same pH decreased from 28% to 11%. Plant height in the ninth week almost followed a pattern similar to that of the seventh week (Fig. 4.6). Most treatments were not significantly different from the bare ZnO NP treatments at pH 6 except for DEX-ZnO NPs and $ZnSO_4$ at pH 6 and control at pH 8, where plants grown in these treatments were around 6-8% shorter. At week 11 however, plants grown in both control and $ZnSO_4$ at pH 6 had similar heights compared to the plants grown in soil amended with the bare ZnO NPs. At week 15, after which stem extension totally halted, all Zn treatments at pH6 were not significantly different from one another while significantly taller than the plants grown in the control treatment at pH 6. Moreover, all Zn amendments at pH 6 produced plants that were

significantly taller compared to plants grown at pH 8 regardless of the amendment. Plant height in pH 8 soils were similar in all treatments (Fig. 4.6).

Although we did not measure soil pH right after spiking the soil with the different forms of Zn, we found that soil pH values generally decreased by at least half to one pH unit across all Zn treatments as compared to the control at both pH levels (Table 4.1).

4.5 Discussion

In the present study, we investigated two different methods for the fertilization of wheat plants using ZnO NPs as compared to $ZnSO_4$. The first method was treating seeds prior to planting and the second was amending the soil. In both studies, we investigated the response to both bare and dextran coated ZnO. Our previous seed germination assay [154] and soil study [83] comparing different ZnO surface coatings suggested that bare ZnO and DEX-ZnO NPs would be the most effective NP treatments.

In the seed coating assay, bare ZnO NP-coated seeds produced grains with the highest Zn concentration and the lowest biomass, albeit not statistically significant, indicating that bare ZnO NP tended to be the most effective Zn treatment in enriching Zn concentration in the grain. Indeed, our previous seed germination assay study showed that treating wheat seeds with 500 mg Zn /L bare ZnO NP resulted in the highest Zn concentration in the shoots compared to other tested particles including DEX-ZnO NPs[154].

Previous studies investigating treating seeds with ZnO NPs prior to growing them in soil are scarce. Taran et al. found that winter wheat seeds which were pre-soaked with ZnO NPs at 120 mg Zn/mL had 61% higher Zn in their leaves compared to the control[121]. In comparison, we did not find significant differences in leaf tissue Zn concentrations

between control and treated seeds, regardless of the Zn form. The duration of the experiment (terminated 10 days after sowing) in the Taran et al. study could possibly explain these differences. As plants grow, the accumulation of biomass and the translocation of nutrients from older to younger plant parts will likely dilute tissue Zn concentration[187-189]. Differences among plants and exposure conditions (Zn concentration and exposure duration) result in different crop responses. For example, compared to the control, treating peanuts seeds with $ZnSO_4$ and ZnO NPs at 400 mg Zn/L produced stems with 2 and 3.5 times greater dry biomass, respectively. Also, bare ZnO NPs enhanced stem and grain biomass by 73% and 13%, respectively, compared to $ZnSO_4$ [55]. Lupin seeds pretreated with a 60 mg Zn/L ZnO NP suspension before being grown in soil had 39, 60, 40, and 66% higher root and shoot lengths, whole plant dry biomass, and Zn concentrations, respectively, compared to the control[122]. Zinc concentrations in barley (*Hordeum vulgare*) shoots were 2.7 times higher than that in the controls when barley seeds were presoaked with bare ZnO NPs at 80 mg Zn/L, although ZnO NPs did not affect plant growth[190].

Soil pH is a key determinant of Zn bioavailability in soil. Despite the clear effect of soil pH on stem and leaf Zn concentrations, it did not affect Zn concentration in the grain regardless of the treatment. However, all Zn treatments, in general, had significantly higher tissue Zn concentration as compared to the controls. In stems and leaves, lower pH resulted in higher Zn accumulation. Watson, et al. found that Zn concentration in wheat shoots grown in an acidic soil was an order of magnitude higher than that when the plants were grown in an alkaline soil [191]. Similarly, tomatoes (*Solanum lycopersicum*) and beans (*Phaseolus vulgaris*) grown in a naturally acidic soil (pH 5.4) had around an

order of magnitude higher Zn in leaf tissue compared to plants grown in a calcareous soil (pH 8.3)[192]. White et al compiled the results from several studies aimed at enhancing Zn concentration in the fruits of edible crops. They found that regardless the crop, or whether Zn was applied on the leaves or as a fertilizer added to the soil, Zn concentration in fruits rarely exceeded 100 µg/g, which was mainly attributed to the tight regulation of Zn inside the plant[31]. This value is comparable to grain Zn concentration we obtained in both experiments suggesting that we most likely reached that limit.

Compared to the bare ZnO NPs, dextran (neutral charge) coating did not affect Zn concentration in wheat grain, leaves, or stems, regardless soil pH, in the soil amendment study. In a study which used a soil mixed with a potting media at a pH of 7.2, Zn concentrations in green pea (*Pisum sativum*) grain, leaves, and stems were all similar when the soil was amended with bare, Al_2O_3 doped, or aminopropyltriethoxysilane coated ZnO NPs at 250 mg Zn/kg soil[104]. This was a very high Zn amendment rate, far greater than what would result from a typical agronomic recommendation. Despite this, there is evidence that particle surface chemistry does influence NP uptake by plants. For example, positively charged CeO_2 and Au NPs adhered better to the roots than neutral or negatively charged ones, but Ce and Au were translocated less efficiently in the plant[130, 131]. The growth medium in these studies was hydroponic, which could explain these differences. Our previous work showed that binding of dissolved organic matter to particles changes the surface chemistry and confers a similar net negative charge regardless the initial surface chemistry[83]. Studies of natural nanoscale colloids in natural soils have also shown that natural organic matter has a controlling influence on particle surface chemistry[193]. Studies in simplified hydroponic media probably don't accurately

predict NP behavior in natural soil due in large part to the absence of natural organic matter.

In the present study, higher tissue Zn concentrations in stems and leaves in experiment B did not increase grain yield except for bare ZnO NP amended soils at pH 6. Likewise, Mukherjee et al. reported no effects of the higher tissue Zn concentration in dry biomass of green pea when the soil was amended with 250 mg Zn/kg soil as ZnO NPs (bare or coated)[104]. Raliya et al showed that bare ZnO NPs at 100 mg/kg enhanced both fruit yield and Zn tissue concentration in tomatoes[194]. However, no soil characterization was provided in this study, which might have an effect on the behavior and uptake of these particles[194]. When a natural soil at pH 6.7 was spiked with 6 mg Zn/kg in the form of bare ZnO NPs or $ZnSO_4$, no significant increase in sorghum (*Sorghum bicolor*) straw or panicle biomass was reported despite higher tissue Zn concentrations. On the contrary, grain yield significantly increased, mirroring an increase in tissue Zn concentration[195]. Again, plant growth conditions and soil spiking concentration have probably resulted in differences among these studies.

Plant height differences among Zn treatments were more pronounced during the vegetative growth stage (vernalization period) in both experiments. However, these differences diminished as soon as the plants moved to the reproductive stage (post-transplant period). Overall, in both experiments, there were no differences in plants height towards the end of the experiment, although in experiment B plants grown in soils at pH 8, regardless of the Zn amendment, tended to be shorter than the ones grown at pH 6. In a previous study, stem elongation of soybean plants grown in a natural soil (pH = 6.78) amended with 5 and 10 mg Zn/kg soil was similar to that of the control whereas at

50 mg/kg, ZnO NPs resulted in shorter plants[119]. In another study performed on soybean, it was found that 50 mg Zn/kg soil of ZnO NPs did not affect the height of the plant while 500 mg Zn /kg decreased stem elongation[117]. These studies used very high concentrations of Zn, and it is likely that Zn toxicity stunted the growth of the plants.

Despite our adjustment of initial soil pH to approximately 6 and 8 in the soil amendment study, the decline in soil pH over the duration of the study minimized our intended treatment difference in pH. This may be reflected in the similar Zn concentration and biomass values observed for wheat plant tissues. Our previous work demonstrated that the dissolution of ZnO NPs was almost 5 times higher at pH 6, as compared to pH 8, in particle free soil water[83]. Given that our final pH values were closer to 6 and 7, differences in the solubility and ζ potential of the NPs were not as great as intended. Based on our previous study of the behavior of these particles in soil, it is possible that the NPs largely dissolved over the long duration of the study, resulting in similar behavior between $ZnSO_4$ and ZnO NPs. Soil pH drift has been reported in previous studies. Over the course of 21 days in a study performed on wheat, it was found that soil pH increased from 7.33 to 7.65 and decreased to 6.32 when soil was spiked with 1000 mg Zn /kg in the forms of ZnO NPs and $ZnCl_2$, respectively[196]. The drift in pH might be due to increases in the pCO_2 from microbial or plant respiration or from CO_2 dissolved in the water used to irrigate the plants or from the atmosphere. It could also be a result of cation exchange when fertilizers were added. Future studies might utilize a more effective soil buffering system to better characterize the effect of pH or examine a large variety of soils with varying native pH values.

Taken together, the results showed that, among all the Zn treatments, only bare ZnO NPs were able to significantly enhance grain tissue Zn concentration, when used as a seed treatment. Therefore, bare ZnO NPs are promising tools to enrich wheat grain with Zn when used as a seed treatment.

Spiking the soil with Zn amendments significantly enhanced stem and leaf Zn concentration at both pH levels. However, there were no differences in tissue Zn concentrations among added Zn amendments (nano and ionic). Both experiments produced grain of similar tissue Zn concentration, although the soil spiking approach produced slightly more robust plants (greater biomass and height). Despite differences in leaf and stem Zn concentrations of plants grown in Zn amended soil as a function of soil pH, no differences in grain Zn concentrations were reported, which could be attributed to physiological limitations which play a major role in the translocation of Zn to the grain.

Future studies should also focus on the concentration response relationship. It is possible that at 15 mg Zn/kg soil, we were well within the adequate range for wheat. Differences between treatments might only be evident within the range over which the crop shows a dynamic response to Zn supplementation. Indeed, even with Zn salts, it was found that yield and tissue Zn concentration were not significantly enhanced when background available soil Zn concentrations were high enough to support the growth of wheat and maize (*Zea mays*)[197]. Future studies should focus on determining what concentration of each of the Zn treatments results in maximum yield. Finally, the response of other crops to dextran coated ZnO remains to be tested.

Tables

Table 4.1: Soil pH values in 1:1 soil in DI water for pot experiment (B). $pH_{initial}$ is the pH of the soil at the beginning of the experiment before sowing the seeds and pH_{final} is the pH of the soil after harvesting the plants. Values are reported as the mean of three replicates ± one standard deviation)

Soil	**Control**		**$ZnSO_4$**		**Bare ZnO**		**DEX**	
pH	**pH initial**	**pH final**	**pH initial**	**pH final**	**pH initial**	**pH final**	**pH initial**	**pH final**
6	6.24±0.01	6.27±0.03	NM	5.29±0.05	NM	5.93±0.06	NM	5.07±0.02
8	7.92±0.05	7.26±0.04	NM	6.58±0.06	NM	6.82±0.01	NM	6.78±0.05

NM: Not Measured

Figures

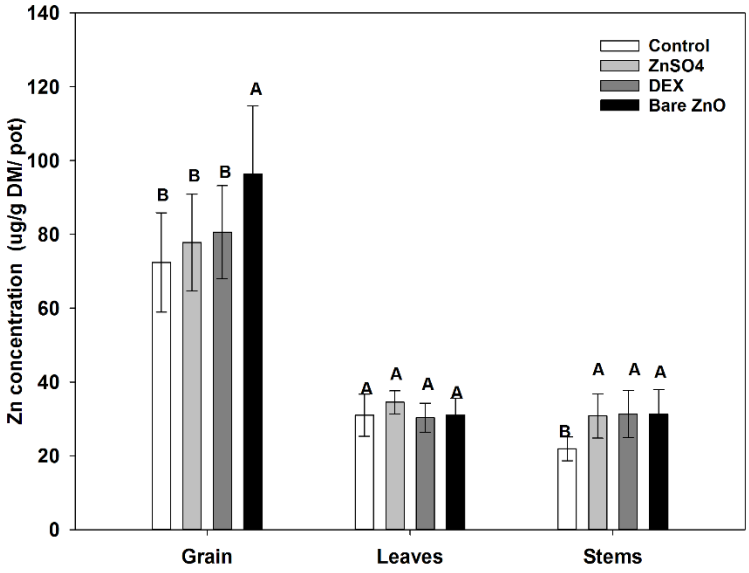


Figure 4.1 Zinc concentrations in grain, leaves, and stems of wheat plants from experiment A. Each bar represents the average of n=9, while error bars represent one standard deviation. Treatments which have the same letter within each tissue type are not significantly different at α=0.05

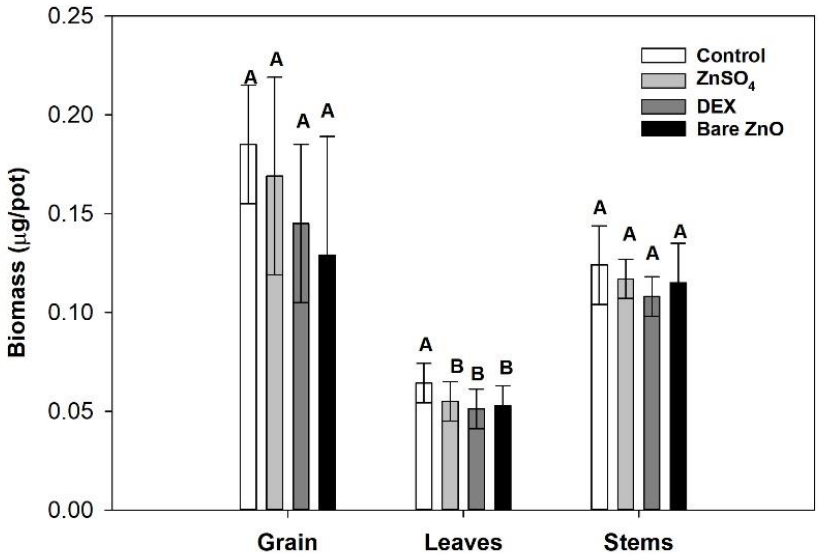


Figure 4.2 Dry biomass of grain, leaves, and stems of wheat plants-Experiment A. Each bar represents the average of n=9, while error bars represent one standard deviation. Treatments connected with the same letter within each plant part are not significantly different at α=0.05

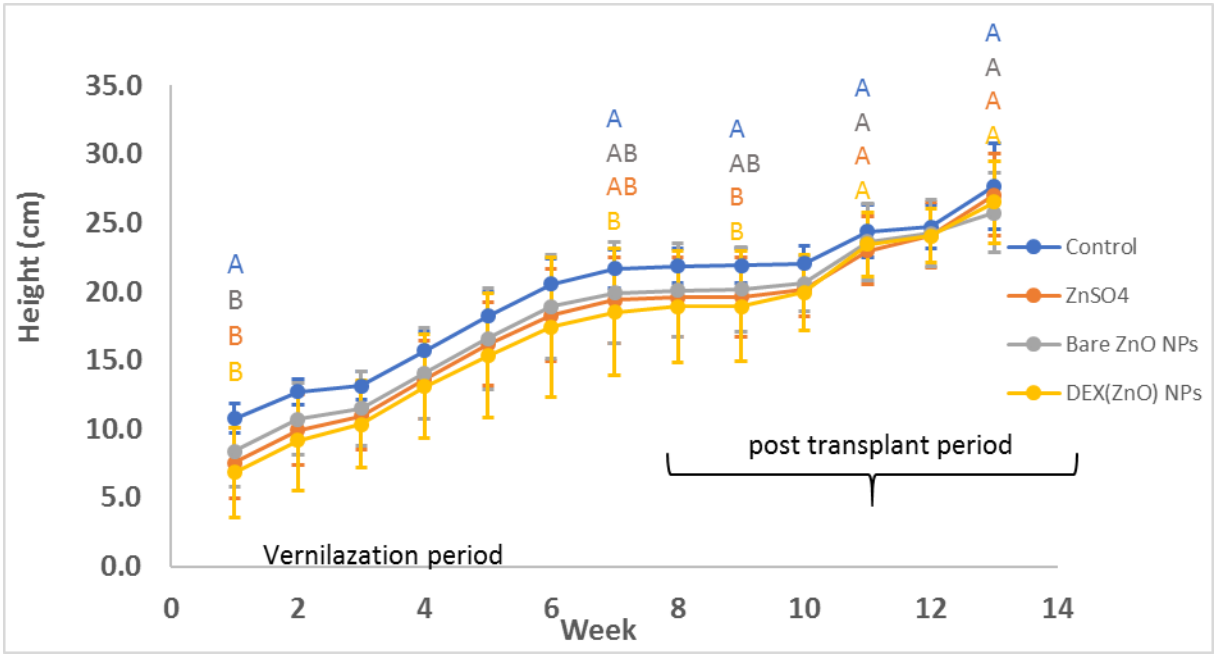
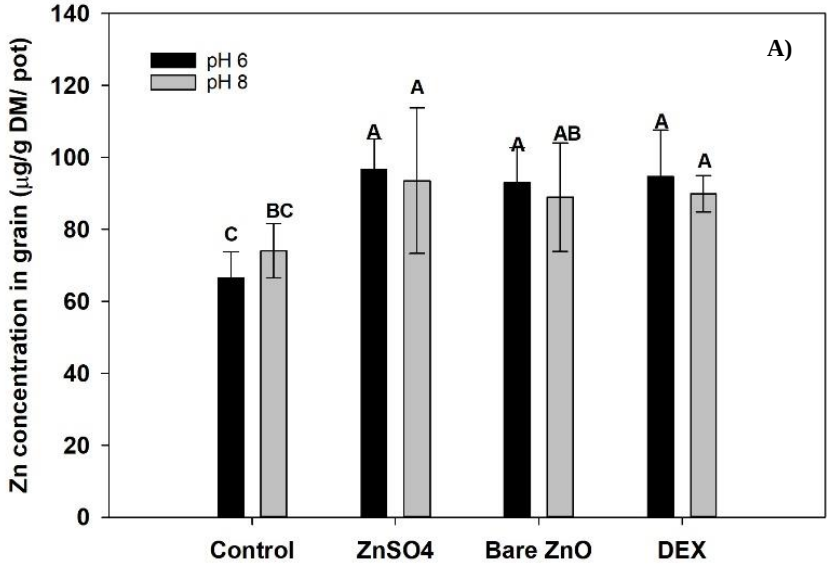
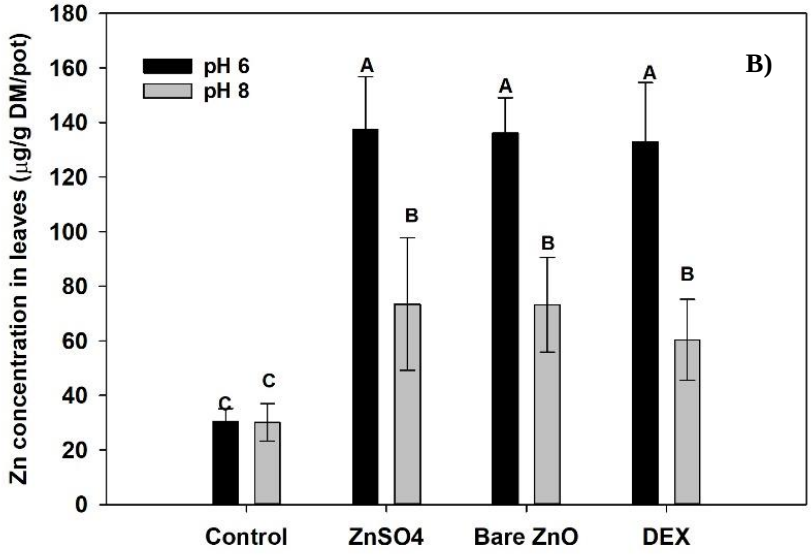


Figure 4.3 Dry biomass of grain, leaves, and stems of wheat plants-Experiment A. Each bar represents the average of n=9, while error bars represent one standard deviation. Treatments connected with the same letter within each plant part are not significantly different at α=0.05.


A)
pH 6
pH 8
Zn concentration in grain (µg/g DM/ pot)
140
120
100
80
60
40
20
0
C
BC
A
A
A
AB
A
A
Control
ZnSO4
Bare ZnO
DEX


B)
pH 6
pH 8
Zn concentration in leaves (µg/g DM/pot)
180
160
140
120
100
80
60
40
20
0
C
C
A
B
A
B
A
B
Control
ZnSO4
Bare ZnO
DEX

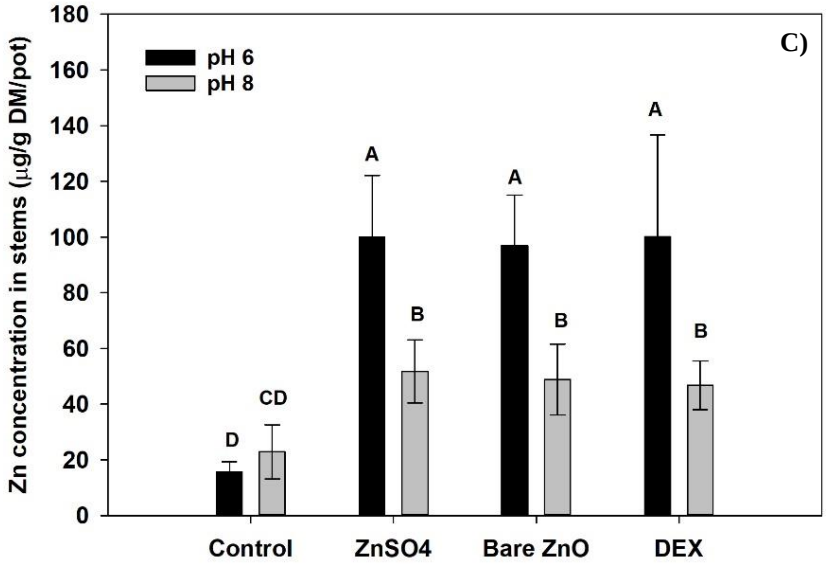
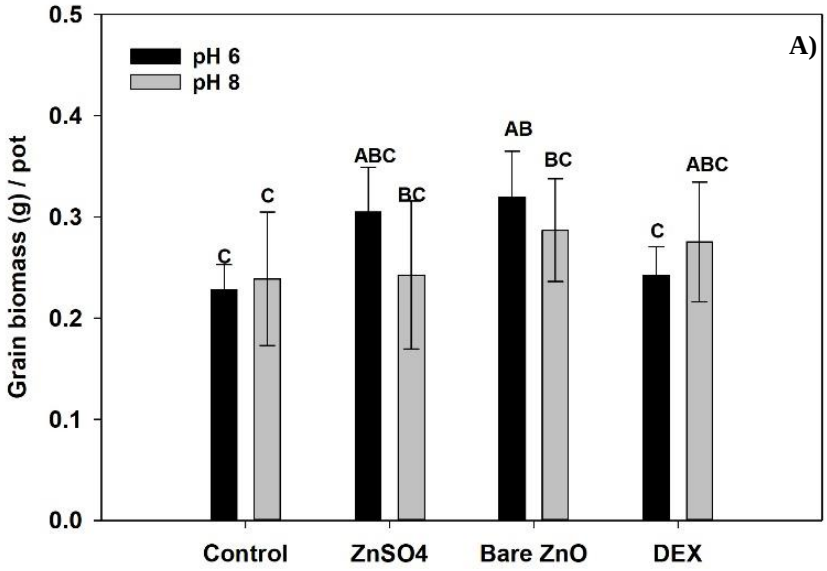
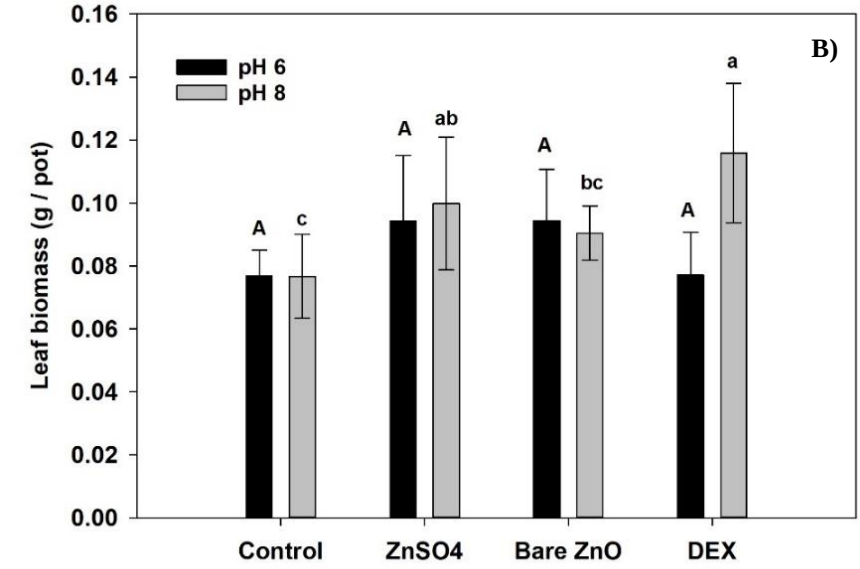


Figure 4.4 Zinc concentration in the grain (A), leaves (B), and stems (C) of wheat plants at each soil pH level-Experiment B. Each bar represents the average of n=11, while error bars represent one standard deviation. Treatments connected with the same letter at each pH level are not significantly different at α=0.05





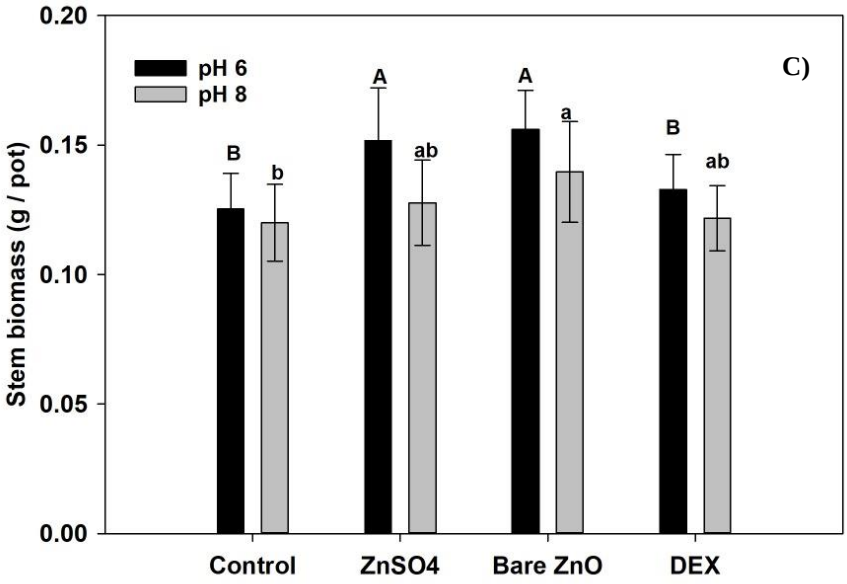


Figure 4.5 Dry biomass of (A) grain, (B) leaves, and (C) of wheat plants at each soil pH level-Experiment B. Error bars represent one standard deviation, n = 11. Treatments connected with the same letter at each pH level are not significantly different at α=0.05. Upper and lower case letters refer to multiple comparisons carried out at each pH separately.

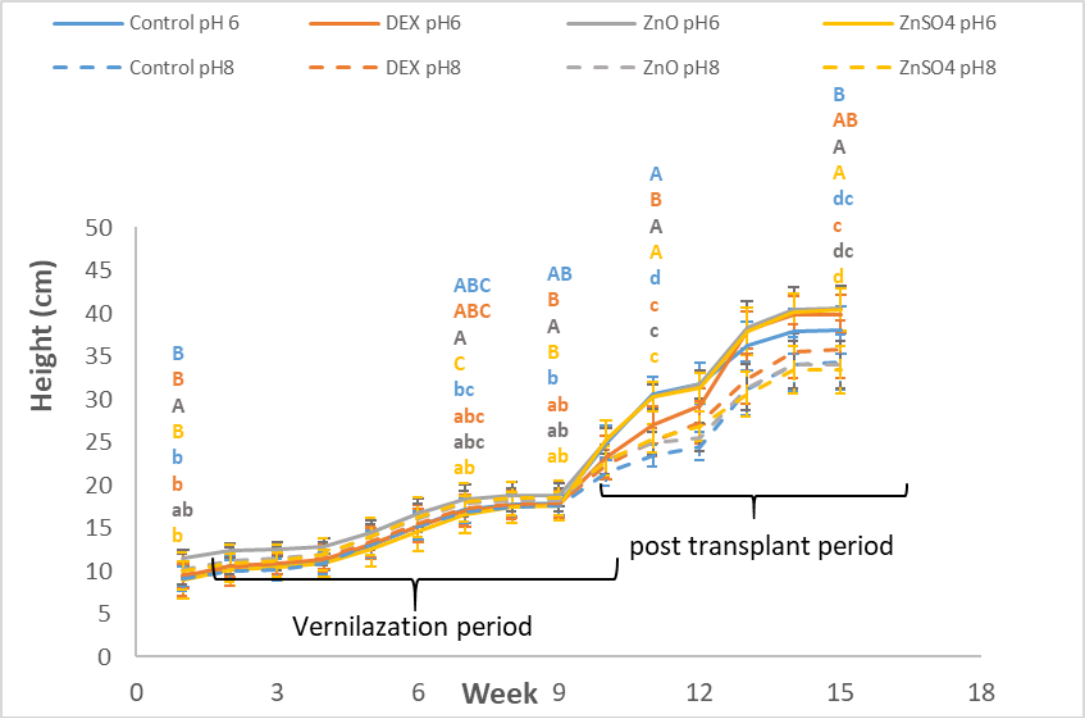


Figure 4.6 Plant height (cm) over time (week)-Experiment B. Error bars represent one standard deviation, n=11. Treatments connected with the same letters, whether in upper or lower cases, are not statistically significant at α=0.05. Letters followed treatment color codes, and uppercase and lowercase letters represent treatments at pH 6 and pH 8, respectively.

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Chapter 5: Conclusions and Future Directions

We performed several experiments to evaluate the potential of using ZnO NPs as nanofertilizers to enhance Zn concentrations and yield in wheat.

In Chapter 2, our results revealed that, compared to $ZnSO_4$, wheat seeds treated with ZnO NPs had higher tissue Zn concentrations and better growth when applied at nontoxic concentrations. We also found that by tuning the surface chemistry of the particles, Zn partitioning patterns in seedling tissue, and growth stimulation differed. We found that treating seeds with DEX-ZnO NPs achieved the highest biomass and Zn concentration in the seedling roots, whereas bare ZnO NPs delivered the highest Zn concentrations to the seedling shoots, with slight growth stimulation.

Data in Chapter 3 showed that particle surface chemistry among the different particles dictated the behavior of the ZnO NPs in simple aqueous solutions whereas the patterns of behavior in natural soil solution were modified by sorption of natural organic matter (NOM). In saturated paste soil extracts, NOM had an immense effect on the partitioning of the particles to the soil solution regardless of the soil pH (acidic or alkaline). In the experiments which involved humic acids, NOM conferred a net negative charge to all NPs regardless their as-synthesized coatings. This enhanced their partitioning to, and stability in, soil solution, resulting in an increase in the total Zn concentration in a saturated paste extract. The higher affinity of the dextran coating for NOM explained the relatively high concentrations of total Zn in saturated paste extracts from the DEX-ZnO NP treatments. Overall, at the very conditions that limit total Zn concentrations in saturated paste extracts for $ZnSO_4$, ZnO nanofertilizers (especially core-shell and DEX-ZnO NPs) had better performance , as demonstrated by the higher total Zn concentration

in soil solution, which in turn would reflect a better bioavailability to crops, assuming that the uptake of Zn from nanoparticulate phases is possible as proved by previous work [154, 163], or that plant roots or associated rhizobacteria can release exudates to solubilize these materials.

Chapter 4 showed that the only seed treatment that resulted in a significant increase in Zn concentration in grain as compared to the control was the bare ZnO NPs. Therefore, bare ZnO NPs can be strong candidates to be used as seed treatments to enrich Zn in grain of wheat and possibly in other staple crops. The results from the second pot experiment showed that there were no differences in grain Zn concentration regardless the soil pH or Zn form (nano and ionic), which could be attributed to physiological limitations to grain Zn loading. It is worth mentioning that the success of this approach to enhancing grain Zn could be related to the maternal supply of Zn in the seeds to begin with. Wheat varieties with seed Zn concentration below 10, equal to 20, and higher than 40 µg Zn/g are considered Zn deficient, sufficient, and recommended for human health, respectively[17] . The seeds we used in this study had apparently high Zn concentration and therefore, only subtle differences in tissue Zn concentrations were observed using different Zn amendments (nano or ionic).

Bare ZnO NPs were more efficiently translocated to the grain. However, further studies are required to explain the enhanced translocation of these NPs especially when compared to $ZnSO_4$, which indicates a nano-specific effect that requires further investigation.

Future work on soil amendments should focus on the dose response relationship between Zn concentration in soil, tissue Zn concentration and yield. The rate of Zn amendment we applied (15 mg Zn/kg soil) was probably well within the range adequate for wheat on the studied soil, so we observed no distinct dynamic response of the plant to Zn supplement. Nutrient studies are more likely to show a difference between several fertilizer types within the linear portion of the concentration response curve. When all treatments result in adequate Zn conditions, no further response in terms of yield or Zn tissue concentrations may be expected. One study showed that yield and tissue Zn concentration were not significantly enhanced when labile Zn concentrations were high enough to support the growth of wheat and maize (*Zea mays*)[197]. Future studies should focus on determining what concentration of each of the Zn treatments results in maximum yield. The response of other crops to dextran coated ZnO remains to be tested as well.

The drift in soil pH that we encountered in the soil spiking pot study suggests the importance of using ZnO NPs on naturally alkaline soils, or soils deficient with Zn to test the performance of these NPs as compared to $ZnSO_4$ under realistic field conditions

Foliar application of ZnO NPs could also be a more effective means of enriching grain with Zn. A few studies reported better performance of ZnO NPs when introduced as a foliar application versus soil amendment[54], [197, 198]. However, to achieve the best results, the surface chemistry of the particles needs to be tuned to enhance the attachment efficiency of the particles to the leaf cuticle[185]. Also, timing of application is important, it was found that higher grain Zn concentrations in wheat were achieved when the plants were sprayed just before the grain filling stage[41, 199].

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199. Cakmak, I.; Kalayci, M.; Kaya, Y.; Torun, A. A.; Aydin, N.; Wang, Y.; Arisoy, Z.; Erdem, H.; Yazici, A.; Gokmen, O.; Ozturk, L.; Horst, W. J., Biofortification and localization of zinc in wheat grain. *J Agric Food Chem* **2010,** *58*, (16), 9092-9102. DOI:10.1021/jf101197h.

VITA
Zeinah Elhaj Baddar

Education

M.S Soil, Water, and Environemnt, University of Jordan, Amman, Jordan, 2007.

B.S. (*Honors*) in Lands and Water Management, The Hashemite University, Zarqa, Jordan, 2003.

Research Experience

- (2012-present): Department of Plant and Soil Sciences, Environmental Chemistry and Toxicology Lab, University of Kentucky, Lexington

Title: Graduate Research Assistant

- (2005-2012): Centre of Environmental Studies at the Hashemite University

Title: Lab supervisor

Awards and Recognition

- 2016 University of Illinois at Urbana-Champaign. "Pioneer Plant Science Symposium". Full travel scholarship: $800.00.
- 2015 2nd place graduate student presentation. "Donald Sparks graduate students minisymposium", University of Kentucky.
- Gamma Sigma Delta-University of Kentucky chapter-Outstanding graduate student.

Publications- Peer reviwed

Elhaj Baddar, Z., and Unrine, J. 2018. Functionalized ZnO nanoparticles as seed coatings to enhance growth and Zn content of wheat (*Tritcum aestivum*) seedlings. Submitted to *Journal of Agricultural and Food Chemistry*.

Elhaj Baddar, Z., Matocha, C., and Unrine, J. 2018. Evaluation of the effect of surface coatings on the sorption and dissolution of ZnO NPs in soil. To be submitted to the SSSAJ.

Elhaj Baddar, Z., and Unrine, J 2018. Effects of soil pH and coating on efficacy of polymer coated ZnO nanoparticulate fertilizers in wheat (*Triticum aestivum*)". In Prep

Starnes, D., Unrine, J., Chen, C., Lichtenberg.,S, Starnes, C., Svendsen, C., Kille, P., Morgan, J., **Elhaj Baddar**, **Z**., Spear, A., Bertsch, P. and Tsyusko. O. 2018. Toxicogenomic responses of Caenorhabditis elegans to pristine and aged Zinc Oxide nanoparticles. To be submitted.

Oral and poster Presentations:

Elhaj Baddar Z, Unrine M. 2018. "The effect of pH and particle surface chemistry on the dissolution and binding of ZnO nanoparticles in soil." **Poster presentation.** Gordon Research Conference on Nanoscale Science and Engineering for Agriculture and Food Systems, South Hadley, MA.

Elhaj Baddar Z, Unrine M. 2017. "Using ZnO nanofertilizers to address Zn malnutrition by biofortifying wheat." **Oral presentation.** Bill Witt graduate students minisymposium, University of Kentucky, Lexington, KY.

Elhaj Baddar Z, Unrine M. 2017. Using ZnO nanofertilizers to address Zn malnutrition by biofortifying wheat. **Poster presentation**. Tracey Farmer Institute for Sustainability and the Environment (TFISE), University of Kentucky, Lexington, KY.

Elhaj Baddar Z, Unrine M. 2017. "Studying the effect of soil pH on the dissolution and binding of engineered ZnO nanoparticles in soil." **Oral presentation.** ASA-CSSA-SSSA-annual meeting, Tampa, FL.

Elhaj Baddar Z, Unrine M. 2017. "Studying the effect of soil pH on the dissolution and binding of engineered ZnO nanoparticles in soil." **Oral presentation.** Donald Sparks graduate students minisymposium, University of Kentucky, Lexington, KY.

Elhaj Baddar Z, Unrine M. 2016. Exploring potential use of ZnO nanoparticles (NPs) to enhance Zn nutrition in wheat (*Triticum aestivum*). **Oral presentation.** ASA- CSSA - SSSA-annual meeting, Phoenix, AZ.

Elhaj Baddar Z, Unrine M. 2016. Exploring potential use of ZnO nanoparticles (NPs) to enhance Zn nutrition in wheat (*Triticum aestivum*). **Oral presentation.** Donald Sparks graduate students minisymposium, University of Kentucky, Lexington, KY.

Elhaj Baddar Z, Unrine M. 2015. "Synthesis, surface modification and characterization of ZnO nanoparticles for use as a micronutrient fertilizer". **Oral presentation.** ASA-CSSA -SSSA-annual meeting, Minneapolis. MN

Elhaj Baddar Z, Unrine M. 2014. "Effect of coatings on the behavior of manufactured zinc oxide nanoparticles in soil and bioavailability to plants". **Oral presentation.** Donald Sparks graduate students minisymposium, University of Kentucky, Lexington, KY.

Elhaj Baddar Z, Unrine M. 2014. Synthesis and characterization of ZnO nanoparticles for biofortification of crops. **Poster presentation**. Tracey Farmer Institute for Sustainability and the Environment (TFISE), University of Kentucky, Lexington, KY.

Poynton H., Unrine, J., Haring, H., **ElHaj Baddar, Z.**, Lazorchak, J. 2014. Characterizing nanoparticle exposure and effects in the epibenthic crustacean, Hyalella Azteca. SETAC/Europe meeting, Basel, Switzerland.

Elhaj Baddar Z, Unrine M. 2013. "Effects of coating on the transformation of ZnO nanoparticles in sewage sludge and soils". **Oral presentation.** Donald Sparks graduate students minisymposium, University of Kentucky, Lexington, KY.

Elhaj Baddar Z, Unrine M. 2013. Method development for the detection and characterization of manufactured zinc oxide nanoparticles in soil-preliminary research results. **Poster presentation**. Tracey Farmer Institute for Sustainability and the Environment (TFISE), University of Kentucky, Lexington, KY.

Invited talks and lectures:
September 13th, 2016. Title:" Exploring potential use of ZnO nanoparticles (NPs) to enhance Zn nutrition in wheat (*Triticum aestivum*)". Pioneer plant sciences symposium-University of Illinois-Urbana-Champaign.

Leadership and outreach:
October 2017: Selected to participate at the "student's leadership conference" at the 2017 ASA-CSSA-SSSA annual meeting, Tampa, FL.
October 2017: Served as a judge for master student oral presentation competition-soil fertility division at the 2017 ASA-CSSA-SSSA annual meeting, Tampa, FL.
July 2017: Served as science projects judge for Kentucky state 4-H communication day, Lexington, KY.
***January 2016*:** Moderator at the Bill Witt graduate students minisymposium. University of Kentucky. Lexington, KY.
April 2015: Participated in "Nano-Days" science educational fair. Kentucky Science Center, Louisville, KY.
***January 2015*:** Moderator at the Bill Witt graduate students minisymposium. Lexington, KY.
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