TRANSFORMATIONS, BIOAVAILABILITY AND TOXICITY OF MANUFACTURED ZnO NANOMATERIALS IN WASTEWTER

Sewwandi Rathnayake

University of Kentucky, sewanndi@gmail.com

Recommended Citation
STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained and attached hereto needed written permission statements(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine).

I hereby grant to The University of Kentucky and its agents the non-exclusive license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless a preapproved embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student’s advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student’s dissertation including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Sewwandi Rathnayake, Student

Dr. Paul M. Bertsch, Major Professor

Dr. Mark S. Coyne, Director of Graduate Studies
TRANSFORMATIONS, BIOAVAILABILITY AND TOXICITY OF MANUFACTURED ZnO NANOMATERIALS IN WASTEWATER

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture, Food and Environment at the University of Kentucky

By

Sewwandi Rathnayake

Lexington, Kentucky

Co-Directors: Dr. Paul M. Bertsch, Professor of Plant and Soil Sciences and Dr. Jason M. Unrine, Assistant Professor of Plant and Soil Sciences

Lexington, Kentucky

2013

Copyright © Sewwandi Rathnayake 2013
ABSTRACT OF THESIS

TRANSFORMATIONS, BIOAVAILABILITY AND TOXICITY OF MANUFACTURED ZnO NANOMATERIALS IN WASTEWATER

In order to properly evaluate the ecological and human health risks of ZnO Manufactured nanomaterials (MNMs) released to the environment, it is critical to understand the likely transformation products in the wastewater treatment process and in soils receiving biosolids. To address this critical knowledge gap, we examined the transformation reactions of 30 nm ZnO MNMs in single component and multi-component systems, with phosphate and natural organic matter (NOM). We also assessed the influence of nano ZnO transformation on the bioavailability and toxicity of ZnO transformation products to *Triticum aestivum*. The data revealed that ZnO MNMs react with phosphate at concentrations expected in wastewater and transform into two distinct morphological/structural phases. A micron scale crystalline zinc phosphate phase (hopeite), and a nano-sized phase that likely consists of a ZnO core with a Zn$_3$(PO$_4$)$_2$ rich shell. Presence of NOM reduces particle aggregation and enhances stability, regardless of the sequence of ligands addition in the aging scenarios. The presence of phosphate and NOM also altered the bioavailability and reduced the toxicity of the ZnO MNMs to *Triticum aestivum*.

KEYWORDS zinc oxide nanoparticle, transformations, phosphate, natural organic matter, toxicity

Sewwandi Rathnayake

June 30, 2013
TRANSFORMATIONS, BIOAVAILABILITY AND TOXICITY OF MANUFACTURED ZnO NANOMATERIALS IN WASTEWATER

By

Sewwandi Rathnayake

Dr. Paul M. Bertsch
Co-Director of Thesis

Dr. Jason M. Unrine
Co-Director of Thesis

Dr. Mark S. Coyne
Director of Graduate Studies

June 30, 2013
ACKNOWLEDGEMENTS

The journey to my Master’s was a whole new experience in my life which made me stronger both academically and personally. I would like to express my appreciation for all those who helped in this successful achievement. First of all, I would like to express my gratitude and special thanks to my thesis Chair, Dr. Paul Bertsch for giving me this golden opportunity, the guidance, support and insight throughout the time at UK. Furthermore, I would like to express my special thanks to my co-advisor, Dr. Jason Unrine for the guidance, support and evaluation in every stage of my thesis process, allowing me to successfully complete it. I am grateful for them for having confidence and faith in me. Next I wish to thank my committee Dr. David McNear for the guidance and directions during the project. I was lucky to have the best research group with me, gave me support all the time and being with me during the ups and downs, thank you Dr. Tsyusko and all my colleagues specially Dr. Jonathan Judy for helping me in success of my work in many ways. Also I would like to thank everyone who supported me to finish a successful research study including, Dr. Anne Francis Miller, Dr. Mark Coyne, William Rao, Yvone Thompson and Jim Nelson. Above all I would like to thank my family for being incredibly supportive in every step of my life, my parents and especially my beloved husband Thushara Ranatunga for all the support and encouragement, I would not be where I am today without him and I don’t have enough words to express my thanks for everything he has done for me. Finally I would like to acknowledge United States Environmental Protection Agency for funding this work through the Science to Achieve Results Grant RD-834574-01.
# TABLE OF CONTENTS

Acknowledgments ........................................................................ iii  
List of Tables ................................................................. vi 
List of Figures ................................................................. vii 

## Chapter One: Introduction and literature review

Nanotechnology and manufactured nanomaterials .................. 1 
Fate and transformations of manufactured nanomaterials ........ 2 
Zinc oxide ........................................................................... 5 
Bioavailability and toxicity of ZnO nanomaterials ................. 6 
Impact of transformations to toxicity ................................... 7 
Characterization techniques ............................................... 7  
   Electron microscopy ...................................................... 7 
   Dynamic light scattering ................................................ 8 
   Mass spectroscopy ......................................................... 9 
   X-ray techniques ........................................................ 9 
   Nuclear magnetic resonance ........................................ 10 
   Objectives/Research outline ........................................ 10 

## Chapter two: A multi-technique investigation of phosphate induced transformation of ZnO nanoparticles

Summary ............................................................................ 11  
Introduction .......................................................................... 12 
Methods and materials .................................................... 14  
   Dispersion and initial characterization of ZnO MNMs .......... 14 
   Aging with phosphate .................................................... 15 
   Dissolved Zn analysis .................................................... 15 
   Synchrotron-based X-ray absorption near edge spectroscopy (XANES) ........................................... 16 
   X-ray diffraction (XRD) ................................................ 16 
   Thermo gravimetric analysis (TGA) ................................. 17 
   Solid state $^{31}$P Nuclear Magnetic Resonance (NMR) ....... 17 
Results and discussion ......................................................... 18  
   Solubility of MNMs in phosphate solutions .............. 18 
   XANES data ............................................................... 19 
   XRD data ................................................................. 20 
   Surface charge of the aged particles ......................... 21 
   Size and of the aged particles .................................... 22
LIST OF TABLES

Table 1-1, Properties of wurtzite (Pearton, Norton et al. 2005)…………………… 5

Table 2-1, Isotropic chemical shifts ($\delta_{\text{iso}}$) and tensors ($\delta_{11}$, $\delta_{22}$, and $\delta_{33}$), $T_1$ relaxation times, cross-polarization buildup times ($\tau_{\text{cp}}$) and $T_{1\text{H},\rho}$ relaxation times for ZnO MNMs aged at pH 6 and pH 8 and standards…………………………………… 25
LIST OF FIGURES

Figure 1-1, Fate and possible environmental modifications of MNMs………………... 3

Figure 2-1, Concentrations of dissolved Zn$^{2+}$ as a function of phosphate concentration, pH, and time…………………………………………………………………………………………………… 19

Figure 2-2, The quantitative distribution of aged material between ZnO and Zn$_3$(PO$_4$)$_2$ estimated from linear combination fit (LCF) analysis of XANES spectra MNMs aged in pH 6 (a) and MNMs aged in pH 8 (b)………………………… 20

Figure 2-3, XRD spectra ZnO MNMs (a) particles aged in 150 mg L$^{-1}$ phosphate concentration for 72 h at pH 8 (b) and pH 6 (c) Zn$_3$(PO$_4$)$_2$ (d) and hopeite (e)… 21

Figure 2-4, Zeta potential of aged MNMs pH 6 (a) aged MNMs pH 8 (b)………… 22

Figure 2-5, TEM images of pristine ZnO MNMs (a) and MNMs aged in 150 mg L$^{-1}$ phosphate concentration for 72 h at pH 6 (b) at pH 8 (c)……………………………. 23

Figure 2-6, Comparison of NMR spectra of hopeite with materials aged at pH 6 vs. at pH 8 in 150 mg L$^{-1}$ phosphates………………………………………………………… 24

Figure 2-7, Effect of $^1$H decoupling and cross-polarization on MNMs aged at pH 6 and pH 8…………………………………………………………………………………………………… 29

Figure 3-1, Changes in hydrodynamic diameter of n-ZnO, p+n-ZnO and pn-ZnO in response to increasing NOM concentration; (1) pH 6 (2) pH 8……………… 40

Figure 3-2, Changes in electrophoretic mobility of n-ZnO, p+n-ZnO and pn-ZnO at (1) pH 6 (2) pH 8 as a function of NOM concentration……………………………………… 41

Figure 3-3a, TEM image of the pristine ZnO MNMs (a) aged MNMs aged in 150 mgL$^{-1}$ phosphate concentration for 72 h at pH 6 (b) at pH 8 (c)………………………… 42

Figure 3-3b, TEM images of n-ZnO at pH 6 (a) p+n-ZnO at pH 6 (b) pn-ZnO at pH 6 (c) n-ZnO at pH 8 (d) p+n-ZnO at pH 8 (e) and pn-ZnO at pH 8 (f)… 42

Figure 3-4, Dissolved Zn$^{2+}$ as a function of NOM concentration and pH……… 43

Figure 3-5, Carbon adsorption to MNM surfaces as determined by dissolved carbon content analysis…………………………………………………………………………………………………… 44
Figure 3-6, The quantitative distribution of aged material estimated from linear combination fit (LCF) analysis of XANES spectra of aged MNMs………………. 45

Figure 3-7, X-Ray diffraction patterns of n-ZnO at pH 6 (a) p+n ZnO at pH 6 (b) pn-ZnO at pH 6 (c) n-ZnO at pH 8 (d) p+n ZnO at pH 8 (e) pn-ZnO at pH 8 (f) 46

Figure 3-8a, Shoot and root elongation (a) Zn$^{2+}$ (b) ZnO MNMs (c) bulk Zn O (d) Zn$^{2+}$ in NOM (e) p-ZnO pH 6 (f) p-ZnO pH 8………………………………….. 47

Figure 3-8a, Shoot and root elongation (g) n-ZnO pH 6 (h) n-ZnO pH 8 (i) pn-ZnO pH 6 (j) pn-ZnO pH 8 (k) p+n-ZnO pH 6 (l) p+n-ZnO pH 8………………………………….. 48
Chapter one: Introduction and literature review

Nanotechnology and Manufactured Nanomaterials

Nanotechnology, the technology exploiting the unique electrical, magnetic, photoactive, and thermal properties of nano-scale materials, is an exciting new field that promises a broad array of benefits [1, 2]. The innovation of the scanning tunneling microscope, which represented a dramatic improvement to our ability to visualize materials at the nano scale and subsequent discovery of fullerenes and carbon nanotubes (graphite sheets or cylinder shape structures with either single or multiple walls) [3] helped generate widespread interest in this new field. Manufactured nanomaterials (MNMs), defined by the National Nanotechnology Initiative as materials with at least one dimension smaller than 100 nm, can be synthesized in a variety of different shapes (spherical, tubular, irregular), sizes, forms (fused, aggregated or agglomerated), compositions, and dimensions [4, 5].

Manufactured nanomaterials may exhibit different properties than their analogue bulk material (>100nm). As particle size decreases below 100 nm, the number of surface atoms increases rapidly, ultimately causing the particles to be extremely reactive [3, 6]. On the other hand, bulk material exhibits properties that depend on density, resistivity and dielectric constant with little contribution from the surface atoms. Large fraction of surface atoms, high surface energy and spatial confinement are among the major characteristics of MNMs that distinguish them from the bulk materials [7].
Fate and Transformation of Manufactured Nanomaterials

Manufactured nanomaterials are components of a steadily increasing number of consumer and industrial products, comprising a market which is expected to grow in size to over a trillion dollars by 2015 [5]. Metal and metal oxide MNMs are manufactured in thousands of tons for use in consumer products [8]. Unfortunately, these particles can be released into the environment during their manufacture, transport, and usage [9]. When MNMs from household products are released into wastewater, they will be subjected to a variety of wastewater treatment regimes. Several studies have demonstrated that about 80-99% of the MNMs found in wastewater partition to biosolids during wastewater treatment [10, 11]. Approximately 60% of biosolids are land applied in the United States, representing a pathway by which MNMs may accumulate in the soil over time [12].

Upon release, MNMs may undergo different physical and chemical reactions and transform into largely unknown end products (Figure 2) [13, 14]. A critical need exists to elucidate what these end products may ultimately be to help determine the potential for environmental implications. For example, several studies have shown that silver (Ag) MNMs are readily transformed to Ag$_2$S during the wastewater treatment process and have been found in wastewater effluent and sludge, in a pilot wastewater treatment plant [15], as well as in field sample sewage sludge [16]. Another recent study reported that zinc oxide (ZnO) MNMs rapidly transformed to ZnS and Zn$_3$(PO$_4$)$_2$ during anaerobic digestion of wastewater and post treatment processing of sewage sludge [17]. These transformations are expected to dramatically alter the fundamental nature of the MNMs
and will likely impact their bioavailability, toxicity, and mobility in terrestrial ecosystems [18].

**Figure 1-1:** Fate and possible environmental modifications of MNMs

Soluble MNMs will release ions via dissolution [19, 20]. Solubility will be affected by MNMs intrinsic properties as well as solution chemistry. For example, smaller MNMs will have a higher rate of dissolution compared to the larger MNMs [21, 22]. Solubility of metal oxide MNMs in solvents such as water is found to be low [23], but can be enhanced at low pH and by the presence of natural organic matter (NOM) [24].

Even though MNMs are produced with initial dimensions less than 100 nm, they will aggregate and produce much larger, colloidal aggregates in many natural waters [25]. Aggregation and sedimentation are the primary processes that control or limit the transport of MNMs in the environment [25]. The rate and extent of aggregation are influenced by the pH and ionic strength of the MNM environment, as well as by MNM
intrinsic properties such as particle size [25-29]. Increased ionic strength, particularly presence of divalent cations, increases the rate and extent of aggregation [26]. Nanomaterials may also be transformed in the environment as a result of the surface adsorption of biologic macromolecules such as NOM. Adsorption of NOM molecules to MNM surfaces has been shown to affect surface charge and aggregation state [25, 27, 29]. The influence of NOM on colloidal stability has been shown to be a function of multiple processes which are likely occurring simultaneously and depend on the type and chemical composition of the aqueous system such as pH and electrolyte composition [29, 30]. The degree to which MNMs will interact with biological organisms remains unclear. These interactions could occur as bioaccumulation, biodegradation, or biotransformation. Many studies have been published demonstrating bioaccumulation of MNMs by plants [31, 32], earthworms [37], and insects [33]. Other studies have reported biodegradation of dextran-coated magnetite MNMs after uptake by rats [34], biotransformation of ZnO and CeO$_2$ in soybean plants [35], and biotransformation of nickel hydroxide MNMs by mesquite plants [36]. The environmental fate and toxicity of MNMs could be influenced by a number of factors. Variations in MNM physicochemical properties such as size, solubility, aggregation state, elemental composition, mass, shape, crystal structure, surface area, and charge are likely to influence potential fate and toxicity [1]. The chemical properties of the environment into which the MNMs are discharged including pH, electrolyte valence, and presence of NOM will also likely be influential [25].
Zinc oxide

Zinc oxide MNMs are one of the most extensively used types of nano metal oxide particles in consumer products. ZnO can occur in two crystal forms: hexagonal wurtzite structured ZnO (also known as “zincite”) and a cubic blend structured ZnO [37]. Hexagonal ZnO is the most stable and most common structure in the ambient temperature.

Table 1-1: Properties of wurtzite (Pearton, Norton et al. 2005)

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lattice parameters at 300K</td>
<td></td>
</tr>
<tr>
<td>a₀</td>
<td>0.32495nm</td>
</tr>
<tr>
<td>c₀</td>
<td>0.52069nm</td>
</tr>
<tr>
<td>a₀/c₀</td>
<td>1.602</td>
</tr>
<tr>
<td>u</td>
<td>0.345</td>
</tr>
<tr>
<td>Density</td>
<td>5.606g/cm³</td>
</tr>
<tr>
<td>Stable phase at 300K</td>
<td>Wurtzite</td>
</tr>
<tr>
<td>Melting point</td>
<td>19750°C</td>
</tr>
<tr>
<td>Thermal conductivity</td>
<td>0.6, 1-1.2</td>
</tr>
<tr>
<td>Static dielectric constant</td>
<td>8.656</td>
</tr>
</tbody>
</table>

Zinc oxide MNMs are of particular interest due to their properties conducive to commercial applications including useful optical and electrical properties, chemical stability towards air, and significant quantum confinement effects [38-40]. For example, ZnO exhibits broad spectrum ultraviolet (UV) blocking and visible transparency when particles are less than 100 nm. As a result, they are widely used in personal care products such as sunscreens and cosmetics [41, 42]. ZnO MNMs are also being used to manufacture solar cells, electronic devices, photodiodes, antifungal agents, and catalysts [40]. Given the high volumes of being manufactured and used, the potential exists for humans, ecological receptors, livestock, and food crops to be exposed to ZnO MNMs.
Bioavailability and Toxicity of ZnO Nanomaterials

Two recent modeling efforts predicted that ZnO MNMs were present in US wastewater treatment plant effluent at 0.22µg L\(^{-1}\) and 0.74µg L\(^{-1}\) (2008), concentrations which may pose a toxicological threat to aquatic organisms [43, 44]. Also, Gottschalk et al. (2009) estimated that based on current usage, the concentration of ZnO MNMs in biosolids increased from 2 to 22 µg kg\(^{-1}\) in sludge treated soil, between 2005 and 2012, although this estimate is based on assumptions that likely underestimate the true concentrations. Toxicity of ZnO MNMs can occur through several different processes. Small particle size and large reactive surface area can lead to toxicological injuries due to production of reactive oxygen species and oxidative stress [45, 46]. Toxicity may also be the result of MNM protein binding that changes the structure of the protein causing adverse biological outcomes [47]. A third mechanism of toxicity is through the release of Zn\(^{2+}\) through dissolution in the media, or within tissues [48, 49].

The toxicity of ZnO MNMs in animals has been studied both in vitro [48, 50-52] and in vivo [53-55] and has consistently been found to be highly toxic compared with other MNMs [48, 51, 53, 56]. Studies indicate that toxicity is the result of both the dissolution and release of Zn\(^{2+}\) ions as well as via particle specific effects [53]. It is still unclear what is the primary source of toxicity and a recent study suggests that different mechanisms are involved with the toxicity with these two species [57]. In plants, it has been observed that, ZnO MNMs cause toxicity by disrupting cellular homeostatis [48] or by inducing reactive oxygen species production [48]. As an example studies indicate that ZnO has caused reduced biomass and growth of wheat seeds [58], affected of root
elongation of ryegrass, radish and rape [59]. Seed germination on corn was affected by ZnO MNMs at a concentration of 2000 mg L$^{-1}$ [59].

**Impact of transformations to toxicity**

According to the literature, sulfidation [60], phosphate induced transformation [17], and stabilization by NOM [61] are the most common types of transformations of ZnO MNMs in wastewater. These transformations alter particle size, chemical composition, and reactivity and the transformed products have been shown to result in highly insoluble products (in the case of sulfidation and phosphatation) [60, 62]. As result that is likely to reduce bioavailability and toxicity [17, 62]. Still, the impacts and toxicity of these transformed materials on plants and other aquatic organisms are poorly understood.

**Characterization techniques**

**Electron microscopy**

Transmission electron microscopy (TEM) can produce images of MNMs, providing information such as shape and size with resolutions as small as 1 nm [63]. A beam of electrons is transmitted through a sample dispersed on a support grid, then magnified and focused by a magnetic objective lens. It is also possible to visualize crystallographic structures with high resolution (HR) -TEM. However, unlike the scanning electron microscope, TEM can produce only two dimensional images [64]. Also, sample preparation includes drying samples on a grid which may lead to changes in particle aggregation [64].
Elemental analysis and chemical composition information of a MNM sample can be obtained with a very high spatial resolution by coupling the HR-TEM with energy dispersive spectroscopy (EDS). The elemental composition of a sample is detected by exciting sample atoms inner shell electrons using an electron beam, generating vacancies in the inner shell. These vacancies are then filled with outer shell electrons, which then emit X-rays characteristic of specific elements [65, 66]. Unfortunately, this technique is not very sensitive, and it provides strictly elemental composition [65].

**Dynamic Light scattering**

Geometric structure and state of motion of small particles can be detected through dynamic light scattering [67]. Routine measurements of this type are often taken with a correlation spectrophotometer. Within this instrument, a detector is placed at a fixed distance at an angle with respect to the direction of an incident light beam. When particles move under Brownian motion, scattered light from the particles varies in intensity with time due to variation constructive and destructive interference. The autocorrelation function can be related to translational diffusion coefficient. This coefficient converted to hydrodynamic radius using the Einstein-Stokes relationship [67]. Often same instrument includes optics and electrodes for performing phase analysis light scattering (PALS). Velocity of the particles under an electric field is measured by measuring the difference in the phase of scattered light relative to the incident beam to calculate the zeta potential [64].
Mass Spectroscopy

Inductive coupled plasma mass spectroscopy (ICP-MS) is a powerful technique with the ability for multi-elemental trace analysis. The main process of the ICP-MS includes sample introduction and aerosol generation, ionization by an argon (Ar) plasma, mass discrimination and finally detection [68]. Aqueous samples are introduced through a nebulizer aspirating the sample with high velocity Ar to form a fine mist. One of the major advantages of ICP-MS is, its speed sensitivity and low detection limits [69].

X-ray techniques

X-ray absorption near edge spectroscopy (XANES) is a widely used characterization technique in nano research that provides information on the local electronic structure of constituent elements. The XANES region of a X-ray absorption spectrum is comprised of the energy region ranging from just below to 50eV above the absorption edge [70]. This technique utilizes the difference in absorption of atoms that arise from interference patterns of photoelectrons scattered from neighboring atoms. High elemental specificity and high spatial resolution are some of the advantages of this technique [70]. This technique can be used to characterize both crystalline and amorphous material [71]. Limited accessibility to synchrotron facilities and the requirement of expert knowledge are among the limitations.

Structural information about a crystalline compound can be obtained by X-ray diffraction (XRD). Information is obtained through passing an X-ray beam through the sample and analyzing the diffracted beam using Bragg’s Law to calculate the spacing between the diffraction planes of the material. Amorphous structures cannot be detected by XRD [72].
Nuclear magnetic resonance

Magnetic resonance occurs when nuclei are subjected to external magnetic field due to the quantum mechanical property of “spin”. Once an external magnetic field is applied, these nuclei align with or against the applied magnetic field. An electromagnetic field is applied and the nuclei absorb and re-emit this energy depending on their environment. This generates signals with information related to the chemical environment, which are subsequently converted into a frequency system and assigned as chemical shift relative to accepted standards [73]. Since these chemical shift values are characteristic of specific nuclear environments, they can be used to identify the structure and composition of the compound. Information for structure determination such as chemical bonds between atoms in a molecule can be obtained through coupling between NMR active nuclei.

Objective/Research outline

Once released to the environment, ZnO MNMs will undergo biogeochemical transformations which control their physicochemical and surface properties and produce stable altered states. The main objective of this study was to understand the physico-chemical transformations of MNMs and evaluate the impact of the transformations on toxicity to *Triticum aestivum*. This study proposes to examine the reaction kinetics between ZnO and both inorganic phosphate (PO$_4^{3-}$) and NOM through a simulated aging process and to subsequently characterize the transformation products. The results of this study will be among the first data characterizing the transformation end products of ZnO MNMs as well as their phytotoxicity, information that will be vital in assessing the potential environmental risk posed by ZnO MNMs.
Chapter two: A multi-technique investigation of phosphate induced transformation of
ZnO nanoparticles

Sewwandi Rathnayake\textsuperscript{1,2}, Jason M. Unrine\textsuperscript{1,2}, Jonathan Judy\textsuperscript{1,2}, Anne-Frances Miller\textsuperscript{3},
William Rao\textsuperscript{1}, Paul M. Bertsch\textsuperscript{1,2,4}

1. Department of Plant and Soil Sciences, University of Kentucky, Lexington, Kentucky, USA
2. Center for Environmental Implications of Nanotechnology, Duke University, Durham, North Carolina, USA
3. Department of Chemistry, University of Kentucky, Lexington, Kentucky, USA
4. Division of Land and Water, CSIRO, Ecosciences Precinct, Brisbane, Australia

Summary
In order to properly evaluate the ecological and human health risks of ZnO manufactured nanomaterials (MNMs) released to the environment, it is critical to understand the likely transformation products in the wastewater treatment process and in soils receiving biosolids. To address this critical knowledge gap, we examined the transformation reactions of 30 nm ZnO MNMs in the presence of different concentrations of phosphate as a function of time and pH using a variety of orthogonal analytical techniques. The data reveal that ZnO MNMs react with phosphate at concentrations expected in wastewater and transform them into two distinct morphological/structural phases: a micron scale crystalline zinc phosphate phase (hopeite) and a nano-sized phase that likely consists of a ZnO core with a Zn\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} rich shell. The Zn species composition was pH dependent, with 82% occurring as hopeite at pH 6 while only 15% occurred as hopeite at pH 8. These results highlight how reactions of ZnO MNMs with phosphate can occur through a variety of mechanisms depending on pH, and the chemical conditions of the discharge.
environment, resulting in heterogeneous structural/morphological characteristics of the transformation products.

**Introduction**

Manufactured nanomaterials (MNMs) are increasingly being employed in consumer products such as pharmaceuticals, biomedical, cosmetics, and electronics, exploiting their novel physical and chemical properties [9, 74]. The commercialization of nanotechnology is expanding rapidly and is expected to be a $1 trillion (U.S. dollars) industry by 2015 [5]. However, MNMs can enter the environment during their manufacturing, transport, use, and disposal [17, 75] and several studies have demonstrated that MNMs will be released into wastewater streams during their use from consumer items, such as textiles and personal care products [58, 76, 77]. Life-cycle inspired material flow analysis has indicated many MNMs will partition to biosolids during wastewater treatment and that soil will be a primary repository for MNMs in areas where biosolids are land applied [9, 77]. The potential risks to environmental and human health posed by MNMs deposited into terrestrial ecosystems in this manner are not fully understood.

Once discharged into the environment, MNMs will be subjected to dynamic physical and chemical conditions which will result in transformation to largely unknown end products [14]. For example, several studies have shown that silver (Ag) MNMs are readily transformed to Ag$_2$S during the wastewater treatment process and Ag$_2$S nanoparticles have been found in wastewater effluent and sludge in a pilot wastewater treatment plant [15], as well as in field sample sewage sludge [16]. Recently, Lombi et al. (2012) [17]
reported that ZnO MNMs rapidly transformed to ZnS and Zn$_3$(PO$_4$)$_2$ during anaerobic digestion of wastewater and post treatment processing of sewage sludge. These transformations are expected to dramatically alter the fundamental properties of the MNMs and will likely impact their bioavailability, toxicity, and mobility in terrestrial ecosystems. Therefore, characterizing the nature of these transformations is essential in assessing the potential risk to the environment [18].

Zinc oxide nanoparticles are among the highest volume MNMs used in consumer products due to their widespread use in semiconductors, pharmaceuticals, paints, personal care products, and sunscreens [43, 44, 78, 79]. Modeling efforts predict ZnO MNMs will be present in wastewater treatment plant effluent in the United States at concentrations of between 0.22 to 0.74 µg L$^{-1}$ as estimated in in 2008 that they may pose a toxicological risk to aquatic organisms [43, 44]. Additionally, Gottschalk et al. [43] estimated that, based on current usage, the concentration of ZnO MNMs in biosolids increased from 2µg kg$^{-1}$ to 22 µg kg$^{-1}$ in sludge treated soil between 2005 and 2012 [43]. The bioavailability and toxicity of ZnO MNMs discharged into terrestrial ecosystems via this pathway is not well understood.

Phosphates are a major constituent of wastewater. Phosphorous input to the municipal wastewater has increased since 1950 as a result of the use of laundry detergents, household cleaning products, cosmetics, and medicated shampoos [80]. A recent study revealed that the interactions between ZnO MNMs and different concentrations of phosphate resulted in the transformation of ZnO MNMs into Zn$_3$(PO$_4$)$_2$ [62]. However, this study was conducted at neutral pH, and according to the literature wastewater pH varies mainly between 6-8 [81], leaving a knowledge gap regarding the influence of
solution chemistry for the final product and the transformation process. The present study differs from previous studies in several key areas. We evaluated the transformation process at different pH values, (both acidic and alkaline conditions), over different time periods (ranging from 24 h to 7 d) and characterized the transformation products (aged MNMs) more extensively using a variety of analytical techniques. Characterization techniques included: X-ray absorption near edge structure (XANES) spectroscopy, X-ray diffraction (XRD), $^{31}$P nuclear magnetic resonance (NMR) spectroscopy, high resolution thermo gravimetric analysis (TGA) and transmission electron microscopy-energy dispersive spectroscopy (TEM-EDS). The results revealed that the chemical speciation and physical form of the reaction products are highly dependent upon pH, phosphate concentration and reaction time.

Methods and materials

Dispersion and initial characterization of ZnO MNMs

Uncoated ZnO-Nanosun MNMs with a nominal particle diameter of 30 nm were obtained by a commercial producer (Micronisers, Melbourne, Victoria, Australia). The ZnO MNMs were dispersed in the various reaction solutions at 100 mg L$^{-1}$ concentration by ultrasonication using a probe sonicator (Misonix, Newtown CT, USA) [74]. Samples were dispersed inside 50 ml polypropylene centrifuge tubes delivering about 2150 J of energy during each sonication treatment. Mean hydrodynamic diameters and electrophoretic mobilities of the MNM suspensions were measured using a Nano-ZS zetasizer (Malvern, Worcestershire, UK). Primary particle size was measured by TEM by drying the particles on 200 mesh formvar/carbon coated Cu TEM grids (Ted Pella,
Redding, CA) and the diameters of 100 randomly selected individual particles were quantified with Image J software (http://rsb.info.nih.gov/ij/).

**Aging with phosphate**

To examine transformations over a pH range typical of wastewaters, 0, 5, 10, 50, 150 mg L\(^{-1}\) \(\text{Na}_2\text{HPO}_4\) solutions were adjusted to pH 6 ± 0.2 or pH 8 ± 0.2 with HCl and NaOH. Then, ZnO MNMs were dispersed in the phosphate solutions at a concentration of 100 mg L\(^{-1}\) and incubated at 25\(^{0}\)C ± 0.1 on a shaker table for 24 h, 72 h or 7 d. Following incubation, an aliquot was removed to measure the mean hydrodynamic diameter and electrophoretic mobility of the aged particles. Samples were then centrifuged at 3250 x g for 2 h to separate the solid fraction from the supernatant. The separated solids were lyophilized and stored at room temperature for analysis. Supernatants were acidified to 0.15 M HNO\(_3\) for analysis of dissolved Zn concentrations by inductively coupled plasma mass spectrometry (ICP-MS).

**Dissolved Zn analysis**

Dissolved Zn\(^{2+}\) concentrations in the supernatants were quantified using an Agilent 7500cx ICP-MS (Santa Clara, CA, USA). Analytical runs contained calibration verification samples, duplicate dilutions, and spike recovery samples. Spike recovery averaged 97.7% ± 6.9 (n=4) and the mean relative percent deference (RPD) between duplicate dilutions was 1.0% ± 2.5 (n=4). Three replicates were prepared for all treatment combinations. Visual MINTEQ was used to predict the thermodynamic speciation and solubility of ZnO as a function of phosphate concentration and pH.
Synchrotron-based X-ray absorption near edge spectroscopy (XANES)

Lyophilized powders were ground with a mortar and pestle and homogenously distributed onto Kapton or cellophane tape for analysis by X-ray absorption near edge spectroscopy (XANES) attempting to eliminate the presence of any particles larger than 1 absorption unit thickness at the Zn K-edge (47 µm). Zinc K-edge XANES spectra were collected at beamline X-26A of the National Synchrotron Light Source, Brookhaven National Laboratory (Upton, New York). The beam was focused to a spot size of approximately 10 x 10 µm$^2$ as previously described [82]. XANES spectra were collected in transmission mode by scanning the monochromator from 9,600 to 9,800 eV. Layers of tape coated with samples and standards were combined to achieve samples that were 1 ± 0.3 absorption units and to eliminate any pinholes or voids in the samples. The energy range was scanned as follows: 9,600 - 9,645 eV in 5 eV steps, 9,645 - 9,710 eV in 0.3 eV steps, 9,710.5 - 9,726 eV in 0.5 steps and 9,726 – 9,800 eV in 0.5 eV steps. The XANES spectra were analyzed using the Athena software package [83]. After normalization, linear combination fits (LCFs) were performed using Zn foil, ZnO MNM, and Zn$_3$(PO$_4$)$_2$ as standards. We verified the quality of the fits by analyzing standards containing known proportions of ZnO and Zn$_3$(PO$_4$)$_2$ performing LCFs.

X-ray diffraction (XRD)

Powder XRD analysis of aged particles performed using an X’Pert PRO MPD (PANalytical, B.V., Almelo, Netherland) diffractometer using a CuKα ($\lambda = 0.154$nm) radiation source in the 2θ scanning range of 2-60$^0$. The XRD patterns were refined according to the high score method with the material analysis using the diffraction
(v.3.0.5) program package [84]. Crystalline phases were identified by comparing experimental data with JCPDS files from the International Center for Diffraction Data.

**Thermogravimetric analysis (TGA)**

Thermogravimetric analysis was performed using a Discovery high resolution thermogravimetric analyzer (TA Instruments, New Castle, Delaware, USA). Approximately 10 mg of sample was weighed into a platinum pan and the weight loss behavior of each sample was measured by heating the sample from 40°C to 1,000°C at 50°C per minute using a high resolution dynamic program.

**Solid state $^{31}$P Nuclear Magnetic Resonance (NMR)**

Samples of 100 - 200 mg were packed into 5 mm zirconia NMR rotors. $^{31}$P magic angle spinning (MAS) spectra were collected at 162 MHz for $^{31}$P (400 MHz for $^1$H) in a 5 mm HX Chemagnetics-type probe using a Varian Inova spectrometer. Unbiased spectra of all $^{31}$P sites were collected via direct polarization (DP) of $^{31}$P using a 5 μs 90° excitation pulse, 80 ms data acquisition with $^1$H decoupling at 50 kHz [85], and a 100 s delay between scans unless otherwise noted. Spectra collected at MAS speeds of 3 kHz, 4 kHz, 5 kHz and 6 kHz were used for measurement of spinning side band intensities. These were used to calculate the principal values of the $^{31}$P chemical shift tensors for each spectral component via Herzfeld-Berger analysis using the software of K. Eichele [86, 87]. Averages of the principal values obtained at three different MAS speeds are reported in Table 1. All $^{31}$P chemical shifts are quoted relative to phosphoric acid (≥85% Fisher A260-500) which was run separately, non-spinning. Spectra obtained at spinning
speeds of 6 kHz were used for deconvolution of contributing signals, using Agilent's VnmrJ 3.2 software. The T1s of each of the 31P signals were evaluated by varying the delay between scans from 900 s to 4 s in 9 steps and fitting the resulting signal amplitudes to the function A=M₀(1-e⁻ᵀ/ᵀ₁) where 'A' is the signal amplitude obtained using an interscan delay of 't', M₀ is the maximum amplitude expected after infinite delay and T₁ is the longitudinal relaxation time.

Spectra emphasizing 31P sites near to ¹Hs were obtained with signal enhancement via ramped cross polarization (CP) from ¹H at a CP field of 50 kHz for ¹H, [88] and ¹H TPPM2 decoupling at 50 kHz during the 80 ms or 50 ms acquisitions [85]. The delay between scans was 20 s. The CP contact time was varied from 0.1 ms to 18 ms in 12 steps to characterize polarization buildup and decay at the different 31P sites due to ¹H-31P cross relaxation and ¹H T₁H,ρ. Signal amplitudes were fit with the expression A = M₀(1-e⁻ᵀ/cp) where τcp is the characteristic time of the buildup of 31P magnetization based on CP from ¹H and T₁H,ρ is the ¹H rotating frame longitudinal relaxation time at a CP field strength of 50 kHz (as used here) [89].

Results and discussion

Solubility of MNMs in phosphate solutions

Measurements of supernatants revealed that the concentrations of dissolved Zn²⁺ in phosphate after 24 h were 23.44 mg L⁻¹ and 18.84 mg L⁻¹ for the pH 6 and 8 treatments, respectively (Figure 1). Concentrations of Zn²⁺ decreased both over time and with increasing phosphate concentration at both pH 6 and 8, providing evidence for transformations of the ZnO that decreased their solubility. The Zn²⁺ concentrations after
72 h and 7 d at both pH 6 and 8 were near thermodynamic equilibrium, as modeled using MINTEQ, suggesting a rapid chemical transformation. The results of our modeling efforts suggest that the dissolution of ZnO MNMs results in an over saturation of Zn$^{2+}$ with respect to a solid phase of Zn$_3$(PO$_4$)$_2$.4H$_2$O (hopeite) at both pH 6 and pH 8.

**Figure 2-1:** Concentrations of dissolved Zn$^{2+}$ as a function of phosphate concentration, pH, and time. Each value represents the mean of three replicates with standard deviation shown by error bars.

**XANES data**

Linear combination fit analysis of Zn K-edge XANES spectra for the solid phases formed during incubation indicates that the ZnO MNMs and phosphate reacted to form Zn$_3$(PO$_4$)$_2$ in various proportions (**Figure 2**). The proportion of Zn$_3$(PO$_4$)$_2$ formed was a function of pH, phosphate concentration and reaction period. **Figure 2** indicates that aging at pH 6 with higher phosphate concentrations for longer reaction times yielded a greater proportion of Zn$_3$(PO$_4$)$_2$. At lower phosphate concentrations (5 mg L$^{-1}$), less than 30% of Zn is present as Zn$_3$(PO$_4$)$_2$ at both pH values. However, at pH 6 more than 60% of Zn is
present as Zn$_3$(PO$_4$)$_2$ at 150 mg L$^{-1}$ phosphate regardless of the reaction time, which is consistent with the previous solubility and modeling data. On the other hand, the pH 8 treatment had 30% or less Zn$_3$(PO$_4$)$_2$, even at 150 mg L$^{-1}$ phosphate concentration in the aging solution.

**Figure 2-2**: The quantitative distribution of aged material between ZnO and Zn$_3$(PO$_4$)$_2$ estimated from linear combination fit (LCF) analysis of XANES spectra MNMs aged in pH 6 (a) and MNMs aged in pH 8 (b).

**XRD data**

XRD analysis revealed sharp, intense diffraction peaks at $2\theta = 31.7$, 34.4 and 36.1 in all aged MNM samples (Figure 3), which are characteristic of the presence of pristine crystalline ZnO MNMs (hexagonal pdf 01-071-6424). In addition to ZnO, at pH 6 orthorhombic hopeite was indicated by peaks at $2\theta$ values of 9.6, 16.6, 17.3, 18.2, 19.3, 20.08, 22.18, 26.17, 31.69 and 46 (pdf 00-037-0465). Surprisingly, at pH 8 we did not observe evidence of crystalline Zn$_3$(PO$_4$)$_2$ in any of the XRD data (Figure 3a). Since
XANES can detect amorphous or crystalline phases, while XRD is only sensitive to crystalline phases, this indicates that the Zn$_3$(PO$_4$)$_2$ formed at pH 8 is amorphous.

**Figure 2-3:** XRD spectra ZnO MNMs (a) particles aged in 150 mg L$^{-1}$ phosphate concentration for 72 h at pH 8 (b) and pH 6 (c) Zn$_3$(PO$_4$)$_2$ (d) and hopeite (e)

**Surface charge of the aged particles**

The zeta potentials calculated from electrophoretic mobility data are shown in **Figure 4a** and **4b**. These data reveal that at both pH values, zeta potential decreases and becomes asymptotic at about -70 mV at phosphate concentrations greater than 50 mg L$^{-1}$. This indicates that, as phosphate concentration increase, surface charge becomes increasingly negative at both pH 6 and pH 8 (**Figure 4**). This could be due to surface sorption of phosphate onto ZnO or due to complete transformation to Zn$_3$(PO$_4$)$_2$. Such an increase in net negative surface charge may stabilize the particles in aqueous environments.
Size of the aged particles

TEM analysis of the un-altered ZnO MNMs used in this study revealed that individual particles were $\sim 34.18 \pm 6.8$ nm in diameter. The $z$-average (intensity weighted) diameter of the particles as measured by Dynamic light scattering (DLS) in DI water was $243 \pm 20$ nm, indicating that at least some aggregates were present.

TEM micrographs of aged particles revealed the presence of two distinct particle morphologies (Figure 5): a micro sized phase with a mean diameter of $1.42 \pm 0.6$ µm and a nano sized phase with a mean diameter of approximately $29.5 \pm 9.1$ nm. EDS of the micro sized phase (EDS, see appendix, Figures A1-A3) indicated a composition of $\sim 35\% \pm 2\%$ P and $64\% \pm 2.6\%$ Zn, consistent with the stoichiometry of $\text{Zn}_3(\text{PO}_4)_2 \cdot 4\text{H}_2\text{O}$. The EDS data of the nano-sized phase revealed a composition of $\sim 6\% \pm 0.7\%$ P and $93\% \pm$
2.1 Zn. No P was detected in EDS spectra collected from the pristine ZnO MNMs. The mean diameter 29.5± 9.1 nm of the nano phase as determined from the TEM images (Figure 5b) is similar in size to the pristine ZnO MNMs. Taken together, the zeta potential data, the TEM images, and the EDS data characterizing the nano phase suggests the formation of a ZnO-Zn₃(PO₄)₂ core-shell structure (Appendix Figure A5).

![Figure 2-5: TEM images of pristine ZnO MNMs (a) and MNMs aged in 150 mg L⁻¹ phosphate concentration for 72 h at pH 6 (b) at pH 8 (c).](image)

**TGA data**

Particles aged at 150 mg L⁻¹ for 72 h had a total weight loss of 34.71% at pH 6 but only 6.6% in the pH 8 treatments. A major weight loss peak was observed between 75° C and 100° C in both treatments, which is likely due to water loss. (Appendix Figure A4). In the pH 6 treatments, two other major weight losses were observed at approximately 135° C and 330° C These weight losses are likely the result of the loss of crystallization water at the lower temperature and either demineralization or polycondensation at 330° C [90].
NMR data

$^{31}$P Solid-State NMR (SSNMR) spectra of samples aged at pH 6 or pH 8 in 150 mg L$^{-1}$ phosphates for 72 h are compared in Figure 6. The numbers and intensities of spinning side bands extending at 4000 Hz (24.7 ppm) intervals in both direction reflects the anisotropy of the different signals (left side of Figure 6), while the isotropic chemical shifts of the different components of each sample are distinguished in a horizontal expansion of the central region (right side of Figure 6). The spectrum of a sample of hopeite ($\text{Zn}_3(\text{PO}_4)_2\cdot4\text{H}_2\text{O}$) is included in both panels for comparison.

![Figure 2-6: Comparison of NMR spectra of hopeite with materials aged at pH 6 vs. at pH 8 in 150 mg L$^{-1}$ phosphates. Spectra shown were obtained at a MAS speed of 4 kHz with direct polarization of $^{31}$P and $^1$H decoupling (TPPM2 at 50 kHz) during 80 ms acquisition times at room temperature with delays of 100 s between scans for hopeite and the material aged at pH 6 but 200 s for the material aged at pH 8.](image)
Table 2-1: Isotropic chemical shifts ($\delta_{iso}$) and tensors ($\delta_{11}$, $\delta_{22}$, and $\delta_{33}$), $T_1$ relaxation times, cross-polarization buildup times ($\tau_{cp}$) and $T_{1H,\rho}$ relaxation times for ZnO MNMs aged at pH 6 and pH 8 and standards.

<table>
<thead>
<tr>
<th>Species</th>
<th>$\delta_{iso}$ (ppm)</th>
<th>$\delta_{11}$ (ppm)</th>
<th>$\delta_{22}$ (ppm)</th>
<th>$\delta_{33}$ (ppm)</th>
<th>span (ppm)</th>
<th>$T_1$ (s)</th>
<th>$\tau_{cp}$ (ms)</th>
<th>$T_{1H,\rho}$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6 B</td>
<td>8.0</td>
<td>46.0 ± 0.5</td>
<td>-7 ± 4</td>
<td>-15 ± 4</td>
<td>61 ± 4</td>
<td>102</td>
<td>1.2</td>
<td>26</td>
</tr>
<tr>
<td>pH 6 A</td>
<td>4.3</td>
<td>38.6 ± 0.3</td>
<td>1.2 ± 0.4</td>
<td>-27.0 ± 0.1</td>
<td>66 ± 0.3</td>
<td>67</td>
<td>.65</td>
<td>5.7</td>
</tr>
<tr>
<td>hopeite</td>
<td>4.2</td>
<td>38.8 ± 0.4</td>
<td>2.5 ± 0.3</td>
<td>-28.6 ± 0.1</td>
<td>67.4 ± 0.6</td>
<td>33</td>
<td>.67</td>
<td>24</td>
</tr>
<tr>
<td>pH 8 A</td>
<td>4.5</td>
<td>37.65 ± 0.07</td>
<td>1.3 ± 0.3</td>
<td>-25.5 ± 0.4</td>
<td>63.2 ± 0.4</td>
<td>67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 8 B</td>
<td>5.3</td>
<td>33 ± 2</td>
<td>-9 ± 1</td>
<td>-9 ± 2</td>
<td>42 ± 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 8 D&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.8</td>
<td>39 ± 2</td>
<td>6 ± 2</td>
<td>-21.6 ± 0.6</td>
<td>60.1 ± 0.9</td>
<td>71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{Zn}_3(\text{PO}_4)_2$</td>
<td>3.9</td>
<td>39.8 ± 0.2</td>
<td>6.7 ± 0.7</td>
<td>-34.9 ± 0.5</td>
<td>74.7 ± 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha$-$\text{Zn}_3(\text{PO}_4)_2$&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.9</td>
<td>37.0</td>
<td>6.4</td>
<td>-31.7</td>
<td>68.7</td>
<td>1948</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-$\text{Zn}_3(\text{PO}_4)_2$&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.6</td>
<td>27.7</td>
<td>3.8</td>
<td>-8.8</td>
<td>36.5</td>
<td>946</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Data for the particles aged at pH 6 and hopeite were obtained using 100 s delays between direct polarization $^{31}$P scans or 20 s between cross-polarized scans and
acquisition times of 80 ms. Data for particles aged at pH 8 were obtained similarly except that they employed 200 s delays between direct polarization scans.

b pH 8 C: a fourth component is needed to describe the spectrum of material aged at pH 8 in 150 mg L\(^{-1}\) phosphate. However due to extensive overlap we do not have a unique description for it at present. A shoulder is seen at 7.1 ppm (Figure 6) but simulation of the pH 8 150 mg L\(^{-1}\) spectrum with four components yields best agreement when the fourth component included is broad and centered at 10.6 ppm (data not shown).

c Our isotropic chemical shifts ranging from 4 - \(\approx\)10 ppm and spans smaller than 70 ppm demonstrate that the phosphate is orthophosphate not a polyphosphate, as Roming et al. [91] report isotropic shifts of 3.9 ppm and 7.6 ppm associated with spans of 68.7 ppm and 36.4 ppm for \(\alpha\)- and \(\beta\)- Zn\(_3\)(PO\(_4\))\(_2\), respectively but spans greater than 80 ppm for most of the components of their Zn\(_2\)P\(_2\)O\(_7\) samples 80.7, 84.4, 84.6, 81.2, 99.1 and 69.3 ppm. Similarly, our relatively modest signal spans are most consistent with un-protonated PO\(_4^{3-}\) groups, as Rothwell et al. [92] found that for Ca\(^{2+}\) salts, HPO\(_4^{2-}\) and H\(_2\)PO\(_4^{-}\) have broader spans of 123 ppm and 127 ppm for HPO\(_4^{2-}\) species and 97 ppm, 125 ppm for H\(_2\)PO\(_4^{-}\) species vs. 34 ppm and 33 ppm for PO\(_4^{3-}\) species.

The multiplicity of signals in each sample is expected. For example, He et al. [93] demonstrated that even samples of ostensibly chemically homogeneous phosphate salts with sodium, magnesium, calcium or zinc produced multiple SSNMR signals, as observed here. We obtained four distinct signals of comparable amplitude from commercial zinc phosphate, which proved heterogeneous by other methods as well (not shown). Therefore the current work employs hopeite as a reference point instead, because
it proved much more homogeneous (Figure 6). Furthermore, the breadth of the $^{31}$P central lines, especially in the sample aged at pH 8, indicate that the material is heterogeneous at an atomic scale.

We estimated the quantities of each the different species present in each sample based on deconvolution and simulation of the central band of spectra obtained at 6 kHz MAS, since the different signals observed have comparable spans and thus similar fractions of total intensity in the central band. For this exercise, spectra obtained upon direct polarization were employed, in order to avoid potential errors introduced by different efficiencies of cross-polarization of different species, and a long delay was employed between scans to avoid biasing the spectra in favor of faster relaxing signals.

Deconvolution and simulation of the spectrum of material aged at pH 6 revealed that the material is dominated by two species: the signal at 8.0 ppm accounts for 18% of the $^{31}$P in the material while the signal at 4.3 ppm accounts for 82% of the material. Deconvolution and simulation of the spectrum of material aged at pH 8 required considerations of four species. Three broad components centered at 10.6 ppm, 7.9 ppm and 5.4 ppm were obtained accounting for 10%, 4% and 27% of the $^{31}$P in the material, respectively. A sharper signal was obtained at 4.5 ppm accounting for 15% of the $^{31}$P in the material. The latter is most likely due to hopeite-like sites since not only its isotropic chemical shift, but even its three principal values agreed qualitatively with those of hopeite ($\delta_{33}$ in particular proved diagnostic, see Table 1). It is not surprising that the three spectra do not produce signals at identical chemical shifts, since the $^{31}$P signals of phosphate are responsive to pH [93] and the two aged particles were isolated from different pHs while the pH from which the hopeite sample was isolated is not known.

27
Thus, the NMR spectra indicate that material aged at pH 6 is 82% hopeite-like while only 15% of the material aged at pH 8 is hopeite-like, in agreement with the findings by other methods.

The NMR spectra provide additional detail regarding particles aged at pH 8, showing that one reason for the low hopeite formation is that a greater number of other species were formed in competition. Whereas the species with the chemical shift of 8.0 ppm (Figure 6B) was the only by-product at pH 6, 47% of the pH 8 material was accounted for by the analogous species (7.8 ppm) but 27% of the material was accounted for by a third component (5.3 ppm) not evident in particles aged at pH 6.

The signals we observe near 4.2 ppm bear qualitative similarities with that of α-Zn$_3$(PO$_4$)$_2$ with respect to both isotropic chemical shift (3.9 ppm) and individual principal values (See Table 1). Our $T_1$s were all considerably shorter than those reported by Roming et al. [91] for α-Zn$_3$(PO$_4$)$_2$, likely because our materials include water molecules (as revealed by TGA, see supplementary material) which place $^1$H near the phosphate $^{31}$P. Indeed Roming et al. [91] report a $T_1$ of 48 s for 'as-prepared' zinc phosphate, in the range of our values ranging from 33 to 67 s for the signal near 4.2 ppm we assign to hopeite.

The dynamics of cross-polarization from $^1$H to $^{31}$P and decay of the $^{31}$P signal provide additional qualitative information on the proximity of $^1$H to the different $^{31}$P sites and thus insight into the environment in which different $^{31}$P species exist in the different materials. In both the pH 6 and pH 8 aged materials from 150 mg L$^{-1}$ phosphate, the species with the chemical shift of 4.3-4.5 appears closer to more $^1$H since this signal
acquires intensity from $^1$H faster than the signal near 8 (Figure 7, right side). The signal near 4.3-4.5 ppm is also the feature that is stronger in spectra obtained using CP than in spectra obtained using DP. This conclusion is supported by the effect of $^1$H decoupling. Decoupling significantly sharpens the signal near 4 ppm but has a very modest effect on the signal near 8 ppm (Figure 7A). Thus, we assign the signal near 4 ppm to $^{31}$P nearer to more $^1$H.

**Figure 2-7:** Effect of $^1$H decoupling and cross-polarization on MNMs aged at pH 6 and pH 8. $^{31}$P spectra were collected following direct polarization (left panel) or cross-polarization from $^1$H for the time shown (right panel) prior to signal collection with $^1$H decoupling (right panel) or decoupling as indicated (left panel). DP spectra were collected at 6 kHz MAS with 100 s delays between scans and acquisition times of 80 ms for material aged at pH 6 and hopeite, but 50 ms of data acquisition for material aged at pH 8. CP spectra were collected with 20 s delays between scans and acquisition times of 80 ms for material aged at pH 6 and 50 ms for hopeite and material aged at pH 8.
The slow decay of the hopeite signal produces better separation of the growth and decay with the result that the growth is more complete when the signal reaches maximal intensity. In contrast, for the 4.3-4.5 ppm signals from the aged MNM, decay has already had significant impact on the signal intensity at the time we observe maximal signal intensity so the actual full intensity is not observed and the half-time for signal development is later than it appears. Thus, fits to the data indicate that the 4.3-4.5 ppm signal in MNM aged at pH 6 in fact has a buildup time similar to that of hopeite (Table 1).

However, the MNM signal at 4.3-4.5 ppm decays more rapidly than that of hopeite under cross-polarization, in behavior that is also clearly distinct from the slower decay of the signals near 8 ppm. We speculate that this reflects a lower amount of water in the hopeite sample overall, which is relatively homogeneous. In the aged MNM, we speculate that the $^{31}$P sites near $^1$H (4 ppm) experienced enhanced relaxation due to spin diffusion from a bath of $^1$H not directly connected to $^{31}$P sites and therefore not contributing efficiently to cross-polarization. This suggests the possibility that the signal near 4 ppm represents a shell exposed to exterior water whereas the signal near 8 ppm represents $^{31}$P with less access to the proposed bath.

**Environmental Implications**

This study reveals that the pH and concentration of phosphate in the wastewater will be influential in determining the final speciation, structure, and morphology of ZnO MNM transformation products depending on whether there is dissolution and re-precipitation of
Zn as $\text{Zn}_3(\text{PO}_4)_3$ (favored at pH 6) or surface modification of the ZnO particles with phosphate to form a core-shell structure (favored at pH 8).

Possible dissolution/precipitation reactions for ZnO MNMs that would result in re-precipitation of hopeite include.

1. $\text{ZnO}_{(s)} + \text{H}_2\text{O} \rightleftharpoons \text{Zn}^{2+}_{(aq)} + 2\text{OH}^-_{(aq)}$

2. $3\text{Zn}^{2+}_{(aq)} + 2\text{PO}_4^{3-} \rightleftharpoons \text{Zn}_3(\text{PO}_4)_2$$_{(s)}$

The data collected using seven orthogonal methods indicate that higher phosphate concentration, lower pH, and increased reaction time will allow greater formation of $\text{Zn}_3(\text{PO}_4)_2$. ZnO MNMs will undergo dissolution and precipitation reactions and dissolution is enhanced at an acidic pH. The dissolved material will then re-precipitate as two $\text{Zn}_3(\text{PO}_4)_2$ phases, hopeite and para-hopiete, which is consistent with previous studies [62]. However, XANES, $^{31}\text{P}$ NMR, and TGA data clearly indicate only minor amounts of $\text{Zn}_3(\text{PO}_4)_2$ formation occurs in MNMs aged at pH 8. The XRD data do not indicate the presence of crystalline $\text{Zn}_3(\text{PO}_4)_2$ in pH 8 aged treatments. We conclude that poorly ordered (amorphous) $\text{Zn}_3(\text{PO}_4)_2$ was present in pH 8 aged MNMs due to partial dissolution and surface transformation of the pristine material. We conclude that this surface transformation resulted in a ZnO/$\text{Zn}_3(\text{PO}_4)_2$ core-shell structure with heterogeneous composition. The NMR data further confirm that the phosphates are a mixture of four different species in pH 8 treatment including hopeite. We speculate that after phosphatation of the outer regions of the pristine MNMs, the ZnO core is protected by the $\text{Zn}_3(\text{PO}_4)_2$ shell, preventing further dissolution.

Due to the low solubility of $\text{Zn}_3(\text{PO}_4)_2$ ($\text{Ksp} \sim 9 \times 10^{-33}$), dissolution in the environment will be limited, limiting the potential for toxicity from $\text{Zn}^{2+}$. The potential toxicity of
ZnO/Zn$_3$(PO$_4$)$_2$ core-shell structures is unknown. Considering that these particles may be more representative of those materials that will be found in the environment, toxicity testing of these materials would be prudent. Further studies examining transformations of these products during wastewater treatment and in soils, sediments, and surface water are also needed. These results highlight the complexity of potential environmental transformations of ZnO and the sensitivity of the transformation process to environmental variables. Finally, this study shows the need for a variety of approaches to probe not just the chemical speciation of transformation products, but their morphological and structural characteristics which could dictate subsequent environmental behavior and toxicity.

**Acknowledgement**

This research was supported by a grant from the U.S. Environmental Protection Agency's Science to Achieve Results (STAR) program (R834574). Also supported by, the National Science Foundation (NSF) and the Environmental Protection Agency (EPA) under NSF Cooperative Agreement EF-0830093, Center for the Environmental Implications of Nanotechnology (CEINT). Any opinions expressed in this paper are those of the authors and do not, necessarily, reflect the official positions and policies of the USEPA. Portions of this work performed at Beamline X26A, National Synchrotron Light Source (NSLS), and Brookhaven National Laboratory. X26A is supported by the Department of Energy (DOE) - Geosciences (DE-FG02-92ER14244 to The University of Chicago - CARS). Use of the NSLS was supported by DOE under Contract No. DE-AC02-98CH10886. The authors gratefully acknowledge the assistance of T. Karathanasis, Y. Thompson and J. Backus.
Chapter three: Influence of phosphate and natural organic matter on transformations and toxicity of ZnO nanomaterials in single and two component systems

Sewwandi Rathnayake¹,², Jason M. Unrine¹,², Jonathan Judy¹,², D. H. McNear¹,²

Paul M. Bertsch¹,²,³

1. Department of Plant and Soil Sciences, University of Kentucky, Lexington, Kentucky, USA

2. Center for Environmental Implications of Nanotechnology, Duke University, Durham, North Carolina, USA

3. Division of Land and Water, CSIRO, Ecosciences Precinct, Brisbane, Australia

Summary

Nano zinc oxide (ZnO) is among the highest volume manufactured nanomaterials (MNMs) currently used in consumer products with estimates up to 1,000t/year around the globe in 2010. Therefore it is critical to understand the transformations and fate of ZnO MNMs in the environment as well as the effects of transformation products to key ecological receptors. In this study, we examine the reaction kinetics and characterize the transformation products of 30 nm ZnO MNMs aged in both single component and multi-component systems using phosphate and natural organic matter (NOM). We also assessed how simulated aging influenced the bioavailability and toxicity of ZnO MNMs to Triticum aestivum. We observed that the presence of NOM reduces particle aggregation and enhances stability, regardless of the sequence of component addition in the aging scenarios. The presence of NOM also altered the uptake and reduced the toxicity of the ZnO MNMs to Triticum aestivum. To our knowledge, this is the first report examining the transformations of ZnO MNMs after simulated aging in both phosphate and NOM (either in separate and mixed component systems) as well as the first report assessing the toxicity of the transformed particles.
Introduction

With the recent growth of nanotechnology and significant increase in use of MNMs in consumer and industrial products, the potential for their release to environment during manufacturing, transport, consumption, and post consumption disposal is of increasing concern [9, 42, 94]. During use, a large quantity of MNMs will be released into wastewater streams and during wastewater treatment, more than 90% of MNMs are expected to be partitioned to the biosolids [12]. A significant proportion of these biosolids (more than 60% in US) are applied to agricultural lands as fertilizers [9]. Although our understanding of the fate, transport, bioavailability, and toxicity of MNMs in the environment has greatly expanded over the last decade, the risk to the environment posed by the entry of MNMs from products to the environment via this pathway remains unclear.

Further complicating this risk assessment is the potential that MNMs will transform into largely unknown end products upon entering the environment [14, 95]. Important potential environmental transformations of ZnO MNMs include dissolution [44, 48, 62], sulfidation [60], phosphorylation [62], and interaction with NOM [96]. Recently, several studies have begun to explore the speciation of MNMs discharged into the environment. For example, a recent study reported that in the presence of inorganic sulfides, complete sulfidation of ZnO MNMs was observed within 5 days at ambient temperature [60]. Similarly, our recent investigations into the transformations of ZnO MNMs as a result of aging in the presence of phosphate provide evidence that ZnO MNMs readily interact with phosphate and transform into end products including a micro sized zinc phosphate (hopeite) phase and nano sized zinc phosphate/zinc oxide core shell structure. Other
studies have indicated that presence of NOM may influence speciation, surface charge and dissolution-precipitation reactions MNMs. [96-99].

However, the research to date has primarily focused on single component systems [96, 99-101], ignoring the complexity and presence of multiple components characteristic of natural systems [102]. Furthermore, although many studies have been published examining the phytotoxicity and bioaccumulation of MNMs [31, 32, 103], to our knowledge, no research has been conducted examining the phytotoxicity of aged MNMs, which is likely to be the much more environmentally relevant.

The objectives of this study were to examine the influence of NOM and a mixed NOM-phosphate multi-component system on ZnO MNM transformations and to assess the phytotoxicity of the transformed particles. We explore the influence of NOM and phosphate on the transformations of ZnO MNMs separately, simultaneously, and sequentially. To the best of our knowledge this is the first attempt to examine MNM transformations in a phosphate-NOM mixed system and to assess the toxicity of transformed/aged ZnO MNMs to *Triticum aestivum*.

**Methods and materials**

Both pristine (ZnO) and phosphate aged MNMs (p-ZnO) were used in the experiments. p-ZnO were prepared by reacting ZnO with Na$_2$HPO$_4$ in the ratio of 1/1.5 for 3 days at 25°C both pH 6 and pH 8 separately. One hundred mg L$^{-1}$ ZnO MNMs and p-ZnO were dispersed in 50 ml polypropylene centrifuge tubes by ultrasonication (at ~2,150 J) using a probe sonicator (Misonix, Newtown CT, USA) [74].
Aging with natural organic matter

To examine the transformations of ZnO MNMs and p-ZnO with NOM, 0, 10, 20 and 40 mg L\(^{-1}\) C solutions, added as International Humic Substances Society (IHSS) reference, Pahokee Peat (hereafter NOM) were adjusted to pH 6 ± 0.2 or pH 8 ± 0.2 with HCl and NaOH. As mentioned above, ZnO MNMs and p-ZnO were dispersed in the NOM solutions at a concentration of 100 mg L\(^{-1}\) and incubated at 25\(^0\)C ± 0.1 on a shaker table for 24 h, 72 h or 7 d. Following incubation, an aliquot was removed to measure the mean hydrodynamic diameter and electrophoretic mobility. Mean hydrodynamic diameters and electrophoretic mobilities of the aged MNM suspensions were measured using a Nano-ZS zetasizer (Malvern, Worcestershire, UK). Samples were then centrifuged at 3250 x g for 2 h to separate the solid fraction from the supernatant (Eppendorf Centrifuge 5810R). The separated solids were lyophilized and stored at room temperature for analysis. Supernatants were acidified to 0.15 M HNO\(_3\) for analysis of dissolved Zn by inductively coupled plasma mass spectrometry (ICP-MS). Dissolved carbon content was measured using a Shimadzu TOC-VCSH total organic carbon analyzer prior and after centrifugation. Aged material will be referred as n-ZnO and p+n-ZnO respectively for ZnO and p-ZnO after aging with NOM.

Aging in mixed system

To examine the reaction kinetics of ZnO in complex multi component systems, ZnO MNMs were added to a system containing both phosphate and NOM. To maintain consistency with the p-ZnO treatment, a fixed Zn/P ratio of 1/1.5 was maintained. 0, 10, 20 and 40 mg L\(^{-1}\) C solutions mixed with phosphate were adjusted to pH 6 ± 0.2 or pH 8
± 0.2 with HCl and NaOH. ZnO MNMs were dispersed in the mixed solutions at a concentration of 100 mg L$^{-1}$ and the experiments were carried out following exactly same procedure as described in the section “Aging with the NOM”. Materials aged simultaneously in the mixed system will be referred as pn-ZnO here after.

**Dissolved Zn analysis**

Dissolved Zn$^{2+}$ concentrations in the supernatants were quantified using an Agilent 7500cx ICP-MS (Santa Clara, CA, USA). Analytical runs contained calibration verification samples, duplicate dilutions, and spike recovery samples. Spike recovery averaged 98.2 % ± 2 (n=3) and the mean relative percent deference (RPD) between duplicate dilutions was 0.98 % ± 1.2 (n=3). Three replicates were prepared for all treatment combinations.

**Synchrotron-based X-ray absorption near edge spectroscopy (XANES)**

Lyophilized powders were ground with a mortar and pestle and homogenously distributed onto Kapton or cellophane tape for analysis by X-ray absorption near edge structure (XANES) spectroscopy, with attempts made to minimize the presence of any particles larger than 1 absorption unit thickness at the Zn K-edge (47 µm). Zinc K-edge XANES spectra were collected at beamline X-26A of the National Synchrotron Light Source, Brookhaven National Laboratory (Upton, New York). The beam was focused to a spot size of approximately 10 x 10 µm$^2$ as previously described [82]. XANES spectra were collected in transmission mode by scanning the monochromator from 9,600 to 9,800 eV. Layers of tape coated with samples and standards were combined to achieve samples
that were 1 ± 0.3 absorption units and to eliminate any pinholes or voids in the samples. The energy range was scanned as follows: 9,600-9,645 eV in 5 eV steps, 9,645 -9,710 eV in 0.3 eV steps, 9,710– 9,800 eV in 0.5 eV steps. The XANES spectra were analyzed using the Athena software package [83]. After normalization, linear combination fits (LCFs) were performed using Zn foil, ZnO MNM, Zn$_3$(PO$_4$)$_2$, ZnSO$_4$ and Zn(CH$_3$COO)$_2$.2(H$_2$O) as standards. We verified the quality of the fits by analyzing standards of known proportions of ZnO and Zn$_3$(PO$_4$)$_2$.

**X-ray diffraction (XRD)**

Powder XRD analysis of aged particles was performed using an X’Pert PRO MPD (PANalytical, B.V., Almelo, Netherland) diffractometer using a Cu Kα (λ = 0.154nm) radiation source in the 2θ scanning range of 2-60°. The XRD patterns were refined according to the high score [84, 104] (v.3.0.5) program package.

**Germination/uptake studies**

*Triticum aestivum* seeds were used in this study. Seeds were surface sterilized with 5% bleach and washed three times with sterilized nanopure water. ZnO MNMs, p-ZnO, n-ZnO, p+n-ZnO, pn-ZnO, bulk ZnO, ZnCl$_2$ and Zn$^{2+}$ in NOM were prepared at concentrations of 0, 10, 100, 1,000, 2,000 mg Zn L$^{-1}$ as treatments. Seeds were soaked in treatment suspensions overnight at 25°C ± 0.1 on a shaker table. A moistened filter paper was placed in a sterilized Petri dish with 10 seeds being transferred into each dish, with three replicates per treatment. Petri dishes were closed, sealed with tape, and placed in an incubator at 25°C ± 0.1. In this study, root and shoot length were measured for each
seedling when 60% of the seedlings in control treatments developed shoots and roots at least 20 mm long in accordance with EPA Ecological effects test guideline, OPPTS 850.4200 Seed Germination/Root Elongation Toxicity Test [105].

Once the roots and shoots were measured oven dried shoots and roots were weighed and placed in micro centrifuge tubes. These samples were digested using a MARS microwave digestion system (CEM, Matthews, NC) for 20 min at 100°C in 750 µL of HNO₃ acid. Samples were allowed to cool down and 250 µL of H₂O₂ was added and heated for another 20 min, afterwards digestate was brought to 15 mL and analyzed by ICP-MS. Additional laboratory control samples (LCS) consisting of spiked digestion blanks and SRM1515 standard reference material (apple leaves) were used to validate the digestion and analytical method for Zn.

Results and discussion

Particle characterization

Dynamic light scattering data (DLS) (Figure 1) provide information on the aggregation state of ZnO, n-ZnO, p+n-ZnO and pn-ZnO at different concentrations. The hydrodynamic particle diameter measured from DLS was 1,264 ± 50 for ZnO and 4,511 ± 165 and 1,222 ± 48 for p-ZnO at pH 6 and 8 respectively in the absence of NOM. At pH 6, hydrodynamic diameter decreased with increasing NOM concentration in all treatments. However hydrodynamic diameter remain was not affected by NOM concentration at pH 8. DLS data of pH 6 treatments reveals that the degree of disaggregation increases with the increasing NOM concentration. At any NOM
concentration, the hydrodynamic diameter of pH 6 treatments followed the order, p+n-ZnO > pn-ZnO > n-ZnO.

Figure 3-1: Changes in hydrodynamic diameter of n-ZnO, p+n-ZnO and pn-ZnO in response to increasing NOM concentration; (1) pH 6 (2) pH 8. Each value represents the mean of three replicates with standard deviation shown by error bars.

For all treatments at both pH values, the addition of NOM results in a strong negative surface charge. At pH 6, as NOM concentration increases from 0 to 10 mg L$^{-1}$, the zeta potential for all three treatments changes from positive to negative. The same is true for the pn-ZnO and the p+n-ZnO at pH 8.
Figure 3-2: Changes in electrophoretic mobility of n-ZnO, p+n-ZnO and pn-ZnO at (1) pH 6 (2) pH 8 as a function of NOM concentration. Each value represents the mean of three replicates with standard deviation shown by error bars.

Considering both the aggregation/disaggregation information from the DLS data and the zeta potential data, NOM absorbed on the particle surfaces result in a highly negative surface charge. This highly negative surface charge leads to individual particles repulsing each other, resulting in disaggregation and potentially enhanced stability in the environment.

TEM micrographs of aged particles reveal the presence of two distinct particle morphologies: a nano size phase with a mean diameter of 40 ± 5 nm and a heterogenous micro size phase with diameters ranging from 0.1-12µm (Figure 3). At both pH values, n-ZnO contained only nano size particles. Micro sized particles are observed only in the presence of phosphate at pH 6.
**Figure 3-3a:** TEM image of the pristine ZnO MNMs (a) aged MNMs aged in 150 mgL⁻¹ phosphate concentration for 72 h at pH 6 (b) at pH 8 (c).

**Figure 3-3b:** TEM images of n-ZnO at pH 6 (a) p+n-ZnO at pH 6 (b) pn-ZnO at pH 6 (c) n-ZnO at pH 8 (d) p+n-ZnO at pH 8 (e) and pn-ZnO at pH 8 (f)
Influence of NOM on particle solubility

Quantification of Zn$^{2+}$ showed a similar pattern of dissolution in all treatments in the presence of NOM (Figure 4). Overall, dissolution of ZnO and p-ZnO was higher at pH 6. The highest amount of dissolution occurred in the n-ZnO at pH 6 (48.76 ± 4.82 mg L$^{-1}$) and the lowest amount of dissolution was observed in pn-ZnO at pH 8 (1.78 ±0.59 mg L$^{-1}$). Aging with phosphate reduce the dissolved Zn$^{2+}$ concentration, on the other hand the presence of NOM increases dissolved Zn$^{2+}$ [97]. Observed dissolution patterns in the presence of NOM at both pH values, at all NOM concentrations were in the order of n-ZnO > p+n-ZnO > pn-ZnO.

Figure 3-4: Dissolved Zn$^{2+}$ as a function of NOM concentration and pH. Each value represents the mean of three replicates with standard deviation shown by error bars.

Higher dissolution may be associated with increased surface area due to disaggregation caused by the NOM adsorption to the particles.

Sorption of NOM (as measured by C content) to nanomaterials followed the order of pn-ZnO > p+n-ZnO > n-ZnO for both pH values at any NOM concentration. Sorption was
enhanced at high pH and with increasing NOM concentration. Presumably, the greater number of de-protonated sites at pH 8 helped to increase adsorption of NOM to ZnO MNMs and p-ZnO.

**Figure 3-5:** Carbon adsorption to MNM surfaces as determined by dissolved carbon content analysis

The linear combination fit analysis of XANES spectra for particles first aged in phosphate at pH 6 and then aged in NOM revealed that the resulting material consists of 60.8% ZnO and 36.7% of Zn$_3$(PO$_4$)$_2$. Particles first aged in phosphate at pH 8 and then exposed to the NOM consist 73.9% of ZnO and 27.6% of Zn$_3$(PO$_4$)$_2$, thus containing more Zn$_3$(PO$_4$)$_2$ than before equilibration with NOM. We speculate that NOM facilitates dissolution and disaggregation of the phosphate aged particles at pH 8, resulting in the observed increase in Zn$_3$(PO$_4$)$_2$ content. In our previous study, aging of ZnO MNMs with phosphate at pH 8 resulted in ZnO /Zn$_3$(PO$_4$)$_2$ core-shell structures (see Chapter 2). This
study provides evidence that NOM likely facilitates dissolution of the core-shell structures. When ZnO MNMs are aged in mixed system (both phosphate and NOM simultaneously), the transformation products consist of 88.8% of ZnO and 17.7% of Zn$_3$(PO$_4$)$_2$ at pH 6 and mostly ZnO at pH 8.

**Figure 3-6:** The quantitative distribution of aged material estimated from linear combination fit (LCF) analysis of XANES spectra of aged MNMs

X-ray diffraction data of the transformation products confirms the presence of crystalline ZnO in all treatments (**Figure 7**). In contrast to our previous study examining phosphate induced transformations of ZnO in the absence of NOM, we observed orthorhombic hopeite, when phosphate aged MNMs (core-shell structures) were exposed to NOM at both pHs. Crystalline hopeite was also formed in the particles aged in the mixed system at pH 6.
**Figure 3-7:** X-Ray diffraction patterns of n-ZnO at pH 6 (a) p+n ZnO at pH 6 (b) pn-ZnO at pH 6 (c) n-ZnO at pH 8 (d) p+n ZnO at pH 8 (e) pn-ZnO at pH 8 (f)

**Phytotoxicity**

No significant change in germination frequency was observed in any treatment. However, root and shoot elongation was clearly reduced in the Zn$^{2+}$ exposure treatment, bulk ZnO treatment as well as pristine ZnO MNM exposure treatment (Figure 3-8a). This observation is similar to results reported in another study that reported exposing reduction in wheat root length by 60% ±10 [100]. When NOM is present in the Zn$^{2+}$ ion media, toxicity of Zn$^{2+}$ ions reduced, but not totally inhibited. (Figure 3-8a). Toxicity as a result of exposure to p-ZnO is lower than all above mentioned treatments.
However, toxicity as a result of exposure to n-ZnO, p+n-ZnO, and pn-ZnO is much lower (Figure 3-8b) than in the treatments that contained no NOM (Figure 3-8a) No significant reduction in shoot elongation was observed in NOM containing treatment, even at 2,000 mg L$^{-1}$. Again, this result is similar to previous findings, that the presence of NOM reduced the toxicity of ZnO MNMs to bacterial cells [97].
Regardless the exposure sequence, when MNMs are aged in phosphate and NOM, the aged MNMs induce the root and shoot elongation (Figure 3-8b). Overall, materials aged at pH 6 exhibited less toxicity to wheat than those aged at pH 8.
Due to the fact, that the seeds are treated only overnight before germination, toxic effects exhibit due to internalization of ZnO MNMs and Zn ions during the imbibitions, retarding the length of both roots and shoots during the germination.

The presence of NOM is a major determinant of aggregation state [95]. Aggregation kinetics is an important parameter which governs the mobility and transport of the MNMs in the environment and will be a primary determinant for which environmental compartment MNMs ends up [102, 106]. For example, aggregated MNMs in surface waters will be more likely to settle out the water column than an individual particle [106]. The dissolution and disaggregation observed in all MNM treatments that contained NOM is consistent with previously published studies [95]. In an aqueous environment, adsorption of NOM will enhance stability facilitating transport and dispersion [107].

The bioaccumulation and toxicity patterns observed here are also consistent with other recent studies, such as one soybean exposure that observed bioaccumulation but no toxicity as a result of exposure to ZnO MNMs. [108, 109] In another study exposing wheat to either ZnO MNMs, ZnO micro particles, or Zn ions, the speciation of bioaccumulated Zn was revealed to be hopeite in all three treatments, suggesting that the observed bioaccumulation was largely the result of uptake of dissolved material, which was subsequently transformed into hopeite [100]. Similarly, a study which exposed soybeans to ZnO MNMs reported XANES spectra suggesting that Zn within the soybean plant was no longer in the form of ZnO MNMs [108]. However, another recent study reported the presence of ZnO nanoparticles in soybean leaves and many studies have reported uptake of MNM species that are unlikely to be dissolved, such as Au or ceria,
suggesting that MNMs are available for uptake in particle form under certain circumstances [110].

**Environmental impact**

In this study, both pristine MNMs and aged MNMs were stabilized by the presence of NOM via disaggregation of small aggregates, resulting in enhanced dispersion. Aging of ZnO MNMs in the presence of NOM and phosphate reduced the toxicity to wheat. Furthermore, the presence of NOM almost completely alleviated toxic effects that were observed in treatments that did not contain NOM. Our findings demonstrate that MNMs present in the environment will be much less toxic than the as manufactured. Further experiments with various types of MNMs and different plants species should be considered.

**Acknowledgement**

This research was supported by a grant from the U.S. Environmental Protection Agency's Science to Achieve Results (STAR) program (R834574). Also supported by, the National Science Foundation (NSF) and the Environmental Protection Agency (EPA) under NSF Cooperative Agreement EF-0830093, Center for the Environmental Implications of Nanotechnology (CEINT). Any opinions expressed in this paper are those of the authors and do not, necessarily, reflect the official positions and policies of the USEPA. Portions of this work performed at Beamline X26A, National Synchrotron Light Source (NSLS), and Brookhaven National Laboratory. X26A is supported by the Department of Energy (DOE) - Geosciences (DE-FG02-92ER14244 to The University of Chicago - CARS). Use
of the NSLS was supported by DOE under Contract No. DE-AC02-98CH10886. The authors gratefully acknowledge the assistance of T. Karathanasis and Y. Thompson in XRD analysis. The authors also acknowledge the assistance of J. A. Nelson, S. Shrestha and B. Collin.
Chapter four: Conclusions

In these studies, we concentrated on the kinetics of the reaction between ZnO MNMs with phosphate and NOM separately as well as in a multi/two component system, that may be expected in wastewater treatment processes. We found that aging ZnO MNMs with phosphate has profound effects on physical, chemical and morphological characteristics of nanomaterials. In chapter 2, we reported how pH plays an important role determining the final composition of the aged products. In this study, we used pH 6 and pH 8 based on the possible pH range in wastewater as well as the fact that ZnO is highly soluble at pH 6 and sparingly so at pH 8. Our data reveal that ZnO MNMs react with phosphate at concentrations expected in wastewater and transform into two distinct morphological/structural phases: a micron scale crystalline zinc phosphate phase (hopeite) formed at pH 6 whereas at pH 8, a nano-sized phase that likely consists of a ZnO core with a Zn$_3$(PO$_4$)$_2$ rich shell was formed. These results highlight how phosphorylation of ZnO MNMs can occur through a variety of mechanisms depending on pH, resulting heterogeneous structural/morphological characteristics in the transformation products.

In chapter 3, we reported how the reaction of ZnO MNMs with NOM reduced MNM aggregation by enhancing the stability of the particles. Also, particle solubility increased at pH 6 in the presence of NOM. We also found that the presence of phosphate and NOM simultaneously cause the competition of ligands with each other and produced intermediate products. Furthermore, we reported that the presence of NOM reduced the toxicity of the ZnO MNMs to Triticum aestivum. A positive effect on root and shoot elongation observed in the presence of NOM.
Considering these results, ZnO MNMs that enter wastewater streams may be transformed into highly insoluble phases depending on the type and concentration of ligands present in the wastewater as well as pH. Several different intermediate and ultimate end products may be formed depending on the conditions and composition of wastewater with important implications for the potential mobility and toxicity of MNMs.

The findings of this work have significant environmental implications. Since the aging reduced the toxicity of the ZnO MNMs to *Triticum aestivum* (materials that will be found in the environment), compared to the MNMs, this study highlights the importance of considering aged MNMs in toxicity testing to properly evaluate the ecological and human health risks. Furthermore, aging will be affected by the type of ligands, concentrations, pH ect. Therefore, attention also needs to be paid to the environmental factors /conditions assessing the risk and regulating MNMs in the environment.
Appendix

Figure A1-A3; EDS graph of pristine material (A1). EDS graph of macro fraction (A2). EDS graph of nano sized fraction (A3).

Figure A4: TGA weight loss graphs.
Figure A5: Schematic diagram of the core–shell structure.
References


Vita

Sewwandi Rathnayake was born in Sri Lanka, received Bachelor of Science degree in Agriculture Management and Technology from University of Peradeniya (Sri Lanka) in 2008. Before she was awarded the Research Fellowship at University of Kentucky (USA) she has worked as a Research Assistant at Institute of Fundamental Studies (Sri Lanka) and University of Cincinnati (USA) and as a Teaching Assistant at University of Peradeniya (Sri Lanka). She won the 1st place in the Soil Science Society of America International annual general conference 2012 with her poster entitled "Transformations of ZnO Nanoparticles in Wastewater Treatment, Biosolids, and Soil".