Fate and Impacts of Contaminants of Emerging Concern during Wastewater Treatment

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ABSTRACT

The purpose of this dissertation was to broadly investigate the fate of antibiotic resistance genes (ARGs) and engineered nanomaterials (ENMs) as representative contaminants of emerging concern in wastewater treatment plants (WWTPs). WWTPs may have their performance impacted by ENMs and may also serve as a reservoir and point of release for both ENMs and ARGs into the environment. Of interest were potential adverse effects of ENMs, such as stimulation of antibiotic resistance in the WWTP, toxicity to microbial communities critical for WWTP performance, and toxicity to humans who may be exposed to effluents or aerosols containing ENMs and their transformation products.

Response of nine representative ARGs encoding resistance to sulfonamide, erythromycin and tetracycline to various lab-scale sludge digestion processes were examined, and factors that drove the response of ARGs were discussed. Mesophilic anaerobic digestion significantly reduced sulI, sulII, tet(C), tet(G), and tet(X) with longer solids retention time (SRT) exhibiting a greater extent of removal. Thermophilic anaerobic digesters performed similarly to each other and provided more effective reduction of erm(B), erm(F), tet(O), and tet(W) compared to mesophilic digestion. Thermal hydrolysis pretreatment drastically reduced all ARGs, but they generally rebounded during subsequent anaerobic and aerobic digestion treatments. Bacterial community composition of the sludge digestion process, as controlled by the physical operating characteristics, was indicated to drive the distribution of ARGs present in the produced biosolids, more so than the influent ARG composition.
Effects of silver (nanoAg), zero-valent iron (NZVI), titanium dioxide (nanoTiO$_2$) and cerium dioxide (nanoCeO$_2$) nanomaterials on nitrification function and microbial communities were examined in duplicate lab-scale nitrifying sequencing batch reactors (SBRs), relative to control SBRs received no materials or ionic/bulk analogs. Nitrification function was only inhibited by high load of 20 mg/L Ag$^+$, but not by other nanomaterials or analogs. However, decrease of nitrifier gene abundances and distinct microbial communities were observed in SBRs receiving nanoAg, Ag$^+$, nanoCeO$_2$, and bulkCeO$_2$. There was no apparent effect of nanoTiO$_2$ or NZVI on nitrification, nitrifier gene abundances, or microbial community structure. A large portion of nanoAg remained dispersed in activated sludge and formed Ag-S complexes, while NZVI, nanoTiO$_2$ and nanoCeO$_2$ were mostly aggregated and chemically unmodified. Thus, the nanomaterials appeared to be generally stable in the activated sludge, which may limit their effect on nitrification function or microbial community structure.

Considering an aerosol exposure scenario, cytotoxicity and genotoxicity of aqueous effluent and biosolids from SBRs dosed with nanoAg, NZVI, nanoTiO$_2$ and nanoCeO$_2$ to A549 human lung epithelial cells were examined, and the effects were compared relative to outputs from SBRs dosed with ionic/bulk analogs and undosed SBRs, as well as pristine ENMs. Although the pristine nanomaterials showed varying extents of cytotoxicity to A549 cells, and gentoxicity was observed for nanoAg, no significant cytotoxic or genotoxic effects of the SBR effluents or biosolids containing nanomaterials were observed.

Studies presented in this dissertation provided new insights in the fate of ARGs in various sludge digestion processes and ENMs in nitrifying activated sludge system in lab-scale reactors. The study also yielded toxicity data of ENMs to biological wastewater treatment microbial communities and human lung cells indicated by a variety of toxicity markers. The results will aid in identifying appropriate management technologies for sludge containing ARGs and will inform microbial and human toxicity assessments of ENMs entering WWTPs.
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Each coauthor is duly credited for his or her contribution to this work, both in their sharing of ideas and technical expertise.

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CHAPTER 1: Introduction

1.1 Background

Contaminants of emerging concern or emerging contaminants (ECs) can be broadly defined as any chemical or biological pollutant that is not commonly monitored in the environment, but has the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects.\(^1\) ECs include contaminants that are newly released into the environment, and also those that have persisted for a long time, but only recently have been recognized due to development of new detection methods or discovery of new source, exposure routes and effects.\(^2\text{-}^4\)

A vast group of chemicals of daily use has been identified as ECs, including pharmaceuticals and personal care products (PPCPs), surfactants and surfactant residues, gasoline additives, hormones and other endocrine disrupting compounds (EDCs), drinking water and swimming pool disinfection by-products (DBPs), engineered nanomaterials (ENMs), etc.\(^5\text{-}^6\) Emerging biological contaminants such as pathogenic microorganisms (bacteria, viruses, and protozoa) and antibiotic resistance genes (ARGs) can also be considered as ECs.\(^7\text{-}^9\)

To fill the data gap in assessing environmental risks of ECs, research can serve to characterize their transport and fate into the environment, exposure routes to humans and ecosystem, and toxicities under various exposure conditions. Wastewater treatment plants (WWTPs) have been an important research focus, for a large portion of ECs are generated from human use and enter wastewater streams. The current wastewater treatment configurations are usually not designed to remove ECs, and thus release of ECs such as PPCPs, EDCs, ENMs, and ARGs into natural waters through treated wastewater effluent has been widely reported.\(^10\text{-}^14\) ECs
can also associate with primary and secondary sludge and persist in biosolids for land-application.\textsuperscript{15-19} Some ECs can be biologically or chemically transformed during treatment processes; however, the transformation products of some pharmaceuticals were reported to be more toxic than the parent compounds,\textsuperscript{20-23} while transformation and toxicity of many other ECs are largely unknown. These findings highlight the need to critically evaluate existing wastewater treatment processes to track transformation and fate of ECs for better assessing their environmental impacts.

On the other hand, biological treatment is the key driving process of wastewater treatment, relying on microbial communities to remove and stabilize contaminants. In particular, nitrifying bacteria, which conduct the critical function of nitrification and oxidize ammonia to nitrite and subsequently nitrate, are highly sensitive to toxic chemicals.\textsuperscript{24} As ECs with identified or suspected toxicities increasingly enter the wastewater streams, their potential adverse impacts on the microbial communities and further on the biological wastewater treatment functions are of concern.

In this dissertation, fate and impacts of two representative ECs during wastewater treatment were examined: 1) antibiotic resistance genes (ARGs), which encode bacterial resistance to antibiotics; and 2) engineered nanomaterials (ENMs), which emerged lately with the expanded application of nanotechnology in consumer products.

1.2 Antibiotic Resistance Genes (ARGs)

Antibiotic resistance is causing increasing failure in therapeutic treatment of bacterial diseases and is a major public health issue.\textsuperscript{25} It occurred as early as shortly after the introduction of penicillin in the 1940s, and the resistance developed in \textit{Staphylococcus aureus} was later spread to methicillin.\textsuperscript{26} In recent years, resistance has been developed in \textit{enterococci} that showed
high-level resistance to the glycopeptides antibiotic vancomycin, which is a drug of “last resort” against multi-resistant *enterococci* and against methicillin-resistant *Staphylococcus aureus*. Multidrug resistance has also been reported to emerge in other organisms.

In the early antibiotic era, mutation in target genes under antibiotic pressure was considered the primary cause of antibiotic resistance. But more recent studies suggest that the majority of antibiotic resistance is most likely acquired through horizontal transfer of ARG from other ecologically and taxonomically distant bacteria. The mobile genetic elements mediating horizontal gene transfer include plasmids, transposons, and integrons. All three classes of elements have been reported to play an important role in spread of ARG amongst bacteria. Spread of ARGs has not been restricted within pathogens, but widely disseminated to common bacteria. Moreover, recent research has revealed that ARGs have evolved to serve broader purposes for the cell beyond fighting antibiotics, and are also induced in response to general toxicities or other environmental stimuli.

WWTPs receive large amount of antibiotics, bacteria, and other chemicals; the complex wastewater constituents are favorable for the development and horizontal transfer of ARGs among bacteria. A wide range of ARGs has been detected in wastewater streams. Tetracycline and sulfonamide resistance genes have been frequently detected in treated wastewater effluent and biosolids, which revealed the potential of WWTPs to disseminate ARGs to the environment.

### 1.3 Engineered Nanomaterials (ENMs)

Engineered nanomaterials (ENMs) are defined as materials with one or more dimensions on the order of 1-100 nm. They are widely applied in many commercial products due to novel properties that are generally not seen in conventional bulk counterparts. The range of
nanotechnology products is now extensive and can be broken down into a number of different compound classes, including metal oxides, zero-valent metals, semiconductor materials such as quantum dots, carbonaceous nanomaterials and nanopolymers.\textsuperscript{43}

The emergence of ENMs inevitably leads to their release into the environment. Studies have documented release of nanosilver from textiles and fabrics during washing,\textsuperscript{44,45} and emission of TiO\textsubscript{2} nanoparticles from exterior façade paints to the discharge and into surface waters.\textsuperscript{46} A study on the life cycle of ENMs indicated that except for a limited amount of recycling, the majority of nanomaterials in manufactured products will end up in disposal systems, e.g. landfill, incineration and wastewater treatment.\textsuperscript{42} In particular, Mueller and Nowack\textsuperscript{47} modeled flow of ENMs (nanosilver, nano-TiO\textsubscript{2} and carbon nanotubes) from products to air, soil, and water in Switzerland, and indicated that up to 95\% of the nanosilver and nano-TiO\textsubscript{2} in textiles, cosmetics, sprays, cleaning agents, coatings, paints and plastics are released into WWTPs. This finding emphasizes the critical role of WWTPs on the pathway of ENMs to the environment.

The fate of silver, silica, copper, titanium dioxide, cerium oxide nanoparticles and fullerenes following wastewater treatment has been studied, and generally indicated their removal from wastewater streams and association with the sludge, although a small portion still retained in aqueous effluent.\textsuperscript{13,17,18,48-51} The extent of different ENMs partitioning into activated sludge was distinct. While ENMs may primarily partition into primary sludge and be routed to anaerobic digestion, partitioning into activated sludge is also important to consider and has been addressed by some studies. For example, a simulated wastewater treatment system removed less carboxy-terminated polymer coated silver nanoparticles (88\%) from wastewaters compared with hydroxylated fullerenes (>90\%), aqueous fullerenes (>95\%), and nano-TiO\textsubscript{2} (>95\%).\textsuperscript{18} The
association of ENMs with activated sludge could be affected by their properties and constituents of the wastewater matrix. Significant influence of surface charge and the addition of dispersion stabilizing surfactants on the removal of cerium dioxide nanoparticles from wastewater were observed in a model wastewater treatment system. In another study, silica nanoparticles coated with Tween 20 underwent rapid flocculation, while the uncoated nanoparticles did not flocculate in wastewater over typical residence times for primary treatment. Effect of extracellular polymeric substances (EPS) and natural organic matters (NOM) on the removal of ENMs by activated sludge was examined, and aqueous fullerenes showed a change in the degree of removal when the nanomaterial suspensions were equilibrated with NOM or EPS was extracted from the biomass.

Due to the limitation of detection and quantification techniques, as well as the complexity of wastewater and activated sludge matrix, transformation of ENMs during wastewater treatment is largely unknown. A few recent studies identified conversion of nanosilver to silver sulfide (Ag$_2$S) and subsequent precipitation into activated sludge. Zinc oxide (ZnO) nanoparticles were observed to be transformed to three Zn-containing species, ZnS, Zn$_3$(PO$_4$)$_2$, and Zn associated Fe oxy/hydroxides in a pilot wastewater treatment plant.

The small nano-size and large surface area of ENMs may increase their reactivity and thus have the potential to exert toxicity to biological systems. Toxicity of ENMs to bacteria pure cultures has been documented in a growing number of studies. Therefore, it is suspected that accumulation of ENMs in activated sludge may cause adverse effects on the efficiency of wastewater treatment due to their toxicity to bacterial community, especially nitrifying bacteria, which are sensitive to toxic chemicals. Inhibition of nanosilver to nitrification has been observed in *Nitrosomonas europaea* pure culture and enriched nitrifying microorganisms. The
Inhibitory effect was further verified by shock loading of 0.75 mg/L nanosilver to a lab-scale Modified Ludzacke Ettinger process, which divided the activated sludge chamber to anoxic and aerobic zones aiming at nitrogen and organic removal. In another study, nitrification efficiency in an anaerobic-low dissolved oxygen sequencing batch reactor was greatly decreased by short-term exposure of ZnO nanoparticles. In the same type of wastewater treatment system, TiO$_2$ nanoparticles had no acute effects on nitrogen removal after short term exposure, but significantly decreased the nitrification efficiency after long-term exposure.

It should be noted that pure culture assays do not always correspond well to complex environments. For example, single walled carbon nanotubes (SWCNTs) were reported to exhibit strong antimicrobial activity in pure cultures. However, in one study, the addition of SWCNTs to activated sludge system did not negatively impact the performance of the reactor, and instead improved sludge settleability and dewaterability. The complex wastewater matrix may alter the toxicity of ENMs by transformations. Furthermore, considering the microbial ecology that drives wastewater treatment, microbial toxicity is particularly relevant at the community level. However, to the author’s knowledge, microbial community response to ENMs in the wastewater treatment process has not been characterized.

ENM residuals and transformed forms can be released into natural water and soil from wastewater treatment plants through discharge of water effluent and land-application of biosolids, and thus has the potential to be exposed to human and ecosystem. Cytotoxicity and genotoxicity of common metal and carbon based ENMs to a variety of human and animal cells or tissues has been reported. However, to the author’s knowledge, the toxicity of environmental samples potentially containing ENMs, such as aqueous effluent and biosolids resulted from
wastewater treatment is rarely examined. It is also unknown whether the transformation of ENMs converts them to non-toxic forms or more toxic chemical structures.

1.4 Research Objectives

The overall objectives of this research are to understand fate of ARGs and ENMs during wastewater treatment processes and to evaluate the toxicity of ENMs during and following wastewater treatment.

To achieve this general objective, the following specific objectives were pursued:

1) Examine effects of various sludge digestion processes on ARG removal and corresponding microbial community compositions in digesters with different SRT, temperature, redox, and feed conditions of untreated biosolids versus thermal hydrolysis pretreatment products.

2) Examine effects of ENMs in nitrifying lab-scale sequencing batch reactors (SBRs) on nitrification function, abundance of nitrifying bacteria, and microbial community compositions, and identify possible relationships of nanomaterial transformation state and the effects observed.

3) Assess cytotoxicity and genotoxicity of wastewater effluent and biosolids from SBRs dosed with ENMs, to examine the toxicity of transformed ENM following biological wastewater treatment, and compare with the toxicities of pristine nanomaterials.

1.5 Annotated Dissertation Outline

Chapter 1: Introduction.

Chapter 2: Effect of Various Sludge Digestion Conditions on Sulfonamide, Macrolide, and Tetracycline Resistance Genes and Class 1 Integrons. This manuscript addressed the first
specific objective, and examined response of nine representative ARGs encoding resistance to sulfonamide, erythromycin, and tetracycline to lab-scale mesophilic, thermophilic anaerobic digesters and a sequential digestion process with thermal hydrolysis pretreatment. Quantitative Polymerase Chain Reaction (Q-PCR) was used to quantify target genes. Microbial communities were characterized by Denaturing Gradient Gel Electrophoresis (DGGE) and sequencing. The role of microbial community compositions in driving the response of ARGs was discussed. The manuscript has been accepted for publication:


Chapter 3: *Microbial Community Response of Nitrifying Sequencing Batch Reactors to Silver, Zero-Valent Iron, Titanium Dioxide, and Cerium Dioxide Nanomaterials*. This manuscript addressed the second specific objective, and examined effect of silver, zero-valent iron, titanium dioxide and cerium dioxide nanomaterials on nitrification function, abundance of nitrifying bacteria (Q-PCR) and microbial communities (Pyrosequencing) in lab-scale SBRs. The effects of nanomaterials in SBRs were compared with SBRs receiving no material and ionic/bulk analogs. Transformation of nanomaterials in SBRs was characterized by Transmission Electron Microscopy (TEM) equipped with Energy Dispersive X-ray Spectroscopy (EDS). The relationship of nanomaterial transformation and the observed effects were discussed. The manuscript is currently under peer-review at *Environmental Science & Technology*. 
Chapter 4: Toxicity of Engineered Nanomaterials and Their Transformation Products Following Wastewater Treatment on A549 Human Lung Epithelial Cells. This manuscript addressed the third specific objective, and evaluated cytotoxicity and genotoxicity to A549 human lung epithelial cells of wastewater effluents and biosolids from SBR conditions studied in Chapter 3. The effects were compared with pristine nanomaterials and ionic/bulk analogs. Cytotoxicity to A549 cells was examined by WST-1 assay, and genotoxicity was analyzed by immunofluorescent labeling of γH2AX foci. This manuscript is currently under peer-review at Environmental Science & Technology Letters.

Chapter 5: Conclusions. This chapter summarized key conclusions drawn from this study, and highlighted the scientific contributions of this study in understanding fate of ARGs and ENMs during wastewater treatment processes, and toxicity implications of ENMs. Future research was suggested based on outputs of this study.

1.6 References


(51) Gomez-Rivera, F.; Field, J. A.; Brown, D.; Sierra-Alvarez, R., Fate of cerium dioxide (CeO$_2$) nanoparticles in municipal wastewater during activated sludge treatment. 


CHAPTER 2: Effect of Various Sludge Digestion Conditions on Sulfonamide, Macrolide and Tetracycline Resistance Genes and Class I Integrons

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2.1 Abstract

Wastewater treatment processes are of growing interest as a potential means to limit the dissemination of antibiotic resistance. This study examines the response of nine representative antibiotic resistance genes (ARGs) encoding resistance to sulfonamide (sulI, sulII), erythromycin (erm(B), erm(F)) and tetracycline (tet(O), tet(W), tet(C), tet(G), tet(X)) to various laboratory-scale sludge digestion processes. The class I integron gene (intI1) was also monitored as an indicator of horizontal gene transfer potential and multiple antibiotic resistance. Mesophilic anaerobic digestion at both 10 and 20 day solids retention times (SRTs) significantly reduced sulI, sulII, tet(C), tet(G) and tet(X) with longer SRT exhibiting a greater extent of removal; however, tetW, ermB and ermF genes increased relative to the feed. Thermophilic anaerobic digesters operating at 47 °C, 52 °C and 59 °C performed similarly to each other and provided more effective reduction of erm(B), erm(F), tet(O) and tet(W) compared to mesophilic digestion. However, thermophilic digestion resulted in similar or poorer removal of all other ARGs and intI1. Thermal hydrolysis pretreatment drastically reduced all ARGs, but they generally rebounded during subsequent anaerobic and aerobic digestion treatments. To gain insight into potential mechanisms driving ARG behavior in the digesters, the dominant bacterial communities were compared by denaturing gradient gel electrophoresis. The overall results suggest that bacterial community composition of the sludge digestion process, as controlled by
the physical operating characteristics, drives the distribution of ARGs present in the produced biosolids, more so than the influent ARG composition.

2.2 Introduction

Numerous studies have demonstrated that antibiotic resistant bacteria are present and can proliferate in wastewater treatment plants (WWTPs).\textsuperscript{1-3} Given the potential detriment to human health as a result of the growing problem of antibiotic resistance, the fate of antibiotic resistant bacteria emanating from WWTPs is of interest. WWTPs produce two primary product streams, reclaimed water, which is typically returned directly to aquatic receiving environments or used for irrigation purposes, and biosolids, of which about 55% are land-applied as a beneficial soil amendment and the remainder disposed of in land-fills or by incineration.\textsuperscript{4}

Antibiotic resistance is imparted to bacteria by their carriage of DNA encoding antibiotic resistance, or antibiotic resistance genes (ARGs). In one study, nine out of ten tetracycline ARGs assayed were commonly detected in WWTP effluent compared to only two out of ten in neighboring Wisconsin lakes,\textsuperscript{5} suggesting that WWTPs may be a significant source of ARGs to the aquatic environment. Further, in a recent study it was found that the molecular signature of ARG occurrence at human-impacted sites along a Colorado river more closely matched those of WWTPs than neighboring animal feeding operation lagoons or the upstream pristine portion of the river.\textsuperscript{6} With respect to biosolids, elevated levels of tetracycline ARGs \textit{tet}(O), \textit{tet}(W) and the sulfonamide ARG \textit{sul}I were observed in certain soil types after land application.\textsuperscript{7} Such findings highlight the need to critically evaluate existing WWTP treatment processes for their potential to attenuate ARGs and limit dissemination to the environment.

In a positive sense, WWTPs may present the opportunity to minimize potential risk to human health, ideally through simple, effective, and economical modifications. In particular,
treatment processes that go beyond traditional disinfection and actually destroy DNA are of interest, given that ARGs can persist and are highly prone to uptake by downstream bacteria via horizontal gene transfer.\textsuperscript{8-10} Characterizing the effectiveness of WWTP processes in terms of the fate of ARGs is also of value considering that pure-culture based methods overlook the vast pool of uncultured organisms.\textsuperscript{11} Although about one to three orders of magnitude of \textit{tet} ARG reduction has been observed in full-scale WWTP, \textit{tet} ARGs are still present at about $10^2$ to $10^6$ copies per milliliter in effluent waters\textsuperscript{5,12} and about $10^8$ to $10^9$ copies per gram of treated biosolids.\textsuperscript{5,7} Thus sludge digestion represents a potentially key process for reducing the spread of ARGs.

The most widely applied sludge digestion technology is mesophilic producing class B biosolids. However, increasing demand for higher quality biosolids and more rigorous inactivation of pathogens has driven interest in alternative methods, such as thermophilic digestion,\textsuperscript{13} dual-stage treatment,\textsuperscript{14} and digestion combined with pretreatments and polishing steps.\textsuperscript{15} Of these, only the effect of thermophilic digestion on ARGs has been reported.\textsuperscript{16} In this study it was observed that anaerobic thermophilic treatment was markedly effective in reducing tetracycline ARGs relative to mesophilic digestion in full-scale digesters. Subsequently the thermophilic phenomenon was confirmed in lab-scale anaerobic digesters operated over a range of temperatures (22 °C, 37 °C, 46 °C, and 55 °C).\textsuperscript{17} While these findings promise a practical approach to reduce the load of ARGs released to the environment, it is important that the effect be demonstrated over a broad range of ARGs and is extended to other treatments. Moreover, the potential for horizontal transfer of ARGs within bacterial communities during various treatment processes is also of interest. Varied response of ARGs to biological treatment has been observed in other studies.\textsuperscript{18,19}
In this study, optimally-functioning lab-scale digesters were investigated under the following conditions: (i) mesophilic anaerobic digestion at 10 and 20 days SRT; (ii) thermophilic anaerobic digestion at 47 °C, 52 °C and 59 °C; and (iii) sequential thermal hydrolytic pretreatment followed by mesophilic anaerobic and aerobic digestion. Nine ARGs encoding sulfonamide resistance (sulI and sulII), macrolide resistance (erm(B) and erm(F)), and three sub-classes of tetracycline resistance (ribosomal protection: tet(O) and tet(W); tetracycline efflux: tet(C) and tet(G); tetracycline transformation: tet(X)) were quantified by quantitative polymerase chain reaction (Q-PCR) during steady-state operation. Additionally, the integrase gene intI1 of Class I integrons was tracked as an indicator of horizontal gene transfer potential. Bacterial communities were characterized by denaturing gradient gel electrophoresis (DGGE) to illustrate their role in driving ARG response to various treatment conditions. Thus, this study provides insight into the potential of a broad range of sludge digestion techniques to reduce several representative antibiotic resistance elements across various classes and resistance mechanisms.

2.3 Materials and Methods

2.3.1 Description of Sludge Treatment Processes

The feed sludge to all treatment processes was from the DCWATER Blue Plains WWTP consisting of a 1:1 ratio of primary and secondary solids, with a combined total solids (TS) concentration of approximately 3%. One mesophilic anaerobic digester was operated at George Washington University at 35 °C with an active volume of 10 L and SRT of 10 days. All other mesophilic and thermophilic digesters were operated at Virginia Tech, using identical reactor configurations and temperature control systems. These included a mesophilic anaerobic digester operated at 35 °C with an active volume of 10L and SRT of 20 days, and three thermophilic
digester operated at 47 °C, 52 °C, and 59 °C, respectively, with active volumes of 12 L and 15 day SRTs.

For the sequential process, the feed sludge was dewatered to approximately 15% TS by DCWATER, shipped on ice to RDP technologies, Inc. (Norristown, PA) and subjected to thermal hydrolysis pretreatment at 150 °C/4.80 bar for 30 min, followed by an immediate flash to ambient temperature and pressure. The sludge hydrolysate (12% TS) was then shipped overnight on ice to Virginia Tech where it was treated by anaerobic digestion at 37 °C with an active volume of 15 L and SRT of 15 days. To further destroy solids and remove ammonia, an aerobic digestion post-treatment followed at 32 °C with an active volume of 5 L and SRT of 5 days.

Feeding and wasting was performed once per day at an amount of 0.5 L for the 20 day SRT mesophilic digester, 0.8 L for thermophilic digesters, and 1 L for all other digesters to maintain the designated SRT. Volatile solids reduction (VSR) was approximately 50%, 30-40%, and 56-62% by the mesophilic digesters, the thermophilic digesters, and the sequential digestion process, respectively. All digesters were operated for over one year and had achieved optimal steady-state performance in terms of VSR and gas production at the time the present ARG reduction study commenced.

2.3.2 Sample Collection and DNA Extraction

Samples collected in this study included the following: (i) feed sludge; (ii) effluent of the two mesophilic digesters; (iii) effluent of the three thermophilic digesters; (iv) effluent of thermal hydrolysis pretreatment and downstream anaerobic and aerobic digesters. Samples were collected over four sampling events with a time interval of at least one SRT. Each sludge sample was centrifuged at 5,000 rpm for 15 min, and 0.4 g of the pellet was subsampled for DNA extraction using a FastDNA SPIN Kit for Soil (MP Biomedicals) and stored at -20 °C for further
analysis. The pellet was also subsampled and dried at 105 °C overnight to measure the dry solid content.

2.3.3 Detection and Quantification of ARGs

PCR assays were performed to detect one methicillin (mecA), one vancomycin (vanA), two sulfonamide (sulI, sulII), three erythromycin (erm(A), erm(B), erm(F)), and twelve tetracycline (tet(A), tetB(P), tet(C), tet(E), tet(G), tet(M), tet(O), tet(Q), tet(S), tet(T), tet(W), tet(X)) ARGs. Nine ARGs (sulI, sulII, erm(B), erm(F), tet(O), tet(W), tet(C), tet(G) and tet(X)) that were frequently detected representing a range of resistance mechanisms, as well as the integrase gene of class 1 integrons (intI1) and 16S rRNA genes were further quantified by Q-PCR. Primers, annealing temperatures, and reaction matrix are described in the Supporting Information (Tables S2.1-S2.3). All standard curves of Q-PCR were constructed from serial dilutions of cloned genes ranging from $10^8$ to $10^2$ gene copies per µL. The presence of PCR inhibitors was checked by serially diluting select samples and comparing PCR efficiencies with standards. It was found that a dilution of 1:50 worked well to minimize inhibitory effects and was applied across samples. Samples were analyzed in triplicate in at least three independent Q-PCR runs, with a standard curve and negative control included in each run.

2.3.4 DGGE Analysis

The bacterial community was characterized by DGGE. 16S rRNA genes in each effluent sample were amplified using primers I-341fGC and I-533r and previously described conditions. The PCR reaction matrix is described in the Supporting Information (Table S2.2). DGGE was performed at 57 °C with a D-Code system (Bio-Rad) employing 8% (w/v) polyacrylamide gels with a denaturant gradient from 20% to 55%. PCR products were electrophoresed at 100 V for
10 min and then 60 V for 15 h. The gel was stained with SYBRGold (Molecular Probes, Inc., Eugene, OR) and documented using the Chemidoc XRS Gel Documentation System (Bio-Rad). Quantity One 1-D analysis software (BioRad) was used to identify the bands in the gel and obtain the intensity profiles for each of the lanes. The relative diversity was calculated using the Shannon diversity index as previously described.21

### 2.3.5 Cloning and Sequencing Analysis

Based on the DGGE profiles obtained, five representative samples were selected for further analysis by cloning: the 52 °C thermophilic anaerobic digester, the 10 and 20 day SRT mesophilic anaerobic digesters, and the sequential anaerobic and aerobic digesters following thermal hydrolytic pretreatment. The 16S rRNA genes were amplified using primers 8F and 1492R (Table S2.1, Table S2.2), and cloned into *Escherichia coli* using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). Fifty clones generated from each sample were analyzed further by DGGE, as described above, to identify inserts that corresponded to dominant bands of each digester sample. The corresponding M13 PCR products were sequenced by the Virginia Bioinformatics Institute sequencing facility. The closest matches to known microorganisms were determined using the BLAST alignment tool at the National Center for Biotechnology Information Website (http://www.ncbi.nlm.nih.gov/BLAST/). The online environmental analysis software FastUniFrac (http://bmf2.colorado.edu/fastunifrac/) was used to perform Principal Coordinate Analysis (PCoA). For data submission of FastUniFrac, a phylogenetic tree was constructed from the sequence alignment generated using Clustal X 2.0.11.
2.3.6 Data Analysis

To report an average performance of various sludge treatment processes, multiple sampling events were treated as replicates. Averages and standard errors of all data were determined using Microsoft Excel 2007. *t* tests were conducted by R 2.8.1 (http://cran.r-project.org/bin/windows/base/old/2.8.1/) to determine whether Q-PCR based ARG and *intI1* quantities were significantly different between samples. Correlation tests were also performed to reveal relevance of *intI1* and ARG occurrence. A *p*-value of <0.05 was considered to indicate significance.

2.4 Results

2.4.1 ARG Occurrence

A broad range of ARGs were detected in the sludge samples, including two *sul* (*sulI* and *sulII*), three *erm* (*erm(A), erm(B), and erm(F)*) and nine *tet* (*tet(C), tetB(P), tet(E), tet(G), tet(O), tet(Q), tet(T), tet(W), and tet(X)) ARGs. The *vanA* and *mecA* genes encoding vancomycin and methicillin resistance, respectively, were not detected in any sludge samples in spite of PCR optimization and serial dilution to eliminate possible inhibition by the matrix.

2.4.2 Response of ARGs to Treatments

Nine ARGs (*sulI, sulII, erm(B), erm(F), tet(O), tet(W), tet(C), tet(G), and tet(X)) that were frequently detected and represented a range of resistance mechanisms were further quantified to examine their response to various treatment conditions. In order to compare absolute reductions of ARGs, gene quantities are presented normalized to grams of dry solids. Quantities of the nine ARGs in the feed sludge ranged from $10^8$ to $10^{10}$ copies/g dry solids (Table S2.4). ARG quantities after anaerobic digestions with varied SRT and temperatures are
compared in Figure 2.1. Quantitative changes of ARGs during the sequential process are shown in Figure 2.2. Gene quantities are also normalized to 16S rRNA genes as an indicator of the proportion of bacteria carrying ARGs (Figure S2.1, Figure S2.2). In terms of ARGs normalized to grams of total solids, highly similar performance was observed between the 10 and 20 day SRT mesophilic anaerobic digesters operated at 35 °C. In both conditions, sulI, sulII, tet(C), tet(G), and tet(X) genes reduced in response to treatment, while erm(B), erm(F) and tet(W) ARGs increased (Figure 2.1). All net changes in concentration were statistically significant (p < 0.05) except tet(C) and tet(W) in the 20 day SRT digester (p = 0.45 and 0.20, respectively). Only tet(O) responded differently between the two mesophilic digesters: it reduced in the 20 day SRT digester (p = 0.0005), but increased in the 10 day SRT digester (p = 0.0014).

The three thermophilic digesters operated at 47 °C, 52 °C and 59 °C also performed similarly to each other. In all three thermophilic digesters, sulI, sulII, erm(B), erm(F), tet(O), tet(W), and tet(G) genes significantly decreased (p < 0.05). The exceptions were tet(C) and tet(X). tet(C) increased after digestion at 52 °C and 59 °C (p < 0.05) and was not significantly different from the feed in the 47 °C digester (p = 0.78). tet(X) decreased in the 59 °C digester (p < 0.05), but increased in the 47 °C digester (p < 0.05) and was not statistically different from the feed after digestion at 52 °C (p = 0.98).

Thermal hydrolysis pretreatment resulted in remarkable reduction of all the nine ARGs (p < 0.05) with the range of reduction from 1.59 to 2.60 log unit (Figure 2.2). However, most of the ARGs were observed to rebound after subsequent anaerobic digestion except sulI and tet(G), which were not significantly different from quantities in thermal hydrolytically treated influent (p = 0.68 and 0.86, respectively). During aerobic digestion following the anaerobic digestion, sulI, sulII, tet(C), tet(G), and tet(X) genes continued to increase (p < 0.05), while erm(B),
*erm*(F), *tet*(O) and *tet*(W) genes decreased (*p* < 0.05).

![Figure 2.1 Quantities of ARG and *int*I1 gene in the feed sludge (Feed) and effluent of the 10 day SRT mesophilic (meso-10d), 20 day SRT mesophilic (meso-20d), and 47 °C, 52 °C and 59 °C thermophilic (thermo) digesters. Error bars indicate standard errors of 9 Q-PCR measurements for each of the 4 sampling events (n=36). The average quantities of ARG and *int*I1 in feed sludge are reported in Table S2.4.
Figure 2.2 Quantities of ARG and \textit{intI1} in the feed sludge (Feed), sludge pretreated by thermal hydrolysis (THydrol), effluent of the mesophilic digester receiving pretreated sludge (AnaeD), and the subsequent aerobic digester (AeroD). Error bars indicate standard errors of 9 Q-PCR measurements for each of the 4 sampling events (n=36). The average quantities of ARG and \textit{intI1} in the feed sludge are reported in Table S2.4.
When ARGs were normalized to 16S rRNA genes, rather than grams of solids, the effect of the various treatment processes on ARGs maintained a remarkably similar trend (Figures S2.1, Figure S2.2), with only two exceptions. Firstly, 20 day SRT mesophilic anaerobic digesters yielded significantly greater reduction of sulI, sulII and tet(G) than the 10 day SRT digester (p < 0.05). Secondly, because all DNA is similarly affected by hydrolysis, normalization of ARGs to 16S rRNA genes masked the effect of the thermal hydrolytic pretreatment on ARGs (Figure S2.2).

2.4.3 Response of intI1 to Treatments

Significant reduction of intI1 was observed in all of the five anaerobic digesters operated at varied SRT and temperatures (p < 0.05, Figure 2.1). During the sequential process, a large portion of intI1 (2.46 log unit) was removed during thermal hydrolysis pretreatment (p < 0.05, Figure 2.2). However, a corresponding rebound and continuous increase of intI1 gene was observed in the following anaerobic and aerobic digestions (p < 0.05).

2.4.4 Characterization of Bacterial Community

The bacterial community was characterized by DGGE to gain insight into the potential role of microbial ecology in driving the ARG response to biological treatment. The mesophilic anaerobic digesters yielded the greatest number of bands and consequently the highest diversity indices (H = 1.45 and 1.51, respectively), relative to the thermophilic digesters (H = 1.17, 1.22, and 1.19 at 47 °C, 52 °C and 59 °C, respectively). Very few bands were yielded by hydrolytically pretreated sludge (H = 0.69), but diversity increased markedly in the subsequent anaerobic and aerobic digesters (H = 1.31 and 1.33, respectively).
Figure 2.3 Denaturing gradient gel electrophoresis (DGGE) of bacterial community in each treatment process. 1) thermophilic digester at 47 °C; 2) thermophilic digester at 52 °C; 3) thermophilic digester at 59 °C; 4) mesophilic digester at 10 day SRT; 5) mesophilic digester at 20 day SRT; 6) sludge pretreated by thermal hydrolysis; 7) mesophilic digester receiving pretreated sludge; 8) aerobic digester receiving feed from 7).

16S rRNA genes that migrated to the same position as bands in the digester DGGE profiles were sequenced (Table S2.5). Relative intensities of the corresponding DGGE bands were used to estimate the fraction of the community represented by each sequence (Figure S2.3). Most identified bacteria belonged to four phyla: *Firmicutes, Proteobacteria, Bacteroidetes*, and *Chloroflexi*, which have previously been reported to be dominant in other sludge samples.22,23 *Firmicutes* and *Proteobacteria* occupied the largest fraction of dominant bacterial community in the thermophilic anaerobic digester. All four phyla were found in the mesophilic anaerobic
digester. Most bacteria identified in the anaerobic digester following thermal hydrolysis pretreatment were *Firmicutes*, while the bacterial community became evenly distributed among phyla in the downstream aerobic digester.

Figure 2.4 First two axes of Principal Coordinate Analysis (PCoA) of 16S rRNA gene sequence data using FastUniFrac. The 52 °C thermophilic digester (thermo-52°C), the 10 day SRT mesophilic digester(meso-10d), the 20 day SRT mesophilic digester (meso-20d), the mesophilic digester following thermal hydrolytic pretreatment (AnaeD), and the subsequent aerobic digester (AeroD) correspond to lanes 2, 4, 5, 7 and 8 in Fig. 2.3.

The phylogenetic distance of samples was characterized by PCoA (Figure 2.4). Digester samples tended to cluster by treatment temperatures as well as by characteristics of the feed sludge. The Phylogenetic make-up of the thermophilic anaerobic digester was distinct from that of the other digesters. Digesters at mesophilic temperature but following thermal hydrolytic
pretreatment were clearly differentiated from mesophilic digesters receiving waste solids directly. There was only a slight distinction between the sequential anaerobic and aerobic digesters following hydrolytic pretreatment, and the 10 day and 20 day SRT mesophilic digesters were clustered closely to each other.

2.5 Discussion

This study demonstrates that sludge digestion stands in a key position to alter the loadings of ARGs exiting wastewater treatment plants. Digesters can act to physically destroy extracellular DNA through hydrolysis and biodegradation, which generally would be expected to occur at higher rates with increased temperature. At the same time, ARGs may be harbored by host bacterial cells and subject to amplification via cell growth or horizontal gene transfer or attenuation via differential survival with respect to the digester operating conditions. The fact that ARG removal did not follow a simple trend with temperature emphasizes the importance of advancing understanding of the role of bacterial community composition in the amplification or attenuation of ARGs in sludge digesters.

2.5.1 Effect of SRT

ARG quantities normalized to 16S rRNA genes revealed a greater removal efficiency of sulI, sulII and tet(G) by the 20 day SRT mesophilic anaerobic digester relative to its 10 day SRT counterpart. The fact that this difference was not observable when ARGs were normalized to grams of solids suggests that a greater proportion of inert solids characteristic of longer SRTs masked the effect. In either case, the two mesophilic anaerobic digesters operated at 10 and 20 day SRT yielded strikingly similar patterns of ARG response to treatment, i.e. the same ARGs increased or decreased in both, with tet(O) the only exception. The similarity of the bacterial
communities was also remarkable, as indicated by their phylogenetic composition (Figure 2.4, Figure S2.3). It is likely that similar bacterial community composition, which largely determines available ARG hosts, drove their congruent response to treatments, with SRT impacting the extent of changes. Future studies incorporating a wider range of SRTs would be of interest, as well as more deeply exploring the potential effects of host range versus the extent of solids destruction.

2.5.2 Effect of Temperature

Results of this study suggest that the thermophilic anaerobic digesters at 47 °C, 52 °C and 59 °C provided superior removal for *erm*(B), *erm*(F), *tet*(O) and *tet*(W) genes, relative to mesophilic digesters. This is consistent with the observations of Ghosh et al.\(^{16}\) and Diehl and LaPara\(^{17}\) with respect to *tet*(O) and *tet*(W) ARGs and further extends their encouraging findings to *erm* ARGs. DGGE revealed markedly lower bacterial diversity in the thermophilic versus the mesophilic digester, as illustrated by the number of bands detected (Figure 2.3) and the phylogenetic distribution of dominant bacteria identified (Figure S2.3). While individual ARG hosts may theoretically maintain abundance in spite of low diversity, this situation diminishes the overall probability of encountering a compatible host via horizontal gene transfer. Thus, restriction of the ARG host range may be a significant mechanism of attenuation in thermophilic digesters. Furthermore, higher temperature offers additional benefits of enhancing biological and chemical reaction rates.\(^{13}\) Such factors may have together contributed to the more effective removal of *erm*(B), *erm*(F), *tet*(O) and *tet*(W) ARGs under higher temperature operation.

Diehl and LaPara\(^{17}\) observed increasing removal rates and efficiencies of all five *tet* ARGs investigated (*tet*(A), *tet*(L), *tet*(O), *tet*(W) and *tet*(X)) as a function of temperature in lab-scale anaerobic digesters operated at 15 day SRT. Although the operating conditions were
similar, the trend was not identical in the present study. Within the thermophilic temperature range (47 °C, 52 °C, and 59 °C), the responses of ARGs in this study were notably consistent. Meanwhile, highly similar bacterial communities within the three digesters were observed by DGGE analysis (Figure 2.3). These observations further support the conclusion that bacterial community composition played an important role in driving similar response of ARGs to treatments. Considering that temperature was the only variable between the three thermophilic digesters, these observations also reveal the high repeatability of the digesters operating in this study.

Furthermore, in this study thermophilic digestion was more effective for \textit{erm}(B), \textit{erm}(F), \textit{tet}(O) and \textit{tet}(W); but did not achieve greater attenuation of the other ARGs relative to mesophilic digestion. While \textit{sul}I and \textit{sul}II genes were reduced to a statistically equivalent extent (p > 0.05) by both digestion processes, quantities of \textit{tet}(C), \textit{tet}(G) and \textit{tet}(X) were generally lower in the effluent of the two mesophilic digesters than in the three thermophilic digesters (p < 0.05, except p = 0.25 for \textit{tet}(C) in 20 day mesophilic versus 47 °C thermophilic digester and p = 0.26 for \textit{tet}(X) in 10 day mesophilic versus 59 °C thermophilic digester). Notably, \textit{tet}(C) increased during the 52 °C and 59 °C digestions, while \textit{tet}(X) increased during the 47 °C digestion (p < 0.05). \textit{tet}(X) in particular exhibited precisely the opposite trend in the present study relative to that of Diehl and LaPara. Although a different forward primer for \textit{tet}(X) was employed (Table S2.1), it is not expected that the specificity would be impacted given that it was designed from the same \textit{tet}(X) sequence variant (GenBank # M37699). Considering that the temperature range of the Diehl and LaPara study (22 °C, 37 °C, 46 °C, and 55 °C) was comparable to the present study, this suggests that other factors may also be important. In addition to a distinct feed sludge, the amount and frequency of sludge replacement in the Diehl
and LaPara study was somewhat atypical, with 33% of the reactor volume replaced every 5 days, whereas in the present study, 5-6.7% of the sludge volume was replaced each day. Although this technically results in the same net SRT, larger and less frequent volume replacement approaches a plug flow reactor regime, which may impose feast and famine conditions on the bacterial community or even subject it to shock loads of toxins.

2.5.3 Effect of Thermal Hydrolysis Pretreatment

Thermal hydrolysis pretreatment was initially introduced to sludge digestion for the purpose of improving dewaterability and accelerating the rate-limiting step of hydrolysis of organic matter. The high temperature and pressure generally acts to sterilize the sludge, destroy cell walls, and release readily degradable components. This study confirmed that DNA is also susceptible to hydrolytic destruction. Interestingly, the anaerobic digester following thermal hydrolysis pretreatment, although also operated at mesophilic temperature, was clearly distinct from the mesophilic digesters receiving untreated sludge (Figure 2.3, Figure 2.4). The dominant bacteria in the anaerobic digester following thermal hydrolysis pretreatment mainly belonged to Firmicutes (Figure S2.3), indicating a favorable environment for fermentation after hydrolysis having largely been executed during pretreatment. It is also noted in the PCoA that the sequential anaerobic and aerobic digesters were clustered closely to each other (Figure 2.4), although they have contrasting redox conditions. This is likely an indication of the persistence of the DNA of dead or inactive bacteria through the relatively short 5 day SRT of the aerobic post-treatment digester.

Arguably, the most significant finding of this study is that most ARGs rebounded during anaerobic digestion following thermal hydrolytic pretreatment. The overall rebound effect following pretreatment illustrates that the microbial community composition of the digester
seemingly dominates the behavior of ARGs relative to influent ARG concentrations. ARG distribution and concentration is likely driven by the characteristics of the seed culture and ARG carriers subsequently respond to the selection pressures imposed by the digester operating conditions. However, the potential for persistence of residual DNA and horizontal gene transfer cannot be precluded. It would be interest to conduct future studies in which the seed culture is carefully selected to be devoid of ARGs, with the feed concentration of ARGs systematically manipulated.

In spite of the rebound effect, all ARGs were still lower in the digester receiving thermal hydrolytically pretreated sludge than in the mesophilic digesters receiving untreated sludge (p < 0.05), except \textit{sul}II and \textit{erm}(B), which rebounded to quantities that were statistically equivalent to the 20 day SRT mesophilic digester (p = 0.07 and 0.28, respectively). Perhaps the lower diversity of bacteria, mostly dominated by \textit{Firmicutes}, provided a narrower host range for ARGs, in a similar fashion to the phenomenon suggested for the thermophilic digesters. An alternate explanation may be that the higher ARG concentrations present in the feed of the conventional mesophilic digesters did provide some contribution to the effluent ARG levels.

Further support for the importance of bacterial community composition is the fact that ARG behavior in subsequent the aerobic digester was distinct: \textit{sul}, \textit{sul}II, \textit{tet}(C), \textit{tet}(G) and \textit{tet}(X) ARGs continued to increase (p < 0.05), while \textit{erm}(B), \textit{erm}(F), \textit{tet}(O) and \textit{tet}(W) ARGs decreased (p < 0.05). Aerobic conditions select for distinct bacteria and generally a wider host range. For example, \textit{tet}(X) encodes an oxygenase enzyme and though it is found in the obligately anaerobic \textit{Bacteroides}, the enzyme itself theoretically requires oxygen to function. Though Diehl and LaPara\textsuperscript{17} observed stable \textit{tet}(X) levels during various aerobic digestions, aerobic
conditions appeared to exert a strong selection pressure on \(tet(X)\) under the conditions of the present study.

2.5.4 Correlation of \(intI1\) with ARGs

The class I integron plays an important role in gene transfer among bacteria. It is frequently reported to carry one or more gene cassettes that encode antibiotic resistance.\(^{25,26}\) Thus documenting behavior of \(intI1\) may provide insight to ARG dissemination potential. In this study, correlation analysis was performed to relate \(intI1\) and ARG occurrence. Only \(sulI\) was found to positively correlate with \(intI1\) occurrence in the feed and effluent of digesters (\(R^2 = 0.88, p < 0.05\)), which is consistent with \(sulI\) typically being associated with class I integrons.\(^{27}\) Significant correlation was not found between \(intI1\) and other ARGs. Notably, \(intI1\) rebounded together with ARGs during the anaerobic digestion following thermal hydrolysis pretreatment, and increased by \(~2\) log units in the aerobic digester, which was the largest increase of any ARG. This suggests that the horizontal gene transfer may be a key process during sludge digestion and is worthy of further study.

2.6 Acknowledgment

Funding for this research was provided by the National Science Foundation, CBET CAREER award # 0547342. The findings of this study do not necessarily reflect the views of the foundation. The authors also thank Dr. Daniel Gallagher (Virginia Tech) for assistance with statistical analysis.
2.7 References


### 2.8 Supplemental Materials

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target gene</th>
<th>Primer / Probe sequence (5’- 3’)</th>
<th>Annealing Temp. (°C)</th>
<th>Source of reference</th>
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<td>(1)</td>
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<td>(5)</td>
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<td>product</td>
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<td>tet(M)</td>
<td>TGGCGTGTCTATGATGTTCAC</td>
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<td>5</td>
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<td></td>
</tr>
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<td>GAGAGCCTGCTTATATGCACC</td>
<td>60</td>
<td>5</td>
<td></td>
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<td>tet(X)</td>
<td>CAATAATGGTGTTGGGACCC</td>
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<td>6</td>
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<td>intI1</td>
<td>CTGGATTTTCGATACCGGACG</td>
<td>60</td>
<td>7</td>
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</tr>
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<td>16S rRNA</td>
<td>CGGTGAATACGTTCYCGG</td>
<td>60</td>
<td>8</td>
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Table S2.2 PCR reaction matrix\(^a\)

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<tr>
<th>Reagent</th>
<th>Amount or concentration in 25 µL master mix</th>
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<tr>
<td>10× buffer</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>5× buffer</td>
<td>5 µL</td>
</tr>
<tr>
<td>2 µL Mg(^{2+})</td>
<td>2 µL</td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.2mM</td>
</tr>
<tr>
<td>Forward and reverse primer</td>
<td>0.25 µM</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>1.75U</td>
</tr>
<tr>
<td>Formamide(^b)</td>
<td>0.25 µL</td>
</tr>
<tr>
<td>DNA template</td>
<td>1 µL</td>
</tr>
</tbody>
</table>

\(^a\)PCR assays were carried out using a MasterTaq kit (Eppendorf, Westbury, NY).

\(^b\)Formamide is added for 16S rRNA PCR only.

Table S2.3 Q-PCR reaction matrix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration in 20 µL master mix</th>
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<tbody>
<tr>
<td>Forward and reverse primers</td>
<td>0.3 µM</td>
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<tr>
<td>SsoFast EvaGreen Supermix</td>
<td>1 ×</td>
</tr>
</tbody>
</table>
### Table S2.4 Average quantities of ARG and intI1 in feed sludge

<table>
<thead>
<tr>
<th>Gene</th>
<th>Quantity (copy of genes / g dry solids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sulI</td>
<td>(1.27 ± 0.13) × 10^{10}</td>
</tr>
<tr>
<td>sulII</td>
<td>(4.55 ± 0.64) × 10^{9}</td>
</tr>
<tr>
<td>erm(B)</td>
<td>(8.92 ± 1.55) × 10^{8}</td>
</tr>
<tr>
<td>erm(F)</td>
<td>(9.20 ± 0.94) × 10^{8}</td>
</tr>
<tr>
<td>tet(O)</td>
<td>(1.39 ± 0.20) × 10^{9}</td>
</tr>
<tr>
<td>tet(W)</td>
<td>(1.75 ± 0.22) × 10^{9}</td>
</tr>
<tr>
<td>tet(C)</td>
<td>(2.67 ± 0.56) × 10^{9}</td>
</tr>
<tr>
<td>tet(G)</td>
<td>(7.69 ± 0.79) × 10^{9}</td>
</tr>
<tr>
<td>tet(X)</td>
<td>(1.50 ± 0.33) × 10^{8}</td>
</tr>
<tr>
<td>intI1</td>
<td>(5.13 ± 0.80) × 10^{8}</td>
</tr>
</tbody>
</table>
Table S2.5 Closest match microorganisms of sequenced clones

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phylogenic affiliation</th>
<th>Closest match microorganism (accession number)</th>
<th>% of identity</th>
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</thead>
<tbody>
<tr>
<td>thermo-52°C</td>
<td>Firmicutes</td>
<td>Posterthermobacter proteolyticus DSM 5265 (CP001145)</td>
<td>98%</td>
</tr>
<tr>
<td>thermo-52°C</td>
<td>Firmicutes</td>
<td>Clostridium sp. ZH11 (HM103331)</td>
<td>87%</td>
</tr>
<tr>
<td>thermo-52°C</td>
<td>Firmicutes</td>
<td>Coprothermobacter proteolyticus DSM 5265 (CP001145)</td>
<td>98%</td>
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<tr>
<td>thermo-52°C</td>
<td>Firmicutes</td>
<td>Clostridium sp. PML3-1 (EF165015)</td>
<td>94%</td>
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<td>Proteobacteria α-Proteobacteria</td>
<td>Rhodobacter sp. TCRI 14 (AB017799)</td>
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<td>Comamonas denitrificans strain 110 (AF233876)</td>
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<td></td>
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<td>Proteobacteria β-Proteobacteria</td>
<td>Acidovorax sp. 3T2-6</td>
<td>100%</td>
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<td>Proteobacteria β-Proteobacteria</td>
<td>Uncultured Methylophilus sp. (FM175198)</td>
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<tr>
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<td>Uncultured soil bacterium clone M22_Pitesti (DQ378242)</td>
<td>97%</td>
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<tr>
<td>Proteobacteria β-Proteobacteria</td>
<td>Uncultured bacterium clone Rap1_6A (EF192877)</td>
<td>98%</td>
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<tr>
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<td>Alcaligenes faecalis strain SAG5 (GQ422443)</td>
<td>98%</td>
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<tr>
<td>TM7 unclassified</td>
<td>Uncultured TM7 bacterium (CU917719)</td>
<td>97%</td>
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<tr>
<td>Planctomycetes Planctomycetacia</td>
<td>Uncultured planctomycete clone PLA10 (AF525965)</td>
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<tr>
<td>Acidobacteria unclassified</td>
<td>Acidobacteria bacterium enrichment culture clone Ac-F6 (GQ902908)</td>
<td>100%</td>
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<tr>
<td>meso-10d</td>
<td>Bacteroidetes Bacteroidia</td>
<td>Uncultured Bacteroidetes bacterium clone QEEA3AF11 (CU918774)</td>
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<td>Species/Clone</td>
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<td>--------------</td>
<td>--------------</td>
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<td>Prevotella sp. 326-8 (FJ848548)</td>
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Figure S2.1 Quantities of ARG and \textit{int}I1 gene normalized to 16S rRNA genes in the feed sludge (Feed) and effluent of the 10 day SRT mesophilic (meso-10d), 20 day SRT mesophilic (meso-20d), and \(47^\circ\text{C}, 52^\circ\text{C}\) and \(59^\circ\text{C}\) thermophilic (thermo) digesters. Error bars indicate standard errors of 9 Q-PCR measurements for each of the 4 sampling events (\(n = 36\)).
Figure S2.2 Quantities of ARG and *int*I1 normalized to 16S rRNA genes in the feed sludge (Feed), sludge pretreated by thermal hydrolysis (THydrol), effluent of the mesophilic digester receiving pretreated sludge (AnaeD), and the subsequent aerobic digester (AeroD). Error bars indicate standard errors of 9 Q-PCR measurements for each of the 4 sampling events (n = 36).
Figure S2.3 Classification of dominant bacteria in the 52 °C thermophilic digester (thermo-52°C), the 10 day SRT mesophilic digester (meso-10d), the 20 day SRT mesophilic digester (meso-20d), the mesophilic digester following thermal hydrolytic pretreatment (AnaeD), and the subsequent aerobic digester (AeroD) corresponding to lanes 2, 4, 5, 7 and 8 in Figure 2.3. 16S rRNA gene clones that migrated to the same position as bands in the digester DGGE profiles were sequenced. Fifty clones were analyzed for each digester.

References


CHAPTER 3: Microbial Community Response of Nitrifying Sequencing Batch Reactors to Silver, Zero-Valent Iron, Titanium Dioxide and Cerium Dioxide Nanomaterials

Yanjun Ma, Jacob W. Metch, Eric P. Vejerano, Ian J. Miller, Elena C. Leon, Linsey C. Marr, and Amy Pruden

3.1 Abstract

As nanomaterials in consumer products increasingly enter wastewater treatment plants, there is concern that they may have adverse effects on biological wastewater treatment. Effects of silver (nanoAg), zero-valent iron (NZVI), titanium dioxide (nanoTiO$_2$) and cerium dioxide (nanoCeO$_2$) nanomaterials on nitrification and microbial community structure were examined in duplicate lab-scale nitrifying sequencing batch reactors (SBRs) relative to control SBRs that received no nanomaterials or bulk/ionic analogs. Nanomaterial/analog doses started at 0.1 mg/L and were sequentially increased every 14 days to 1, 10, and 20 mg/L. Nitrification function was not measurably inhibited in the SBRs by any of the dosed materials based on ammonia, nitrite, or nitrate levels. However, decreased nitrifier gene abundances (qPCR of amoA and *Nitrobacter* 16S rRNA genes) and distinct bacterial communities (pyrosequencing of 16S rRNA genes) were observed in SBRs dosed with nanoAg, Ag$^+$, nanoCeO$_2$, and bulkCeO$_2$, relative to controls, with the most pronounced effects associated with Ag$^+$. There was no apparent effect of nanoTiO$_2$ or NZVI on nitrification, nitrifier gene abundances, or microbial community structure. Because nanoAg and nanoCeO$_2$ indicated the greatest potential effects, experiments were repeated for these two nanomaterials and their analogs at a continuous high load of 20 mg/L over 42 days. While the high load Ag$^+$ condition rapidly lost nitrification functionality, there was no
measureable effect of continuous high loads of the other dosed materials on nitrification function or levels of nitrification gene markers. TEM-EDS analysis indicated that a large portion of nanoAg remained dispersed in the activated sludge and formed Ag-S complexes, while NZVI, nanoTiO$_2$ and nanoCeO$_2$ were mostly aggregated and chemically unmodified. Thus, the nanomaterials appeared to be generally stable in the activated sludge, which may have limited their effect on nitrification function and microbial community structure. Experiments carried out over longer term or shock load conditions may elicit a stronger effect.

3.2 Introduction

As nanotechnology-based consumer products enter into widespread use, a large portion of nanomaterials will enter wastewater treatment plants.$^{1-2}$ As a gateway of controlling nanomaterial release into the natural environment, wastewater treatment processes have been observed to remove the majority of nanomaterials in aqueous effluents, primarily concentrating them into the activated sludge phase.$^{3-4}$ Compared with traditional bulk materials, the nano-size range imparts unique properties, including greater reactivity and thus more potential toxicity to biological systems.$^5$ Toxicity of nanomaterials to microbes has been well-documented in pure culture studies.$^{6-8}$ Therefore, there is concern that the accumulation of nanomaterials in activated sludge could have adverse effects on microbes responsible for biological wastewater treatment.

Nitrifying bacteria, including ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), conduct the critical function of nitrification in biological wastewater treatment to covert ammonia to nitrite and subsequently to nitrate. Nitrifying bacteria are known for slow growth rate and high sensitivity to toxic chemicals.$^9$ As increasing amounts of nanomaterials enter wastewater, they have the potential to inhibit nitrification function due to toxicity to nitrifying bacteria. Toxic effects of silver nanoparticles have been observed on Nitrosomonas.
Europeae in pure culture\textsuperscript{10-11} and enriched nitrifying microorganisms.\textsuperscript{12} However, the effects of other nanomaterials on nitrifying bacteria, especially under the real-world complex wastewater matrix and aeration conditions of biological treatment, have not been established.

Impacts of a few nanomaterials, including Ag, TiO\textsubscript{2} and ZnO, on nitrification have been examined in laboratory-scale reactors.\textsuperscript{13-16} However, the extent of reported nitrification inhibition has varied and inconsistent trends have been observed with respect to effects of nanomaterial concentrations and individual wastewater treatment processes. In one study, nitrification inhibition was observed in response to a 12-h period of silver nanoparticle shock loading to reach a final peak silver concentration of 0.75 mg/L in a lab-scale Modified Ludzacke-Ettinger process.\textsuperscript{13} In another study, continuous loading of 0.1 and 0.5 mg/L silver nanoparticle into sequencing batch reactors (SBRs) reduced ammonia removal rate on the first day, but the SBRs recovered quickly.\textsuperscript{14} Nitrification efficiency decreased significantly in an anaerobic-low dissolved oxygen SBR following short-term exposure of 10 and 50 mg/L ZnO nanoparticles,\textsuperscript{15} but a similar effect was not observed for short-term exposure of 1 or 50 mg/L TiO\textsubscript{2} nanoparticles in the same type of reactor.\textsuperscript{16} Thus, there is a need for broad comparison of a range of nanoparticles on nitrification in complex bioreactors under a common set of conditions. Furthermore, considering that wastewater treatment is a microbial-ecologically driven process, insight into effects of nanomaterials on the broader microbial community is needed.

In this study, effects of silver (nanoAg), zero valent iron (NZVI), titanium dioxide (nanoTiO\textsubscript{2}) and cerium dioxide (nanoCeO\textsubscript{2}) nanomaterials were investigated in duplicate lab-scale SBRs simulating typical nitrifying activated sludge wastewater treatment. Effects of nanomaterials were compared to control SBRs that were undosed or received bulk or ionic counterparts (Ag\textsuperscript{+}, Fe\textsuperscript{2+}, bulkTiO\textsubscript{2}, or bulkCeO\textsubscript{2}). SBRs were re-started prior to each set of
experiments, which were conducted in two phases: 1.) sequentially increased loading after each 14 days with nanomaterial concentrations of 0.1, 1, 10 and 20 mg/L and 2.) continuous high loading at 20 mg/L for nanoAg, nanoCeO$_2$, and their analogs, which indicated the greatest potential effects based on the sequential loading experiments. Nitrification performance was monitored along with the abundance of gene markers of nitrification by quantitative polymerase chain reaction (q-PCR), while response of microbial communities was characterized by pyrosequencing of bacterial 16S rRNA genes.\textsuperscript{17,18} Transmission Electron Microscopy (TEM) equipped with Energy Dispersive X-ray Spectroscopy (EDS) was used to examine aggregation state and interaction of nanomaterials with activated sludge to understand potential relationships between nanomaterial transformation and toxicity effects.

### 3.3 Materials and Methods

#### 3.3.1 Preparation of Nanomaterial Suspensions

NanoAg was synthesized by the sodium citrate reduction method, yielding a concentration of 100 mg/L.\textsuperscript{19} NZVI dispersion with a combination of a biodegradable organic and inorganic stabilizer was purchased from NANO IRON s.o.r. (NANOFER 25S, Rajhrad, Czech Republic). Anatase TiO$_2$ and CeO$_2$ nanopowder (Sigma-Aldrich, Saint Louis, MO) were dispersed into nanopure water by sonication for 20 min (90 W, 20 KHz, 20 °C) at the concentration of 100 mg/L. Freshly prepared nanomaterial dispersions were used to dose SBRs. The properties of the prepared nanomaterial dispersions were confirmed and characterized by TEM. The average sizes of the nanoparticles were 52±12 nm for nanoAg, 46±10 nm for NZVI, 21±12 nm for nanoTiO$_2$, and 33±12 nm for nanoCeO$_2$. 
3.3.2 Set-up of Sequencing Batch Reactors (SBRs)

Lab-scale SBRs were set up in 3 L glass beakers (Fisher, Suwanee, GA) with an active volume of 2 L in a temperature controlled room at 20 °C. Each SBR was operated with two 12 h cycles per day with solids retention time (SRT) of 23 days and hydraulic retention time (HRT) of 2 days. Each cycle consisted of 0.1 h feeding, 10.82 h aeration, 0.05 h mixed liquor wasting (solids wasting), 0.75 h settling, and 0.33 h decant. Waste sludge was returned at a rate of 0.10 to maintain the biomass. The SBRs were seeded with return nitrifying activated sludge from a local municipal wastewater treatment plant. A synthetic wastewater (Table S3.1 and S3.2) served as feed with an initial chemical oxygen demand (COD) of 450 mg/L and total nitrogen (TN) of 55 mg/L. Upon reaching steady state, pH of the SBRs was in the range of 7.4-7.7, mixed liquor suspended solids (MLSS) 3000-4000 mg/L, and mixed liquor volatile suspended solids (MLVSS) 2200-3400 mg/L. SBRs were acclimated until ammonium nitrogen removal of >98% was achieved (typically 3-5 weeks) before dosing of nanomaterials. Each nanomaterial was tested individually, with the SBRs re-started prior to the next experiment in order to attain equivalent performance among the SBRs and avoid effects of prior nanomaterials before testing the subsequent nanomaterial.

3.3.3 Sequential Load of Nanomaterials

Three conditions were examined in duplicate SBRs: nanomaterial, corresponding bulk or ionic material [Ag⁺ as AgNO₃ (Fisher, Suwanee, GA); Fe²⁺ as FeSO₄ (Fisher, Suwanee, GA); bulkTiO₂ (Fisher, Suwanee, GA); bulkCeO₂ (Sigma-Aldrich, Saint Louis, MO)], and undosed controls. The dosing of nanomaterials and bulk/ionic materials was initiated at an influent concentration of 0.1 mg/L and sequentially increased after each 14 days to 1 mg/L, 10 mg/L and
finally 20 mg/L. Nanomaterial and bulk/ionic material dispersions or solutions were directly dosed to the reactors at the feeding time of each cycle.

3.3.4 High Load of Nanomaterials

Based on results of sequential load experiments, nanoAg, nanoCeO$_2$ and the bulk/ionic analogs were selected for high load experiments. Upon reaching steady-state, 20 mg/L nanoAg, Ag$^+$, nanoCeO$_2$ and bulkCeO$_2$ dispersions or solutions were dosed to each reactor at the feeding time of each cycle for a total of 42 days.

3.3.5 Analytical Methods

Nitrification was monitored by quantifying concentrations of nitrite nitrogen (NO$_2^-$-N), nitrate nitrogen (NO$_3^-$-N) and ammonia nitrogen (NH$_3$-N) in the aqueous effluent by Ion Chromatography (Thermo Scientific, Tewksbury, MA) and using an Ammonia Nitrogen Test Kit (Hach, Loveland, CO). pH, MLSS and MLVSS of SBRs were monitored regularly as indicators of reactor performance using standard methods.$^{20}$ Partitioning of nanomaterials and bulk/ionic materials in effluents and activated sludge were analyzed using ICP-MS (Thermo Scientific, Tewksbury, MA) by quantification of Ag, Fe, Ti and Ce. Aqueous effluents were filtered through 0.45 µm mixed-cellulose ester (MCE) membrane filters (EMD Millipore, Billerica, MA) prior to analysis. Activated sludge containing nanoAg was digested with 1:3 hydrochloric to Nitric acid (v/v), and activated sludge containing NZVI, nanoTiO$_2$, and nanoCeO$_2$ was digested by 1:1 sulfuric to nitric acid (v/v). The digested samples were diluted and filtered through 0.45 µm MCE membrane filters (EMD Millipore, Billerica, MA) for ICP-MS.
3.3.6 q-PCR

Activated sludge was sampled from each SBR just prior to commensing dosing and every 7 days during both sequential load and high load experiments. Total nucleic acid was extracted in duplicates from 175 µL activated sludge using a MagMAX™ Total Nucleic Acid Isolation Kit (Life technologies, Grand Island, NY) following the manufacturer’s instruction. To monitor the abundance of nitrifying bacteria, amoA genes encoding the active-site polypeptide of ammonia monooxygenase, 16S rRNA genes of Nitrobacter and Nitrospira (two common genera of NOB), and universal bacterial 16S rRNA genes were quantified by q-PCR using previously reported primers.²¹⁻²⁴ All q-PCR standard curves were constructed from serial dilutions of cloned genes ranging from 10⁸ to 10² gene copies per µL. The presence of PCR inhibitors was checked by serially diluting a representative sub-set of samples and comparing PCR efficiencies with standards. It was found that a 1:50 dilution performed well to minimize inhibitory effects and was applied across samples. Samples were analyzed in triplicate with a standard curve and negative control included in each run. Gene copy numbers of amoA and Nitrobacter and Nitrospira 16S rRNA genes were normalized to universal bacterial 16S rRNA genes. Averages and standard deviations of all data were determined using Microsoft Excel® 2007. t tests were conducted using R 2.8.1 software (http://cran.r-project.org/bin/windows/base/old/2.8.1/) to determine statistical differences between samples at at a significance level of 0.05 (p < 0.05).

3.3.7 Pyrosequencing

Nucleic acid extracts corresponding to just prior to dosing and at the end of both sequential load and high load experiments were diluted to 20 ng/µL and subjected to pyrosequencing of 16S rRNA genes using primers 341F/907R on a Roche 454 FLX Titanium platform (Roche, Nutley, NJ) by the Research and Testing Laboratory (Lubbock, TX). Duplicate
extracts were combined for pyrosequencing analysis. The retrieved sequences were processed using MOTHUR 1.32.1 software according to published methods \(^{25}\) and on-line 454 SOP accessed in 2013 (http://www.mothur.org/wiki/454_SOP). In brief, primers and barcodes were removed, and sequences with ambiguous base pairs, more than two mismatches in the primer sequence, more than one mismatch in the barcode sequence, more than nine homopolymers, or less than 150 base pairs were filtered out. Alignment of sequences was carried out using SILVA reference database (http://www.mothur.org/wiki/Silva_reference_alignment). Chimeras were removed using UCHIME. Sequences corresponding to mitochondria, chloroplast, Archaea, Eukarya or unknown were removed. This processing resulted in an average of 3,541 sequences per sample (\(n = 64\), \(sd = 2108\)). The sequences were subsampled to the smallest library size and the phylotype was assigned at the genus level. A total of 315 genera were identified. Inverse Simpson diversity index \((1/\lambda = 1/\sum_{i=1}^{S} p_i^2)\) was calculated by MOTHUR for each sample.\(^{26}\)

Multidimensional Scaling (MDS) analysis was performed using Primer 6 software (Primer-E, Plymouth, UK) to compare similarities of microbial community compositions between samples based on the calculation of Bray-Curtis coefficient.\(^{27}\)

3.3.8 TEM-EDS mapping

Activated sludge from SBRs dosed with nanoAg, NZVI, nanoTiO\(_2\), and nanoCeO\(_2\) were freeze-dried for TEM grid preparation as previously described.\(^{28}\) A JEOL 2100 TEM operated at 200 kV was used to detect and characterize aggregation state of nanoparticles in the activated sludge. The microscope was equipped with EDS which was used to analyze the chemistry of the particles of interest under the scanning TEM (STEM) mode.
3.4 Results and Discussion

3.4.1 Effects of Nanomaterials on Nitrification

In the sequential load experiments, NH$_3$-N and NO$_2^-$-N effluent concentrations remained near the detection limit in all SBRs throughout the dosing (data not shown). This indicates that nitrification functionality was not impacted by dosing of nanomaterials or bulk/ionic analogs when the load was sequentially increased. There was similarly no effect of nanomaterials in the high load experiments for nanoAg or nanoCeO$_2$, with NH$_3$-N and NO$_2^-$-N consistently near the detection limit (data not shown). Nitrification was also not significantly impacted by high load bulkCeO$_2$; however, in the high load Ag$^+$ condition, effluent NH$_3$-N rose above 10 mg/L and NO$_3^-$-N decreased to $\sim$15 mg/L within seven days (Figure 3.1A). NO$_2^-$-N concentrations remained near the detection limit all through the dosing, indicating that NO$_2^-$ oxidization was not impacted even by Ag$^+$ (Figure 3.1B). The SBRs receiving the high load of Ag$^+$ failed at about 14 days, with high COD (109 and 92 mg/L) and turbid effluent, while the remaining SBRs continued to operate at high load with no apparent inhibitory effects on nitrification.

Overall, the impacts of the nanomaterials and bulk/ionic analogs on nitrification appeared to be negligible under the conditions of this study, with the exception of Ag$^+$. Although nanoAg was reported to elicit greater inhibition to nitrification than Ag$^+$ in studies of nitrifying bacterial enrichments,$^{12-13}$ inhibition of nitrification by nanoAg was not observed under the complex SBR conditions in this study. In a lab-scale Modified Ludzack-Ettinger process, nitrification was inhibited by a 12-h period of nanoAg shock loading to reach a final peak silver concentration of 0.75 mg/L.$^{13}$ However, nanoAg did not elicit an apparent effect on nitrification function in this study, even when the concentration was as high as 20 mg/L. The present study was consistent
with another study that dosed 0.1 and 0.5 mg/L of nanoAg to lab-scale SBRs and reported no significant effect on NH₃ removal.¹⁴

![Figure 3.1](image)

**Figure 3.1** Concentrations of (A) NH₃-N, NO₃⁻-N and (B) NO₂⁻-N in duplicate SBRs dosed with continuous high load of 20 mg/L nanoAg or Ag⁺ relative to undosed controls.

Although nanoTiO₂ was observed to inhibit nitrification at 50 mg/L in lab-scale anaerobic-low dissolved oxygen SBRs dosed over a 70 day period,¹⁶ no effects were observed in this study of SBRs dosed with nanoTiO₂ or bulkTiO₂. García and colleagues²⁰ reported strong inhibition of enriched AOB cultures by nanoCeO₂, but no effects of nanoCeO₂ or bulkCeO₂ on nitrification function were observed in this study. To the authors’ knowledge, the effects of NZVI on nitrification and nitrifying bacteria have not been previously reported. In this study, there were no apparent toxic effects of NZVI or Fe²⁺ on nitrification function.
3.4.2 Effects of Nanomaterials on Abundance of Nitrifying Bacteria

In the sequential load experiments, *amoA* gene copy numbers decreased in SBRs dosed with nanoAg and Ag\(^+\) at the 20mg/L level (Figure 3.2A, p < 0.05) and also in NZVI, Fe\(^{2+}\), nanoCeO\(_2\) and bulkCeO\(_2\) SBRs at the end of 20mg/L dosing (Figure 3.2C-D, p < 0.05). *amoA* genes were not significantly impacted by dosing of nanoTiO\(_2\) or bulkTiO\(_2\) (Figure S3.1A, p > 0.05). *Nitrobacter* 16S rRNA gene copy numbers decreased significantly in SBRs dosed with nanoAg from 10 to 20 mg/L (Figure S3.2A, p < 0.05), but were not significantly impacted by Ag\(^+\) or the other three nanomaterials/bulk/ionic analogs relative to undosed controls (Figure S3.2, p > 0.05). *Nitrospira* 16S rRNA gene levels were not impacted by any of the four nanomaterials or bulk/ionic materials relative to undosed controls (Figure S3.3, p > 0.05).

A recent study reported no significant changes of *amoA*, *Nitrobacter* and *Nitrospira* 16S rRNA genes during long-term (more than 60 days) continuous loading of 0.1 mg/L nanoAg to a membrane bioreactor.\(^{30}\) In this study, dosing of nanoAg at 0.1 mg/L in the sequential load also did not noticeably impact *amoA*, *Nitrobacter* and *Nitrospira* 16S rRNA gene levels. However, *amoA* and *Nitrobacter* 16S rRNA gene copy numbers did decrease as the nanoAg loading concentration increased to 20 mg/L. Yang and colleagues\(^{31}\) also noted *amoA* expression to be sensitive to nanoAg and Ag\(^+\) in pure cultures of *Nitrosomonas europaea*. Nonetheless, in this study, nitrification function was not observed to correspond to decrease in nitrification marker genes monitored in any of the sequential load experiments. It is possible that the observed decrease in nitrifier marker genes had not reached the threshold of causing actual nitrification inhibition and thus inhibition of nitrification functionality could eventually be observed over longer time scales. It is also possible that other microbes that were not monitored, such as ammonia oxidizing Achaea and other genera of NOB, played an important role in nitrification.

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Quantities of *amoA* genes normalized to 16S rRNA genes in conditions that indicated an effect of the dosed materials: (A) sequential load of nanoAg and Ag$^+$; (B) high load of nanoAg and Ag$^+$; (C) sequential load of NZVI and Fe$^{2+}$; and (D) sequential load of nanoCeO$_2$ and bulkCeO$_2$. Error bars represent standard deviation of duplicate DNA extracts with triplicate q-PCR runs.

In the high load experiment, *amoA* genes decreased significantly in SBRs dosed with Ag$^+$ compared with SBRs dosed with nanoAg and undosed controls (Figure 3.2B, *p* < 0.05). However, *Nitrobacter* and *Nitrospira* 16S rRNA genes were not significantly impacted (Figure S3.4A-B, *p* > 0.05). The results corresponded well with the observed accumulation of ammonia during high load of Ag$^+$, while no accumulation of nitrite was observed (Figure 3.1). Although significant decreases of *amoA* genes and *Nitrobacter* 16S rRNA genes were observed at 20 mg/L nanoAg in the sequential load experiments, no effects of nanoAg on any of the three nitrifier
marker genes were observed in the high load experiments (Figure 3.2B, Figure S3.4A-B, p > 0.05). Similarly, amoA genes decreased by the end of the nanoCeO₂ and bulkCeO₂ sequential load experiments, but none of the three genes were significantly impacted by the high loads (Figure S3.1B, Figure S3.4C-D, p > 0.05). Thus, it appeared that sequential load of nanomaterials had a greater impact on the abundance of nitrifying bacteria than the high load condition. Considering that the exposure time of the sequential load (56 days) was longer than the high load (42 days), it may be that the longer exposure time had a stronger influence than exposure concentration or the total nanomaterial loaded (435 versus 840 mg total load). It is also possible that spikes in concentration in the sequential loading conditions triggered a shock response that was not observed under the stable loading conditions of the high load experiment.

In the above-referenced study that observed nitrification inhibition by shock loading of 0.75 mg/L nanoAg to a Modified Ludzacke-Ettinger process, decreases of *Nitrosomonas europaea* and *Nitrospira* populations were observed, while *Nitrobacter* completely washed out. However, in the present study, AOB appeared to be more sensitive than *Nitrobacter* and *Nitrospira*. amoA genes were impacted by nanoAg, NZVI, nanoCeO₂ and the bulk/ionic analogs. By contrast, *Nitrobacter* was only impacted by sequential loading of nanoAg, while *Nitrospira* was not impacted by any of the nanomaterials or bulk/ionic materials. Considering the distinct operating conditions of Modified Ludzacke-Ettinger process versus SBR, different process configurations likely vary in their susceptibility to potential microbial nanotoxicity.

### 3.4.3 Effects of Nanomaterials on Microbial Community Structure

To the knowledge of the authors, pyrosequencing was used for the first time to gain insight into the response of wastewater microbial communities to nanomaterials. Shifts in diversity can serve as an indicator of the overall health of a microbial community. According to
the inverse Simpson diversity index, microbial diversity was the lowest in SBRs dosed with Ag\(^+\), in both sequential and high load experiments (Figure S3.5A-B). In the sequential load, one SBR dosed with nanoAg was significantly lower than the initial diversity, but the duplicate SBR maintained the initial level of diversity (Figure S3.5A). The diversities of SBRs dosed with high loads of nanoAg were lower than the undosed controls, but higher than the SBRs dosed with Ag\(^+\) (Figure S3.4B). No significant changes in microbial diversity were observed in SBRs dosed with NZVI, nanoTiO\(_2\), nanoCeO\(_2\) or their bulk/ionic analogs (Figure S3.4C-F).

Figure 3.3 Multidimensional Scaling (MDS) analysis of microbial community similarities derived from pyrosequencing of bacterial 16S rRNA genes at the beginning (Begin) and at the end (End) of dosing: (A) sequential load and high load of nanoAg and Ag\(^+\); (B) sequential load and high load of nanoCeO\(_2\) and bulkCeO\(_2\); (C) sequential load of NZVI and Fe\(^{2+}\); (D) sequential load of nanoTiO\(_2\) and bulkTiO\(_2\).
MDS analysis was used to examine relative similarities of microbial communities at the beginning and the end of dosing. In general, microbial communities at the beginning were clustered closely, with similarities greater than 70%, which confirmed that initial conditions were relatively consistent prior to dosing (Figure 3.3). However, by the end of dosing, microbial communities in all SBRs shifted notably, with similarities less than 70% relative to the initial communities (Figure 3.3).

In the sequential loading experiment, SBRs dosed with Ag\(^+\) were noted to house distinct microbial communities, with similarities less than 60% relative to SBRs dosed with nanoAg and undosed controls (Figure 3.3A). The SBRs dosed with nanoAg, nanoCeO\(_2\), and bulk CeO\(_2\) also diverged relative to undosed controls, with similarity between 60% and 70% (Figure 3.3A-B). Although the microbial communities at the end of dosing shifted compared with the initial communities in SBRs dosed with NZVI, Fe\(^{2+}\), nanoTiO\(_2\) and bulkTiO\(_2\), with similarities less than 70%, there was no apparent difference among dosed and control SBRs at the end of the experiment (similarities greater than 70%) (Figure 3.3C-D).

Similar to the sequential load experiments, SBRs dosed with the high load of Ag\(^+\) contained distinct microbial communities relative to SBRs dosed with nanoAg and undosed controls (similarity less than 60%) (Figure 3.3A). SBRs dosed with nanoAg were also distinct from undosed controls, with the similarity between 60% and 70% (Figure 3.3A). For SBRs dosed with nanoCeO\(_2\) and bulkCeO\(_2\) at high load, although there was shift in microbial community structure at the end of dosing relative to the beginning, there was no significant difference relative to undosed controls (similarities greater than 70%) (Figure 3.3B).

Ag\(^+\) showed the strongest effect on the microbial community diversity and composition, corresponding well with the impacts of nanomaterials on nitrification function and abundance of
nitrifying bacteria. This suggests that nitrification inhibition was associated with reduction of microbial diversity and changes of microbial community compositions, an association observed in one previous study. While nitrification function was associated in microbial shifts in the other conditions investigated in this study, a threshold response might be achieved with longer exposure times.

![Multidimensional Scaling (MDS) analysis of bacterial community similarities derived from pyrosequencing in the beginning (Begin) and at the end (End) of dosing in all sequential load and high load experiments. Green: sequential load of nanoAg and Ag⁺; Red: sequential load of nanoTiO₂ and bulk TiO₂; Blue: sequential load of NZVI and Fe²⁺; Yellow: sequential load of nanoCeO₂ and bulk CeO₂; Gray: high load of nanoAg and Ag⁺; Pink: high load of nanoCeO₂ and bulk CeO₂.](image)

**Figure 3.4** Multidimensional Scaling (MDS) analysis of bacterial community similarities derived from pyrosequencing in the beginning (Begin) and at the end (End) of dosing in all sequential load and high load experiments. Green: sequential load of nanoAg and Ag⁺; Red: sequential load of nanoTiO₂ and bulk TiO₂; Blue: sequential load of NZVI and Fe²⁺; Yellow: sequential load of nanoCeO₂ and bulk CeO₂; Gray: high load of nanoAg and Ag⁺; Pink: high load of nanoCeO₂ and bulk CeO₂.

Since SBRs were re-started with each experiment to avoid bias of prior dosed materials, it was of interest to compare the similarities of SBR microbial communities across experiments. Interestingly, variation in the initial microbial community structures were even greater than the
variation associated with dosing of nanomaterials and bulk/ionic analogs, except SBRs dosed with Ag\(^+\) (Figure 3.4). Considering that stable nitrification was achieved at the beginning of each experiment, the specific microbial composition of the communities appeared not to be critical for performance, suggesting functional redundancy. Also, considering that microbial diversity was affected only by Ag\(^+\), while many of the materials influenced microbial community composition, it may be that shift in diversity is a superior indicator of shift in performance.

### 3.4.4 Nanomaterial Transformation and the Toxicity Effects

Results of ICP-MS indicated that >99% of nanomaterials and bulk/ionic analogs dosed to the SBRs partitioned into the activated sludge, except that the Ag concentration increased to 1.49 and 0.74 mg/L in the aqueous effluent of duplicate SBRs receiving high loads of Ag\(^+\) (data not shown). The results were consistent with other studies indicating that the majority of nanoAg, nanoTiO\(_2\) and nanoCeO\(_2\) is captured by activated sludge during biological wastewater treatment,\(^{3,4,14,30,33}\) while the fate of NZVI has not previously been reported to the authors’ knowledge. However, no functional inhibition to nitrification was observed in spite of accumulation of the nanomaterials in activated sludge. In order to identify possible relationships between nanomaterial transformation state and the effects observed, TEM-EDS was used to detect nanoAg, NZVI, nanoTiO\(_2\) and nanoCeO\(_2\) in activated sludge and to identify nanomaterial transformation by mapping the chemical compositions of particles.

The finding that a large portion of nanoAg remained dispersed was unexpected (Figure S3.6). This was possibly due to the high colloidal stability of nanoAg coated with citrate, which was reported in another study.\(^{10}\) EDS mapping revealed good spatial correlation of silver with sulfur, but not with chlorine, phosphorus, oxygen or carbon, which could also form complexes with silver (Figure 3.5, Figure S3.7). Studies have shown that sulfidation of nanoAg and
formation of Ag-S complexes could reduce toxicity of nanoAg to microbes, and that Ag-S complexes are stable under aerobic conditions. Another recent study also reported that all nanoAg and Ag⁺ dosed to a pilot-scale activated sludge plant was converted to Ag₂S. In this study, sulfate was rich in the synthetic wastewater and pyrosequencing showed high abundance of sulfate-reducing bacteria in the activated sludge. Thus Ag-S complexes may have formed and limited the toxicity of nanoAg. Moreover, monodispersed nanoAg has been observed to result in a greater extent of sulfidation than polydispersed, aggregated particles. Therefore, in this study the dispersed particles could favor the occurrence of sulfidation and effectively stabilize nanoAg.

This study supported observations of others that Ag⁺ is more toxic than nanoAg. The toxicity of Ag⁺ could also be reduced by reaction with sulfide, but one study reported that sulfide could only restrict Ag⁺ toxicity within a narrow concentration range. This restriction might explain nitrification inhibition at the high loading of 20 mg/L Ag⁺; however, Ag⁺ did not inhibit nitrification at 20 mg/L in the experiment with sequential loading. It is possible that as the concentration was sequentially increased from 0.1 mg/L to 20 mg/L, microbes adapted to resist the toxicity under sublethal conditions. Such an effect was suggested in a recent study in which the abundance of the silver resistance gene silE gradually increased during continuous loading of 0.1 mg/L nanoAg to a membrane bioreactor.

In contrast to nanoAg, the other three nanomaterials; NZVI, nanoTiO₂, and nanoCeO₂, were mostly aggregated (Figure 3.6, Figure S3.9-S3.11). According to a previous study, NZVI could be rapidly oxidized under aerobic conditions and inactivate microbes by physical attachment, disruption of the membrane and generation of reactive oxygen species. However, in this study EDS mapping did not indicate correlation of iron with oxygen or other elements.
(Figure S3.8), indicating that the particles were not chemically modified. Particle aggregation could possibly reduce the reactivity of NZVI and overall toxicity effects.

Figure 3.5 EDS maps of silver (Ag), sulfur (S), chlorine (Cl), phosphorus (P), oxygen (O) and carbon (C) in activated sludge after dosing with nanoAg. Color intensity is indicative of the number of X-ray counts. Corresponding STEM images appear in the gray insets.
EDS maps of titanium and cerium spatially correlated only with oxygen, and not with chlorine, sulfur, phosphorus or carbon, suggesting that nanoTiO$_2$ and nanoCeO$_2$ remained chemically unmodified (Figure S3.10-S3.11). This was expected considering that nanoTiO$_2$ and nanoCeO$_2$ are highly insoluble and thus not likely to be altered in the SBRs. Toxicity of nanoTiO$_2$ and nanoCeO$_2$ has mainly been attributed to generation of reactive oxygen species and penetrating the cells due to the small size.$^{38,39}$ However, in this study, the toxic effects might have been reduced by aggregation of the nanomaterials.

![Figure 3.6](image)

**Figure 3.6** EDS maps of: (A) iron, (B) cerium and (C) titanium in activated sludge after dosing of NZVI, nanoCeO$_2$ and nanoTiO$_2$. Color intensity is indicative of the number of X-ray counts. Corresponding STEM images appear in the gray insets.

The overall results suggest cautious optimism regarding disposal of nanowaste into wastewater treatment plants and their potential to inhibit key microbial processes. However, considering the effects of nanoAg and nanoCeO$_2$ on the abundance of nitrifying bacteria and microbial community compositions, long-term study is necessary to verify if continuous exposure to nanomaterials will eventually exert adverse effects on the nitrification. Also, shifting nanomaterial concentrations may be more of a concern than stable loading conditions and is worthy of further investigation.
3.5 Acknowledgments

Funding for this research was provided by the U.S. Environmental Protection Agency Star Grant #834856, the National Science Foundation (NSF) Center for the Environmental Implications of Nanotechnology (CIENT) (EF-0830093), and the Microbiology in the Post-Genome Era NSF REU site at Virginia Tech (NSF Award #1156954), and the Virginia Tech Institute for Critical Technology and Applied Science (ICTAS).

3.6 References


3.7 Supplemental Materials

Table S3.1 Chemical composition of the synthetic wastewater

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Formula</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactopeptone</td>
<td>/</td>
<td>0.300</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>C_{18}H_{32}O_{2}</td>
<td>0.090</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>(NH_{4})<em>{2}SO</em>{4}</td>
<td>0.030</td>
</tr>
<tr>
<td>Aluminum sulfate octadecahydrate</td>
<td>Al_{2}(SO_{4})<em>{3}·18H</em>{2}O</td>
<td>0.037</td>
</tr>
<tr>
<td>Calcium chloride dihydrate</td>
<td>CaCl_{2}·2H_{2}O</td>
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</tr>
<tr>
<td>Iron chloride</td>
<td>FeCl_{3}</td>
<td>0.017</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>MgSO_{4}</td>
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</tr>
<tr>
<td>Potassium hydrogen phosphate</td>
<td>K_{2}HPO_{4}</td>
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</tr>
<tr>
<td>Sodium acetate</td>
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</tr>
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<td>Sodium bicarbonate</td>
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<tr>
<td>Micronutrients</td>
<td>Table S2</td>
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Table S3.2 Chemical composition of micronutrients

<table>
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<th>Formula</th>
<th>Concentration (g/L)</th>
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<td>Citric acid</td>
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</tr>
<tr>
<td>Hippuric acid</td>
<td>C_{9}H_{9}NO_{3}</td>
<td>2</td>
</tr>
<tr>
<td>Trisodium ethylenediaminetetraacetic acid tetrahydrate</td>
<td>Na_{3}(EDTA)·4H_{2}O</td>
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</tr>
<tr>
<td>Boric acid</td>
<td>H_{3}BO_{3}</td>
<td>0.25</td>
</tr>
<tr>
<td>Zinc sulfate heptahydrate</td>
<td>ZnSO_{4}·7H_{2}O</td>
<td>0.15</td>
</tr>
<tr>
<td>Manganese chloride tetrahydrate</td>
<td>MnCl_{2}·4H_{2}O</td>
<td>0.12</td>
</tr>
<tr>
<td>Copper sulfate pentahydrate</td>
<td>CuSO_{4}·5H_{2}O</td>
<td>0.06</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>KI</td>
<td>0.03</td>
</tr>
<tr>
<td>Sodium molybdate dihydrate</td>
<td>Na_{2}MoO_{4}·2H_{2}O</td>
<td>0.03</td>
</tr>
<tr>
<td>Cobalt chloride hexahydrate</td>
<td>CoCl_{2}·6H_{2}O</td>
<td>0.03</td>
</tr>
<tr>
<td>Nickel chloride hexahydrate</td>
<td>NiCl_{2}·6H_{2}O</td>
<td>0.03</td>
</tr>
<tr>
<td>Sodium tungstate tetrahydrate</td>
<td>NaWO_{4}·2H_{2}O</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Figure S3.1 *amoA* gene copy numbers normalized to universal bacterial 16S rRNA genes in duplicate nitrifying SBRs during: (A) sequential load of nanoTiO$_2$ and bulkTiO$_2$; and (B) high load of nanoCeO$_2$ and bulkCeO$_2$ (at 20 mg/L). Error bars represent standard deviation of duplicate DNA extracts with triplicate q-PCR runs.
Figure S3.2 *Nitrobacter* 16S rRNA gene copy numbers normalized to universal bacterial 16S rRNA genes in duplicate nitrifying SBRs during sequentially increased loading of: (A) nanoAg and Ag⁺, (B) NZVI and Fe²⁺, (C) nanoTiO₂ and bulkTiO₂, (D) nanoCeO₂ and bulkCeO₂ Error bars represent standard deviation of duplicate DNA extracts with triplicate q-PCR runs.
Figure S3.3 *Nitrospira* 16S rRNA gene copy numbers normalized to universal bacterial 16S rRNA genes during sequential loading of: (A) nanoAg and Ag⁺, (B) NZVI and Fe²⁺, (C) nanoTiO₂ and bulkTiO₂, (D) nanoCeO₂ and bulkCeO₂. Error bars represent standard deviation of duplicate DNA extracts with triplicate q-PCR runs.
Figure S3.4 *Nitrobacter* (A) and *Nitrospira* (B) 16S rRNA gene copy numbers normalized to universal bacterial 16S rRNA genes in the high load experiments for nanoAg and Ag⁺ and *Nitrobacter* (C) and *Nitrospira* (D) 16S rRNA gene copy numbers normalized to universal bacterial 16S rRNA genes in the high load experiments for nanoCeO₂ and bulkCeO₂. Error bars represent standard deviation of duplicate DNA extracts with triplicate q-PCR runs.
Figure S3.5 Inverse Simpson diversity index of microbial communities at the beginning and at the end of dosing in (A) sequential load of nanoAg and Ag$^+$; (B) high load of nanoAg and Ag$^+$; (C) sequential load of nanoCeO$_2$ and bulkCeO$_2$; (D) high load of nanoCeO$_2$ and bulkCeO$_2$; (E) sequential load of NZVI and Fe$^{2+}$; and (F) sequential load of nanoTiO$_2$ and bulkTiO$_2$. The calculation was conducted by MOTHUR based on genus level classification of pyrosequencing data.
Figure S3.6 TEM images of nanoAg in activated sludge from SBRs dosed with nanoAg.
Figure S3.7 Additional EDS maps of silver (Ag), sulfur (S), chlorine (Cl), Phosphorus (P), oxygen (O) and carbon (C) in activated sludge after dosing of nanoAg. Greater color intensity indicates higher X-ray counts. Corresponding STEM images appear in the gray insets.
Figure S3.8 EDS maps of iron (Fe), sulfur (S), chlorine (Cl), Phosphorus (P), oxygen (O) and carbon (C) in activated sludge after dosing of NZVI. Greater color intensity indicates higher X-ray counts. Corresponding STEM images appear in the gray insets.
Figure S3.9 EDS maps of titanium (Ti), sulfur (S), chlorine (Cl), Phosphorus (P), oxygen (O) and carbon (C) in activated sludge after dosing of nanoTiO$_2$. Greater color intensity indicates higher X-ray counts. Corresponding STEM images appear in the gray insets.
Figure S3.10 EDS maps of cerium (Ce), sulfur (S), chlorine (Cl), Phosphorus (P), oxygen (O) and carbon (C) in activated sludge after dosing of nanoCeO$_2$. Greater color intensity indicates higher X-ray counts. Corresponding STEM images appear in the gray insets.
CHAPTER 4: Toxicity of Engineered Nanomaterials and their Transformation Products following Wastewater Treatment on A549 Human Lung Epithelial Cells

Yanjun Ma, Subbiah Elankumaran, Linsey C. Marr, Eric P. Vejerano, and Amy Pruden

4.1 Abstract

Here we characterize the toxicity of environmentally-relevant forms of engineered nanomaterials (ENMs), which can transform during wastewater treatment and persist in aqueous effluents and biosolids. In an aerosol exposure scenario, cytotoxicity and genotoxicity of effluents and biosolids from lab-scale sequencing batch reactors (SBRs) to A549 human lung epithelial cells were examined. The SBRs were dosed with nanoAg, nano zero-valent iron (NZVI), nanoTiO₂ and nanoCeO₂ at sequentially increasing concentrations from 0.1 to 20 mg/L. Toxicities were compared to outputs from SBRs dosed with ionic/bulk analogs, undosed SBRs, and pristine ENMs. Pristine nanoAg and NZVI showed significant cytotoxicity to A549 cells in a dose-dependent manner from 1 to 67 µg/mL, while nanoTiO₂ and nanoCeO₂ only exerted cytotoxicity at 67 µg/mL. Only nanoAg induced a genotoxic response, at 9, 33 and 53 µg/mL. However, no significant cytotoxic or genotoxic effects of the SBR effluents or biosolids containing nanomaterials were observed.

4.2 Introduction

The concern that engineered nanomaterials (ENMs) may have adverse effects on human health is increasing as application of nanotechnology in consumer products expands.¹ ² Humans can be exposed to ENMs in the workplace and during use of nano-products, and also through
contact with water, soil, or air to which ENMs may have been released. Comprehensive risk assessment of ENMs requires characterization of the toxicity of ENMs under a wide range of exposure conditions, including environmental routes. Cytotoxicity and genotoxicity of common metal and carbon nanomaterials, such as nanoAg, nanoTiO₂, and carbon nanotubes have been widely studied in human lung, dermal and visceral cells. However, in assessing the risks of ENMs released into the natural environment, available data on the toxicity of environmentally-relevant forms of ENMs is lacking. Extrapolation of toxicity data based on testing of pristine ENMs may not be appropriate because ENMs are highly reactive by nature and can be chemically, physically and biologically transformed in the environment, potentially altering their toxicity.

Wastewater treatment plants (WWTPs) are a critical route of ENM receipt and release into the natural environment. The complex wastewater matrix is likely to favor transformation of ENMs. A small portion of ENMs will remain in the wastewater effluent, while the majority will associate with the sludge and eventually be disposed of by land-application, landfill, or incineration. During the reuse of treated wastewater and land-application of waste sludge (biosolids), there is potential for humans to be exposed to transformed ENMs, especially through inhalation of aerosols generated. However, to the knowledge of the authors, impacts of human exposure to transformed ENMs following wastewater treatment has not previously been reported.

This study examined cytotoxicity and genotoxicity of effluents and biosolids from lab-scale sequencing batch reactors (SBRs) receiving ENMs to A549 human lung epithelial cells. The SBRs were dosed with nanoAg, nano zero-valent iron (NZVI), nanoTiO₂ and nanoCeO₂ at sequentially increasing concentrations from 0.1 to 20 mg/L. Toxicities were compared to outputs
from SBRs dosed with ionic/bulk analogs (Ag⁺, Fe²⁺, bulkTiO₂ and bulkCeO₂), outputs from undosed SBRs, and pristine ENMs.

### 4.3 Materials and Methods

#### 4.3.1 Preparation of Samples

NanoAg (52±12 nm, prepared using citrate reduction method), NZVI (46±10 nm, NANOFER 25S, Rajhrad, Czech Republic), nanoTiO₂ (21±12 nm, anatase nanopowder, Sigma-Aldrich, Saint Louis, MO), nanoCeO₂ (33±12 nm, Sigma-Aldrich) and their ionic/bulk analogs [Ag⁺ as AgNO₃ (Fisher, Suwanee, GA), Fe²⁺ as FeSO₄ (Fisher), bulkTiO₂ (Sigma-Aldrich) and bulkCeO₂ (Sigma-Aldrich)] were prepared/purchased and characterized as described in Ma et al. As previously reported, lab-scale nitrifying SBRs fed with synthetic wastewater were set up and operated at steady state under three conditions in duplicate: (1) SBRs dosed with nanoAg, NZVI, nanoTiO₂, or nanoCeO₂; (2) SBRs dosed with Ag⁺, Fe²⁺, bulkTiO₂, or bulkCeO₂; and (3) undosed SBRs. The dosing was initiated at 0.1 mg/L and sequentially increased to 1, 10 and 20 mg/L. The SBRs were actively nitrifying at the time of this study; further details on SBR performance are reported in Ma et al. Aqueous effluents and biosolids were sampled at the end of 20 mg/L dosing. In preparation for toxicity tests, samples from duplicate SBRs were combined and sterilized, as described in the Supporting Information. Concentrations of nanomaterials in SBR effluents and biosolids were quantified by inductively coupled plasma mass spectrometry (ICP-MS) as described elsewhere.

#### 4.3.2 Cell Culture and Treatment

Human lung alveolar epithelial cells A549 were obtained from ATCC (#CCL-185, Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo...
Scientific HyClone, Logan, UT) containing 10% heat inactivated fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin-streptomycin (Thermo Scientific HyClone, Logan, UT), and were maintained in a humidified incubator at 37 °C and 5% CO₂. Cells were seeded at a density of $1 \times 10^4$ in 100 µL culture medium in each well of 96-well plates. At 80% confluency of adherent cells, they were treated for 24 h either with pristine nanomaterials, ionic/bulk analogs, SBR aqueous effluents, or SBR biosolids as indicated. The exposure concentrations of the pristine materials (Table S4.1) varied from 1 to 67 µg/mL for the cytotoxicity assay to estimate IC₅₀ values. Genotoxicity assays were carried out at key concentration points according to results of the cytotoxicity assays (Table S4.1). The exposure concentrations of nanomaterials and ionic/bulk materials in SBR effluents and biosolids are shown in Table 4.1. All the samples were diluted with the culture medium to target concentrations.

4.3.3 Cytotoxicity Assay

Cell viability was measured using the WST-1 assay (Roche, Indianapolis, IN) based on quantification of mitochondrial activity as an indicator of cytotoxicity. After the A549 cells were treated with the samples for 24h, WST-1 reagent was added to each well at 1/10 volume of the medium. The absorbance was quantified after incubating at 37 °C for 3 h using a Tecan Safire² Microplate Reader (Tecan US In., Research Triangle Pa, NC) at 440 nm with a reference wavelength of 660 nm. All samples treated with A549 cells (a) were tested in triplicate in three independent experiments with three controls: (b) untreated A549 cells in culture medium; (c) samples in culture medium without A549 cells; and (d) culture medium only. In a single experiment, cell viability was calculated as percentage of the average absorbance derived from triplicate runs of treated cells relative to untreated control cells, with absorbances of
corresponding controls subtracted out to address possible matrix interferences: fraction cell viability = [(a-c)/(b-d)].

Table 4.1 Concentration of nanomaterials and ionic/bulk materials in SBR effluents and biosolids, and exposure concentration in cytotoxicity and genotoxicity assays.

<table>
<thead>
<tr>
<th></th>
<th>SBR effluents (µg/L)</th>
<th>Exposure concentration (µg/L)</th>
<th>Biosolids (µg/g total solids)</th>
<th>Exposure concentration (µg/mL)</th>
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</thead>
<tbody>
<tr>
<td>NanoAg</td>
<td>28.43</td>
<td>9.48</td>
<td>2332.34</td>
<td>0.47</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>43.67</td>
<td>14.56</td>
<td>2212.61</td>
<td>0.44</td>
</tr>
<tr>
<td>NZVI</td>
<td>7.10</td>
<td>2.37</td>
<td>38327.68</td>
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</tr>
<tr>
<td>Fe²⁺</td>
<td>4.70</td>
<td>1.57</td>
<td>48061.38</td>
<td>9.61</td>
</tr>
<tr>
<td>NanoTiO₂</td>
<td>0.95c</td>
<td>0.32</td>
<td>49201.98</td>
<td>9.84</td>
</tr>
<tr>
<td>BulkTiO₂</td>
<td>1.02c</td>
<td>0.34</td>
<td>67649.03</td>
<td>13.53</td>
</tr>
<tr>
<td>NanoCeO₂</td>
<td>0.25</td>
<td>0.08</td>
<td>22360.00</td>
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</tr>
<tr>
<td>BulkCeO₂</td>
<td>0.86</td>
<td>0.29</td>
<td>26454.21</td>
<td>5.29</td>
</tr>
</tbody>
</table>

a SBR effluents were diluted approximately 1:3 in the culture medium.

b SBR biosolids were exposed to A549 cells at 200 µg total solids/mL.

c Concentrations of nanoTiO₂ and bulkTiO₂ in SBR effluents were near the detection limit of Ti by ICP-MS and not significantly different from concentrations in the undosed SBR.

4.3.4 Genotoxicity Assay

DNA damage in A549 cells was detected using immunofluorescent labeling of γH2AX foci as described elsewhere.²⁰ At sites of DNA double strand breaks, H2AX, a minor nucleosomal histone protein, is rapidly phosphorylated and forms γH2AX.²¹ The experimental and imaging procedures are provided in the Supporting Information. Three independent experiments were conducted with at least 200 cells imaged in a single test. Untreated cells and
cells treated with 100 µM H$_2$O$_2$ for 10 min were included as negative and positive controls, respectively. Images were analyzed using ImageJ 1.47 (http://rsbweb.nih.gov/ij/) with a macro designed to subtract background and count the number of foci within the defined nucleus masks.

**4.3.5 Statistical Analysis**

The data were presented as mean ± standard deviation of three independent experiments. The student’s $t$ test or pairwise $t$ test was conducted in R-2.8.1 (http://cran.r-project.org/bin/windows/base/old/2.8.1/) to determine statistical differences between samples at a significance level of 0.05 ($p < 0.05$).

**4.4 Results and Discussion**

**4.4.1 Toxicity of Pristine Nanomaterials**

Based on the WST-1 assay, the viability of cells exposed to nanoAg, Ag$^{+}$, NZVI and Fe$^{2+}$ for 24 h decreased significantly ($p < 0.05$) in a dose-dependent manner (Figure S4.1). The IC$_{50}$ values for these materials were estimated to be 53±2, 21±0.1, 38±2 and 55±2 µg/mL, respectively. In particular, the IC$_{50}$ of Ag$^{+}$ was significantly lower than that of nanoAg ($p = 5\times 10^{-4}$), a result that is in agreement with other studies using A549 cell targets.$^{4,22}$ By contrast, NZVI was more toxic than its ionic analog, Fe$^{2+}$ ($p = 6\times 10^{-4}$). In another study, the cytotoxicity of NZVI (synthesized through reduction of FeCl$_3$ by NaBH$_4$ and coated with Pd) and Fe$^{2+}$ to human bronchial epithelial cells 16HBE14o was not significantly different.$^{23}$ Differences between the studies could relate to differences in NZVI coatings (manufacturer in present study reports biodegradable organic and inorganic stabilizers) or the different cells used in the assays (A549 versus 16HBE14o). The viability of cells exposed to nanoTiO$_2$, nanoCeO$_2$ and their bulk analogs only decreased at 67 µg/ml by 10-20% (Figure S4.1, $p < 0.05$). But in a few other studies, no
significant cytotoxicity of nanoTiO$_2$ and nanoCeO$_2$ to A549 cells was observed even at concentrations up to 100 µg/ml.$^{5,24,25}$

Only cells that were exposed to 33 and 53 µg/mL nanoAg and 21 µg/mL Ag$^+$ showed significantly higher levels of average γH2AX foci per cell (Figure S4.2A) and greater percentages of cells containing γH2AX foci (Figure S4.2B) ($p < 0.05$) relative to untreated control cells, suggesting genotoxicity resulting from DNA double strand breaks. The number of γH2AX foci per cell exposed to 9 µg/mL nanoAg was not significantly different from that of the control cells (Figure S4.2A, $p = 0.12$), but the percentage of cells containing γH2AX foci was significantly higher than the control cells (Figure S4.2B, $p = 0.03$). Although IC$_{50}$ concentrations of NZVI and Fe$^{2+}$ (38 and 55 µg/mL, respectively), and nanoTiO$_2$, nanoCeO$_2$ and the bulk analogs at 67 µg/ml induced significant cytotoxicity to A549 cells, the number of γH2AX foci were not significantly different in cells treated with these materials (Figure S4.2, $p > 0.05$). To the authors’ knowledge, genotoxicity of NZVI to human cells has not been investigated previously. Genotoxicities at concentrations of 2.5-15 µg/mL of nanoAg$^4$, 10-50 µg/mL of nanoTiO$_2$,$^{26}$ and 0.5-100 µg/mL of nanoCeO$_2$,$^{25}$ have been observed in A549 cells in previous studies. However, in this study, the genotoxicity of nanoAg could be demonstrated, but not nanoTiO$_2$ or nanoCeO$_2$ at 67 µg/mL.

4.4.2 Toxicity of SBR Effluents and Biosolids

Based on a previous study,$^{19}$ >99% of nanomaterials and ionic/bulk analogs dosed into the SBRs partitioned into the sludge relative to the influent concentrations. Concentrations of nanomaterials in SBR aqueous effluents and biosolids, as well as exposed to A549 cells are shown in Table 4.1. No significant decrease of cell viability or induction of γH2AX foci were observed in A549 cells treated with SBR effluents (Figure 4.1A, Figure 4.2, $p > 0.05$). The
exposure concentration of nanoAg in this study (9.48 µg/L) was much higher than predicted concentrations in WWTP effluents (< 0.5 µg/L),\textsuperscript{12-14} while the exposure concentrations of NZVI (2.37 µg/L), nanoTiO\textsubscript{2} (0.32 µg/L) and nanoCeO\textsubscript{2} (0.08 µg/L) were within or lower than the lower bound concentrations predicted (0.7-20 µg/L for NZVI\textsuperscript{14}, 1-70 µg/L for nanoTiO\textsubscript{2}\textsuperscript{12-14}, and 0.5×10^{-4}-2 µg/L for nanoCeO\textsubscript{2}\textsuperscript{13-14}). Results of this study indicated limited toxicity of nanoAg in wastewater effluents at higher than environmentally-relevant concentrations to A549 cells, while the effects of higher concentrations of NZVI, nanoTiO\textsubscript{2} and nanoCeO\textsubscript{2} remains to be determined.

Figure 4.1 Characteristic cytotoxicity of A549 cells exposed to (A) wastewater effluents and (B) biosolids from undosed SBR, and SBRs dosed with nanoAg, Ag\textsuperscript{+}, NZVI, Fe\textsuperscript{2+}, nanoTiO\textsubscript{2}, bulkTiO\textsubscript{2}, nanoCeO\textsubscript{2} and bulkCeO\textsubscript{2} for 24 h by WST-1 assay. Exposure concentrations of materials are shown in Table 4.1. Error bars represent standard deviations of three independent experiments. “*” indicates significant decrease of viability compared with untreated control cells (p < 0.05).

The concentrations of nanomaterials in biosolids in this study (>2000 µg/g dry mass, Table 4.1) were significantly higher than concentrations predicted in biosolids from WWTP (<1000 µg/g dry mass).\textsuperscript{12-14} Moreover, 200 µg total solids/mL represented a higher rate of
exposure relative to other studies of effects of aerosolized biosolids to human lung cells. The viability of cells exposed to biosolids at 200 µg total solids/mL decreased by 7-10% relative to untreated control cells (p < 0.05), except for biosolids containing NZVI (p = 0.08) (Figure 4.1B). But, there was no significant difference between cells exposed to biosolids from undosed versus dosed SBRs (p > 0.05), indicating that the decrease in cell viability was unlikely due to the nanomaterials or ionic/bulk materials, but probably due instead to the high concentration of total solids. Cytotoxicity of biosolids was also examined at 50 and 100 µg total solids/mL, and no significant effects were observed relative to control cells (p > 0.05, Figure S4.3).

Figure 4.2 γH2AX foci in untreated control A549 cells; cells treated with wastewater effluents from undosed SBR and SBRs dosed with nanoAg, Ag⁺, NZVI, Fe²⁺, nanoTiO₂, bulkTiO₂, nanoCeO₂ and bulkCeO₂ for 24 h; and cells treated with 100 µM H₂O₂ for 10 min. Exposure concentrations of materials are shown in Table 4.1. Data are presented as (A) number of γH2AX foci per cell, and (B) percentage of cells containing γH2AX foci. Error bars represent standard deviations of three independent experiments. “*” indicates significant difference compared with untreated control cells (p < 0.05).
Genotoxicity of biosolids was examined at 200 µg total solids/mL. However, no significant differences were observed in terms of the number of γH2AX foci per cell or in the percentage of cells containing γH2AX foci in cells treated with biosolids relative to untreated control cells (Figure 4.3, p > 0.05), suggesting little or no DNA damage to A549 cells even at concentrations exceeding most likely aerosol exposure levels.

![Figure 4.3 γH2AX foci in untreated control A549 cells; cells treated with biosolids from undosed SBR and SBRs dosed with nanoAg, Ag⁺, NZVI, Fe²⁺, nanoTiO₂, bulkTiO₂, nanoCeO₂ and bulkCeO₂ for 24 h; and cells treated with 100 µM H₂O₂ for 10 min. Exposure concentrations of materials are shown in Table 4.1. Data was presented as (A) number of γH2AX foci per cell, and (B) percentage of cells containing γH2AX foci. Error bars represent standard deviations of three independent experiments. “*” indicates significant difference compared with untreated control cells (p < 0.05).](image)

Uptake of pristine nanoAg, nanoTiO₂, and nanoCeO₂ by human cells⁵,₂²,₂₄,₂₈ and NZVI by mammalian nerve cells²⁹ has been observed in previous studies and the most commonly identified mechanism of toxicity was the generation of reactive oxygen species, which induced
oxidative stress\(^4,23,26,28\). Release of Ag\(^+\) was considered another potential cause of nanoAg toxicity.\(^30\) Based on TEM-EDS mapping carried out in a previous study of the SBR biosolids,\(^19\) while a large portion of nanoAg remained dispersed, it mainly formed Ag-S complexes. Sulfidation has been reported to reduce toxicity of nanoAg to microbes, aquatic and terrestrial eukaryotic organisms due to low solubility of Ag-S complexes.\(^31,32\) Similarly, transformation of nanoAg in this study may limit their reactivity and result in little toxicity of SBR biosolids to A549 cells. The majority of NZVI, nanoTiO\(_2\) and nanoCeO\(_2\) were aggregated, but not chemically modified. Epithelial cells are impervious to aggregated nanomaterials by diffusion or macropinocytosis.\(^33\) Therefore, the absence of cytotoxicity and genotoxicity of SBR biosolids in this study could be attributed to the inability of aggregated NZVI, nanoTiO\(_2\) and nanoCeO\(_2\) to enter cells. However, the size of the nanomaterial aggregate can affect its physiological distribution and kinetics, cellular distribution (for example within the draining lymph node for an aerosolized particle), cellular uptake and intracellular processing pathways.\(^34\) Also, it is difficult to ascertain the fate of aerosolized nanomaterials from biosolids in the respiratory tract as particles can diffuse and convect during interstitial transport depending on their size.\(^35\) Thus, future work may consider the toxicity under *ex vivo* and *in vivo* conditions.

### 4.5 Acknowledgments

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4.6 References


### 4.7 Supplemental Materials

**Preparation of SBR effluents and biosolids for toxicity assays**

Wastewater effluents from SBRs were filtered through 0.45 µm mixed-cellulose ester membrane filters (EMD Millipore, Billerica, MA) to remove bacteria, and biosolids were freeze-dried (FreeZone Plus 4.5, Labconco, Kansas City, MO). A subsample of 1.6 mg freeze-dried biosolids was sterilized with 0.5 mL 100% ethanol and air-dried in a fume hood. The ethanol-treated biosolids were suspended in 1.6 mL 0.1% PBS-Tween solution by sonication for 30 min.

**Immunofluorescent labeling of γH2AX foci**

A549 cells were exposed to samples for 24 h, followed by washing with PBS and fixation in 4% paraformaldehyde for 15 min and permeabilization in 0.25% Triton X-100 for 15 min. After being blocked with 1% bovine serum albumin in PBS for 1 h, cells were incubated with a rabbit
polyclonal anti-γH2AX antibody (1:500; Santa Cruz Biotechnology Inc., Paso Robles, CA) for 1 h. Following washing with PBS 3x, the cells were incubated with Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) Antibody (1:500, Life Technologies Corporation, Grand Island, NY) and Hoechst 33342 (10 µg/mL, Life Technologies Corporation, Grand Island, NY) for 1 h. After washing the cells three times with PBS, fluorescence was visualized under 40× objective of an EcLipse TS 100 fluorescent microscopy (Nikon, Melville, NY). γH2AX foci emitted green fluorescent and the nuclei were stained blue with Hoechst 33342.

Supporting tables and figures

Table S4.1 Exposure concentration of pristine nanomaterials and ionic/bulk materials to A549 cells

<table>
<thead>
<tr>
<th>Assay</th>
<th>Exposure concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NanoAg</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td></td>
</tr>
<tr>
<td>(µg/mL)</td>
<td>1, 9, 33, 50, 60 and 67</td>
</tr>
<tr>
<td>Genotoxicity*</td>
<td></td>
</tr>
<tr>
<td>(µg/mL)</td>
<td>67</td>
</tr>
</tbody>
</table>

*Genotoxicity of nanoAg, Ag⁺, NZVI and Fe²⁺ was examined at IC₅₀ obtained from cytotoxicity assay (53, 21, 38 and 55 µg/mL, respectively); nanoAg was also examined at 9 and 33 µg/mL because significant genotoxicity were observed at 53 µg/mL; nanoTiO₂, bulkTiO₂, nanoCeO₂ and bulkCeO₂ were examined at 67 µg/mL, because cytotoxicity was only observed at 67 µg/mL.
Figure S4.1 Characteristic cytotoxicity of A549 cells exposed to nanoAg, Ag⁺, NZVI, Fe²⁺, nanoTiO₂, bulkTiO₂, nanoCeO₂ and bulkCeO₂ for 24 h by WST-1 assay. Six concentrations were tested: 1, 9, 33, 50, 60 and 67µg/mL. Error bars represent standard deviations of three independent experiments. “*” indicates significant decrease of viability compared with untreated control cells (p < 0.05).
Figure S4.2 γH2AX foci in untreated A549 control cells; cells treated with nanoAg, Ag⁺, NZVI, Fe²⁺, nanoTiO₂, bulkTiO₂, nanoCeO₂ and bulkCeO₂ at different concentrations for 24 h; and cells treated with 100 µM H₂O₂ for 10 min. Data are presented as (A) number of γH2AX foci per cell, and (B) percentage of cells containing γH2AX foci. Error bars represent standard deviations of three independent experiments. “*” indicates significant difference compared with untreated control cells (p < 0.05).
Figure S4.3 Characteristic cytotoxicity of A549 cells exposed to 50 and 100 µg total solids/mL biosolids from undosed SBR, and SBRs dosed with nanoAg, Ag⁺, NZVI, Fe²⁺, nanoTiO₂, bulkTiO₂, nanoCeO₂ and bulkCeO₂ for 24 h by WST-1 assay. Error bars represent standard deviations of three independent experiments. There were no significant differences of viability between cells treated with biosolid samples compared with untreated control cells (p > 0.05).
CHAPTER 5: Conclusions

Antibiotic resistance genes (AGRs) and engineered nanomaterials (ENMs) were recently identified as contaminants of emerging concern. Their fate in the environment and potential impacts on human and ecosystem health have not been fully characterized. This study aimed to improve understanding of the fate of AGRs and ENMs during wastewater treatment processes, which is an important recipient, reservoir, and source of AGRs and ENMs to the natural environment. The toxicity of ENMs during biological wastewater treatment was further examined with respect to impacts on the functionality of microbial communities, as well as toxicity of ENMs transformed during wastewater treatment to human lung epithelial cells in an aerosol exposure scenario. The study explored a variety of advanced molecular biological tools and toxicity indicators to facilitate quantification of AGRs, characterize the profile of microbial communities, and assess toxicity of ENMs. Specific contributions of this study to further understanding of the fate and impacts of AGRs and ENMs during wastewater treatment processes include:

1) Identification of the important role of bacterial community composition of sludge digestion processes in driving the distribution of AGRs present in the produced biosolids. Response of nine representative AGRs to various sludge digestion conditions revealed that ARG removal did not follow a simple trend: Similar performance was observed among mesophilic anaerobic digesters at 10 and 20 day SRT, and also among thermophilic anaerobic digesters at 47 °C, 52 °C and 59 °C, with thermophilic digestion exhibit more effective reduction to \textit{erm}(B), \textit{erm}(F), \textit{tet}(O), and \textit{tet}(w). Thermal hydrolysis pretreatment remarkably reduced all the AGRs; however, in the downstream anaerobic digestion, all the AGRs rebounded, and in the following aerobic digestion, different behavior of AGRs was observed. DGGE and sequencing analysis
verified distinct diversities and bacterial community compositions in the mesophilic, thermophilic digesters and digesters following thermal hydrolysis pretreatment, which supported the argument that bacterial community compositions in the digesters played an important role in driving the response of ARGs.

2) **Thermophilic anaerobic digestion and thermal hydrolysis pretreatment are promising for reducing ARGs present in produced biosolids.** Thermophilic anaerobic digesters operated at 47 °C, 52 °C and 59 °C provided more effective removal of *erm*(B), *erm*(F), *tet*(O), and *tet*(w) compared with mesophilic digesters. The reduction of ARGs by thermal hydrolysis pretreatment was remarkable. Although ARGs rebounded in the following anaerobic and aerobic digesters, the final concentration of most ARGs was still lower than in the produced biosolids of mesophilic anaerobic digesters. The advantages of thermophilic anaerobic digestion and thermal hydrolysis pretreatment were likely due to the lower diversity of bacterial communities which provided a narrower host range of ARGs.

3) **Limited adverse effects of silver (Ag), zero-valent iron (NZVI), titanium dioxide (nanoTiO$_2$) and cerium dioxide (nanoCeO$_2$) nanomaterials during and following simulated nitrifying activated sludge system.** In sequential loading of nanoAg, NZVI, nanoTiO$_2$ and nanoCeO$_2$ to SBRs from 0.1 to 20 mg/L for 56 days, and high loading of nanoAg and nanoCeO$_2$ at 20 mg/L for 42 days, nitrification function was not measurably inhibited by any of the nanomaterials, although decreased nitrifier gene abundances and distinct bacterial communities were observed in SBRs receiving nanoAg and nanoCeO$_2$. Also, no significant cytotoxicity and genotoxicity were observed to A549 human lung epithelial cells for SBR effluents and biosolids containing nanomaterials. The Ag-S complexes transformed from nanoAg and the aggregated NZVI, nanoTiO$_2$ and nanoCeO$_2$ appeared to be generally stable in the activated sludge, which
may have limited their effects on nitrification function, microbial community structure and A549 cells.

4) Diversity of microbial community is likely to be a superior indicator of shift in wastewater treatment performance than specific microbial compositions. One result of interest in this study was the variation of initial microbial community structures when SBRs were re-started for the testing of subsequent nanomaterials, although stable nitrification was achieved in all the SBRs at the beginning of each experiment, which indicated functional redundancy. Moreover, while many of the materials impacted the microbial community structure, only Ag⁺ significantly decreased the microbial diversity and inhibited nitrification function.

Overall, results of this study will assist characterizing release of ARGs and ENMs from WWTPs to the environment, and assessing potential risks of ENMs to biological wastewater treatment and human health. Based on this work, the following research is suggested as next steps:

1) Further investigate horizontal transfer of ARGs during sludge digestion. Response of ARGs to various sludge digestion conditions in this study indicated an important role of horizontal gene transfer, especially rebound of ARGs in anaerobic digesters following thermal hydrolysis pretreatment, and the correlation of occurrence of sulI genes with class I integron gene intI1. Future research may be conducted in conditions which the seed culture is carefully selected to be devoid of ARGs, with the feed concentration of ARGs systematically manipulated. Better understanding of horizontal gene transfer will facilitate innovation of current sludge treatment processes for extensive removal of ARGs.

2) Examine long-term effects of nanomaterials in nitrifying activated sludge systems. Although inhibition to nitrification function by nanomaterials was not observed in the time
period examined in this study (42-56 days), decrease of nitrifier gene abundances and distinct bacterial communities in SBRs dosed with nanoAg and nanoCeO$_2$ should be of concern. Long-term study is necessary to verify if continuous exposure to nanomaterials will eventually exert adverse effects on the nitrification.

3) Examine effects of nanomaterials on nitrifying activated sludge systems in more complex wastewater matrix. This study used simplified synthetic wastewater during operation of SBRs and dosing of nanomaterials. However, behavior of nanomaterials in real-world wastewater streams may be impacted by a broader range of chemicals. It is necessary that the research is extended to a more complicated wastewater matrix to identify potential effects of nanomaterials associated with other wastewater constituents.

4) Extend study of transformation and toxicities of ENMs to other groups. The list of ENMs is expanding with the rapid development of nanotechnology. This study only examined four metal and metal oxide nanomaterials. For thoroughly assessing the risks of ENMs, it is necessary to extend the study to other groups, and with a variety of properties.