An investigation into metal oxide nanoparticle toxicity to bacteria in environmental systems using fluorescence based assays

Olga Mileyeva-Biebesheimer

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A Dissertation

entitled

An Investigation into Metal Oxide Nanoparticle Toxicity to Bacteria in Environmental Systems Using Fluorescence Based Assays

by

Olga Mileyeva-Biebesheimer

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Engineering

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May 2011
While photocatalytic bactericidal effect of titanium dioxide (TiO$_2$) and zinc oxide (ZnO) nanoparticles (NPs) is routinely studied, there is very limited information regarding the toxic properties of these NPs in the absence of light. This research was focused on the evaluation of possible toxic effect of TiO$_2$ and ZnO NPs on pure planktonic cultures (Gram-Negative bacteria *Escherichia coli* and Gram-Positive bacteria *Enterococcus faecium*), single culture biofilm (*E. coli*), and anaerobic digester microorganisms in the absence of light. Fluorescent cell membrane integrity stain propidium iodide and dsDNA specific counter stain PicoGreen® were applied to distinguish damaged and intact cells. The pure culture and diluted sludge samples were incubated for 1 h with 0 – 500 mg/L of TiO$_2$ and ZnO NPs. Biofilm was exposed to 0 – 100 mg/L of TiO$_2$ and ZnO NPs for 24 h. Undiluted sludge samples were incubated for 144 h with 0 – 500 mg/L of TiO$_2$ and ZnO NPs (0.31 – 15.43 μg NP /mg total suspended solids) or as titanium (Ti) in TiO$_2$ calculated by weight: 375, 750, and 3752 μg /L or 0.01,
0.023, and 0.12 µg/mg TS. A statistically significant increase in the percentage of damaged cells was detected for pure culture of *E. coli* and diluted sludge samples at concentration 100 mg/L of TiO_2_ NPs. A pure culture of *E. faecium* and diluted sludge samples incubated with ZnO NPs did not exhibit an increase of damaged cells. The toxic effect was not significant for biofilm or undiluted sludge samples incubated with TiO_2_ NPs for 24 and 144 h respectively. Biofilm samples incubated with 100 mg/L of ZnO NPs demonstrated a statistically significant increase in the percent of damaged cells. Undiluted sludge samples incubated with 500 mg/L of ZnO NPs significantly reduced biogas production. This study demonstrated that fluorescent microscopy and cell membrane integrity stain propidium iodide may be utilized for evaluation of the toxic impact of NPs on microorganisms. This method enables evaluation of NPs impact on fastidious microorganisms and eliminates conventional plating techniques that strongly depend on culturability of the microorganisms.
To my Mother, who taught me to read, to learn and supported me in all my study.

And to my husband, who provided technical IT support for all my PC troubles, proofreading my writing and learned more about bacteria than he ever wanted to.
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Chapter 1

1 Introduction

1.1 Background

Nanoparticles (NPs), which can occur naturally in the environment (e.g., soot and products of volcanic eruptions), have been recently expanded to include engineered nanomaterials, products of a rapidly growing industry – nanotechnology. The standard definition provided by the U. S. Environmental Protection Agency (EPA) and the National Nanotechnology Initiative (NNI) states that materials in the range of 1 to 100 nanometers are considered to be nanomaterials. Nanotechnology enables control, manipulation, modeling and imaging materials in this size range. New materials and technologies require new approaches to regulations and standards to ensure a safe workplace, safe handling procedures, and appropriate disposal of engineered nanoparticles.

The necessity to coordinate research strategies in nanotechnology lead to the establishment of many oversight groups such as the Interagency Working Group on Nanotechnology in September 1998. In 2001, nanoscience and technology received federal status as the National Nanotechnology Initiative (NNI). The Nanoscale Science, Engineering and Technology (NSET) Subcommittee, the National Science and
Technology Council’s (NSTC) Committee on Technology (CT), The National Nanotechnology Coordination Office (NNCO) were established to serve as centers for communication, contact, cooperation, research and development for 25 Federal Agencies (www.nano.gov). The US Food and Drug Administration (FDA) established a Nanotechnology Task Force (Task Force) in 2006 and a NanoTechnology Interest Group (NTIG) that was announced in July 2007 (www.fda.gov). On September 29, 2009 the US Environmental Protection Agency (EPA) announced the Nanoscale Materials Stewardship Program (NMSP). This program outlined EPA strategies in understanding, assessing and minimizing potential risks from manufactured nanomaterials and products to human health and the environment, while at the current time EPA considers nanomaterials as "chemical substances" referring to the Toxic Substances Control Act (TSCA) (www.epa.gov).

Nanotechnologies have become an area of intensive research and development and the applications of NPs are constantly increasing. Annually, industries produce tons of nanoparticles and the number of the products incorporating nanotechnologies is rapidly increasing. Investments in nanotechnology including education, research and development are growing at a rapid pace. Funding for the National Nanotechnology Initiative (NNI) is constantly increasing and cumulatively has reached nearly $12 billion since 2001, that includes $1.6 billion for the 2010 budget (www.nano.gov), while for example in 2005 estimated budget was $11 million (Maynard, 2006). It was predicted that the production of nanoparticles would reach 58,000 tons by 2011 – 2020 (Maynard, 2006).
The Project on Emerging Nanotechnologies summarized and published on a website the Consumer Products Inventory that listed 54 products which incorporated nanotechnology in 2005. As of August 25, 2009 nanotechnologies were incorporated in 1015 products, among which 605 products are related to health and fitness. These products are manufactured by 485 companies located in 24 countries. In the USA alone 540 products are manufactured and that number is constantly growing. The prevalent numbers of nanotechnologies are applied in manufacturing materials, instruments, and health related products (www.nanotechproject.org). The Consumer Products Inventory provides numbers of health and fitness products incorporating nanotechnology (Table 1.1) and identifies six most common nanomaterials (Table 1.2) as of August 25, 2009 (www.nanotechproject.org).

Table 1.1: Health and fitness products incorporating nanotechnology.

<table>
<thead>
<tr>
<th>Type of the products</th>
<th>Personal care products</th>
<th>Clothing</th>
<th>Cosmetics</th>
<th>Sporting goods</th>
<th>Sunscreen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of products in inventory</td>
<td>193</td>
<td>155</td>
<td>137</td>
<td>93</td>
<td>33</td>
</tr>
</tbody>
</table>

(Data adopted from the Consumer Products Inventory, www.nanotechproject.org).

Table 1.2: Major nanomaterials incorporated in various products.

<table>
<thead>
<tr>
<th>Nanomaterials</th>
<th>Silver</th>
<th>Carbon</th>
<th>Titanium</th>
<th>Silicon / Silica</th>
<th>Zinc</th>
<th>Gold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of products in inventory</td>
<td>259</td>
<td>82</td>
<td>50</td>
<td>35</td>
<td>30</td>
<td>27</td>
</tr>
</tbody>
</table>

(Data adopted from the Consumer Products Inventory, www.nanotechproject.org).
Nanotechnology provides unique opportunities for the development of medical devices and various sensors (Biswas and Wu, 2005; Tsuang et al., 2008). Metal oxide nanoparticles are utilized in the production of many consumer products including sunscreens, dye-sensitized solar cells, paint, textiles, electronics, and pharmaceuticals (Adams et al., 2006; Oskam, 2006; Borm and Berube, 2008).

A report on nanotechnologies by The Royal Academy of Engineering (2004) indicated that industries manufacturing skin care products annually utilized 1,000 – 2,000 tons of NPs such as titanium dioxide, zinc oxide, silicon dioxide, aluminum oxide, zirconia and iron oxide. The focus of this research is the metal oxides: zinc oxide (ZnO) and titanium dioxide (TiO₂). Rapidly developing nanotechnologies and continuously increasing areas of applications of metal NPs result in consequently expanding volumes of engineered NPs introduced to the environment. These nanomaterials have properties that are advantageous for product development, but those same properties may be problematic in the environment. For example, the photocatalytic properties of some metal oxides are responsible for the development of self-cleaning coatings for surfaces. The same photocatalytic properties produce a reactive oxygen species (ROS), which may result in NPs toxicity to microorganisms (Rincon and Pulgarin, 2005; Liu et al., 2007; Borm and Berube, 2008; Heinlaan et al., 2008; Xia et al., 2008; Alrousan et al., 2009; Aruoja et al., 2009).

The environmental fate of these applied NPs and their possible impact on the environment has yet to be studied. Recent research has demonstrated that aging exterior paint released TiO₂ NPs in quantities that were traceable with facade runoff to surface waters (Kaegi et al., 2008). It was reported that in presence of fulvic acid or natural
organic matter (NOM), at concentrations equivalent naturally occurring in the surface waters, TiO$_2$ NPs were stabilized and disaggregated, thus increasing the travel distance of TiO$_2$ NPs in surface waters (Neal, 2008; Domingos et al., 2009; Keller et al., 2010). Consumer products such as clothing, cosmetics, and sunscreens eventually get discarded to the landfills, flushed to waste water treatment systems, emitted into the air through combustion, and as a result enter the environment. Considering the increasing volumes of NPs deposited in the environment it is necessary to estimate not only the benefits from the application of NPs, but also to evaluate the possible negative impacts on the environment from the accumulation and transformation of deposited NPs.

### 1.2 Structure and properties of ZnO and TiO$_2$ nanoparticles

Nanoparticles have a very high surface to volume ratio. This feature places 40 – 50% of atoms on the surface of NPs and provides more opportunities for reactions in comparison to micro and macro scale (bulk) materials (Christian et al., 2008; Farré et al., 2009). The surface chemistry of the materials in nano scale might differ from the surface chemistry the same materials in micro and macro scales (Christian et al., 2008). For example, it was reported that catalytic activity of gold NPs was size dependent (Sau et al., 2001).

Commercially available TiO$_2$ NPs might have either rutile or anatase tetragonal crystal structure (Brown et al., 1999; Fu et al., 2005; Banerjee et al., 2006; Allen et al., 2008). Titanium dioxide NPs are considered to be hydrophobic and insoluble in water or organic solvents (Brown et al., 1998; Lomer et al., 2000; Lovern and Klaper, 2006; Kotsokechagia et al., 2008).
Up to recent time the prevalent volume of manufactured ZnO NPs have a wurtzite structure (hexagonal close packing) (Brayner et al., 2006). Rapidly developing nanotechnology at the present time is able to control the shape of ZnO NPs during manufacturing to include the following: nanoparticles, microspheres, nanorods, nanowires, nanocombs, branched hierarchical structures, nanohelixes and nanorings (Wang, 2004; Wang et al., 2006; Han and Gao, 2009). While the surface of ZnO might be reported as hydrophilic (Steele et al., 2010), production of ZnO NPs under controlled conditions allows to manufacture superhydrophobic ZnO NPs (Zhang et al., 2006; Changsong et al., 2009). It was reported that TiO₂ and ZnO NPs exhibit reversible wettability from hydrophobic to hydrophilic depending on an exposure to UV light or an absence of illumination (Miyauchi et al., 2002; Han and Gao, 2009).

It was reported that at lower pH (1.5 – 5.0) TiO₂ NPs display positive zeta potential (Fu et al., 2005; Gogniat et al., 2006). Reported isoelectric point for anatase structure of TiO₂ NPs is in the range of 4.0 – 5.5 pH depending on manufacturer (Mandzy et al., 2005). Accordingly, at higher pH values TiO₂ NPs exhibit negative zeta potential. Reported isoelectric point of ZnO NPs was in the range of 7.13 – 9.2 pH (Berg et al., 2009; Brayner et al., 2010; Fang et al., 2010; Keller et al., 2010).

1.3 Bacterial role in the environmental processes

Bacteria exist in water environments as freely floating (planktonic) cells or as biofilms attached to surfaces (Brown et al., 1998; Dunne, 2002; Ciston et al., 2008) and play a significant role in biological nutrient removal, maintaining self-cleaning abilities
of the creeks, rivers, and lakes by digesting organic matter, pollutants and herbicides (Iwamoto and Nasu, 2001; Dean-Ross *et al.*, 2002; Bellinaso *et al.*, 2003).

Wastewater treatment plants (WWTP) utilize bacteria to remove excessive nitrogen, phosphorous and other various organic compounds from municipal sewage. The toxic impact on microorganisms involved in nutrient removal in these engineered processes might result in disruption of these processes, and release an insufficiently treated effluent into the environment during these events. The EPA fact sheet (2007) states that nearly 25% of water quality reduction cases are nutrient related including algal bloom, oxygen depletion, and turbidity.

Microbiological nutrient removal applied to wastewater treatment is based on aerobic and anaerobic treatment of sewage. Aerobic bacteria are mainly used to remove nitrogen and phosphorous from the wastewater. Anaerobic digestion technology is based upon the activity of hydrolytic, fermentative, acidogenic, and methanogenic microorganisms that convert complex organic compounds into more simple substrates and reduce the volume of the sludge. During these processes microorganisms not only remove organic compounds, but also produce biogas that contains carbon dioxide, 65 to 70 % methane, and other gases (Metcalf & Eddy. *et al.*, 2003). Methane is a useful byproduct that can be collected and utilized for heating or energy production.

After being flushed in sewage, the toxic compounds are transported to the WWTP where these compounds might damage microorganisms at any stage of wastewater treatment and can be found in the effluent or in biosolids. Studies conducted by Limbach and coworkers (2008) demonstrated that some metal oxide NPs might be found at significant levels in outflow from the model WWTP that was used in that study. Kiser
and coworkers (2009) reported that the investigated WWTP raw sewage contained 100 – 3000 μg Ti/L, biosolids contained titanium (Ti) on an average of 1.1±0.42 mg Ti/(g suspended solids), and with a Ti removal rate around 80%, effluent contained 5 – 15 μg Ti/L. Researchers estimated that at sampled WWTP the daily load of Ti was 4.2 (mg/person)/day.

Many environmental processes, including nitrogen and sulfur cycling, are carried out by bacteria existing in communities – biofilms (Davey and O'toole G, 2000). Currently the impact of NPs on biofilms has not been well studied. In general, biofilm is composed of bacterial cells attached to the surface and extracellular polymeric substances (EPS) produced by bacteria (Brown et al., 1998; Donlan and Costerton, 2002; Ciston et al., 2008). It was reported that biofilms are more resistant than planktonic bacterial cells to various stress factors including heavy metals, toxins, and bactericidal agents (Brown and Gauthier, 1993; Gilbert et al., 1997; Davey and O'toole G, 2000; Teitzel and Parsek, 2003; Ryu and Beuchat, 2005; Uhlich et al., 2006). However, the properties of materials in nano scale are different from properties of the same materials in micro and macro scale (National Nanotechnology Initiative, 2007; Christian et al., 2008; Farré et al., 2009). Since some of these properties are not yet fully understood, researchers are intensely investigating the possible toxic impact of NPs on microorganisms and trying to pinpoint what toxicity mechanisms are involved (Adams et al., 2006; Zhang et al., 2007; Neal, 2008; Jiang et al., 2009).
Chapter 2

2 Research objectives and Hypotheses

The goal of this research is the evaluation of the toxic effect of TiO$_2$ and ZnO nanoparticles on bacteria utilizing cell membrane integrity stain – propidium iodide. This stain targets only bacterial cells with damaged membranes, thus bacteria damaged during exposure to NPs will emit a red fluorescent color and will be distinguishable under fluorescent microscopy. This method is based on reports that bacterial cells have damaged cell membranes after exposure to NPs (Stoimenov et al., 2002; Fu et al., 2005; Morones et al., 2005; Adams et al., 2006; Pal et al., 2007; Li et al., 2008)

**Objective 1:** To evaluate the cell membrane integrity of a pure culture of bacteria following exposure to metal oxide nanoparticles (TiO$_2$ and ZnO) in the absence of light. To determine the impact of these nanoparticles on Gram-negative and Gram-positive bacteria.

**Hypothesis:** Nanoparticles damage the cell membrane of planktonic Gram-negative and Gram-positive cells.
Objective 2: Determine impact of metal oxide nanoparticles (TiO$_2$ and ZnO) on bacteria in anaerobic digester sludge from a wastewater treatment plant.

Hypothesis: Metal oxide NPs will result in damage to cell membranes and a reduction in the activity of bacteria in anaerobic digesters, impacting the anaerobic digestion process.

Objective 3: Evaluate impact of TiO$_2$ and ZnO nanoparticles on single culture biofilm.

Hypothesis: Metal oxide NPs will have minimal effect on bacterial biofilms due to the protection afforded by EPS.
Chapter 3

3 Literature review

3.1 The impact of nanoparticles on bacteria

Growing concern over the potentially negative effect of engineered NPs on living organisms and our habitat has motivated scientists to analyze the toxic impact of NPs on cells and has driven the scientist efforts to identify NP toxicity mechanisms. Bactericidal effect of metal oxide NPs was reported towards *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas fluorescens*, and *Staphylococcus aureus* (Morones et al., 2005; Rincon and Pulgarin, 2005; Choi et al., 2006; Huang et al., 2008). Toxic effects were also reported towards algae (Navarro et al., 2008; Aruoja et al., 2009), protozoa (Gelover et al., 2006) and crustaceans (Lovern and Klaper, 2006; Warheit et al., 2007; Heinlaan et al., 2008). The accumulation of NPs in the environment might shift ecological niches and damage ecological systems. While the bactericidal effect for deliberate disinfection of pathogenic bacteria via application of antimicrobial coating to various surfaces (Pitkethly, 2004; Daoud et al., 2005) is a desirable feature, the uncontrolled and unmanageable potential bactericidal effect from NPs released into the environment could damage the ecosystems of creeks, rivers, wetlands and other ecological systems that harbor bacterial communities.
There are conflicting reports that attribute the toxicity mechanism of metal oxide NP to their size, morphology, or electrostatic attraction (Stoimenov et al., 2002; Morones et al., 2005; Adams et al., 2006; Zhang et al., 2007; Heinlaan et al., 2008; Huang et al., 2008; Neal, 2008; Aruoja et al., 2009). Jiang and coworkers (2009) hypothesized that the tendency of NPs to aggregate and attach to bacterial surface might contribute to NP toxicity. It was reported that NPs were observed inside of bacteria (Morones et al., 2005; Huang et al., 2008), furthermore, changes were detected in the crystalline structure of internalized ZnO NPs (Huang et al., 2008). Prevalent number of reports stated that smaller NPs demonstrated a higher toxicity rate in comparison to bigger NPs or bulk (micro scale) materials (Morones et al., 2005; Jones et al., 2008; Padmavathy and Vijayaraghavan, 2008). However, it was also observed that materials in micro scale were more toxic than NPs (Karlsson et al., 2008). Researches hypothesized that toxic effect might be specific to the type of metal oxide NP (Aruoja et al., 2009) and may vary depending upon species of microorganisms (Jiang et al., 2009).

Conflicting reports linking the toxicity of ZnO NPs to dissolved Zn ions raise more questions regarding the toxicity mechanism of ZnO NPs. It was reported that the toxicity of NPs to bacteria was higher than the toxicity of the same compound with equivalent ion concentrations (Navarro et al., 2008; Jiang et al., 2009; Wu et al., 2010). Ferris and coworkers (2009) reported that the presence of Zn ions does not explain the toxicity of ZnO NPs, since samples incubated with ZnCl₂ at concentrations higher than the concentrations of ZnO NPs did not exhibit a bactericidal effect. Other studies credited observed toxicity to soluble metal ions (Aruoja et al., 2009; Fang et al., 2010).
Reactive oxygen species (ROS) were reported as a key to the photocatalytic bactericidal mechanism (Gelover et al., 2006; Mitoraj et al., 2007; Zhang et al., 2007; Alrousan et al., 2009; Aruoja et al., 2009). Gogniat and coworkers (2006) concluded that if ROS modify cell membrane this should occur in proximity to the cell membrane, since ROS have a very short life time \((10^{-9}\) s), and therefore the cell adsorption to NPs is a key to the bactericidal effect of TiO$_2$ NPs. However, since ROS production is triggered by photons, this toxicity mechanism cannot explain the toxic impact of ZnO or TiO$_2$ NPs in the dark.

Brown and coworkers (1999) proposed that surface defects such as kink, step, terrace, vacancy and adatom on single-crystal surfaces of metal oxides are the sites of active chemical reactions. It was hypothesized that the attachment of metal nanoparticles to bacteria may involve extracellular metal-binding proteins and polypeptides produced by bacteria (Moreau et al., 2007). It was reported that polysaccharides (O-antigen and dextrans) produced by bacteria were forming hydrogen bonds with hydroxyl groups of water molecules adsorbed on TiO$_2$ surface (Jucker et al., 1997).

### 3.2 Bacterial resistance to the toxic impact of NPs

Differences in the cell wall structures between Gram-negative bacteria, which have a cell wall and an outer membrane composed of complex macromolecules, and Gram-positive bacteria, which have a thicker cell wall composed of peptidoglycan and lack the outer cell membrane (Mitoraj et al., 2007; Huang et al., 2008) might be one of the factors contributing to bacterial resistance to the toxic impact of NPs. However, some studies that have focused on the toxicity of TiO$_2$ NPs to Gram-positive as compared to
Gram-negative bacteria reported conflicting findings (Fu et al., 2005; Rincon and Pulgarin, 2005; Adams et al., 2006). Data from previous studies suggest that NP toxicity may vary significantly between Gram-positive and Gram-negative bacteria, and in some cases, vary by organism (Jiang et al., 2009).

### 3.3 Toxicity assessments

Inconsistent reports regarding the bactericidal effect of ZnO and TiO$_2$ NPs indicate the necessity to collect additional information that will provide a foundation for the evaluation of NPs impact on the environment. Many previous microbial toxicity studies have utilized plating techniques, which are labor intensive and rely on cell culturability rather than cell membrane integrity (Stoimenov et al., 2002; Daoud et al., 2005; Fu et al., 2005; Adams et al., 2006; Liu et al., 2007; Pal et al., 2007; Rampaul et al., 2007). This might be a contributing factor to the conflicting reports assessing bacterial viability. Employing different assessment methods, such as cell membrane integrity stains and direct cell count, might overcome culturability issues. The direct count method would eliminate a time required for growing bacterial cultures on adopted media, minimize time required for estimating damaged/dead and live/intact cells, and would result in more precise and reliable data that will not be affected by culturability issues of the fastidious environmental bacteria.
4 Objective 1: Pure culture. Assessing the impact of titanium dioxide and zinc oxide nanoparticles on bacteria using a fluorescent based cell membrane integrity assay


4.1 Abstract

The aim of this research was to determine if metal oxide nanoparticles damage bacterial cell membranes. In this research, bacterial cell membrane integrity was determined following 1 hour exposure to titanium dioxide (TiO$_2$) and zinc oxide (ZnO) nanoparticles (NPs) (0, 10, 50, 100, and 500 mg/L) in ultrapure water and in the presence of natural organic matter (NOM). Samples were stained with propidium iodide and counterstained with a non-specific DNA stain for cell membrane integrity assessment using fluorescence microscopy. Results of this experiment revealed a measurable impact on the cell membrane integrity of Gram-negative (*E. coli*) bacteria at 100 and 500 mg/L
TiO$_2$ even in the presence of NOM (2mg/L DOC). On the other hand, no statistically significant ($p= 0.05$) change in cell membrane integrity was observed with ZnO which aggregated and precipitated at concentrations at or above 100 mg/L. In addition, Gram-positive bacteria (*E. faecium*) were not impacted in the presence of ZnO or TiO$_2$.

Transmission electron microscopy images show an interaction between TiO$_2$ NPs and *E. coli* cells. This interaction was not observed for ZnO NPs or Gram-positive cells. The cell membrane integrity assay indicated NP impact on bacterial cells that were not detected by plating techniques. Cell membrane integrity assays show promise for broader application in environmental matrices where traditional growth-based approaches are limited due to their bias toward easily cultured organisms.

**KEY WORDS:** nanoparticles, bacteria, zinc oxide, titanium dioxide, viability
4.2 Introduction

Nano-sized particles possess properties that are unique and markedly different than larger particles and bulk material. Distinguishing attributes, such as their high surface to volume ratio, have been exploited to advance emerging technologies. Due to the broad range of application of nanomaterials, nanoscale materials are expected to be manufactured at an increasing rate over the next decade, from an estimated 2,000 tons in 2004 to approximately 58,000 tons in 2011-2020 (Maynard et al., 2006). Emerging nanoparticles, such as metal oxides, are being utilized in the production of many consumer products including sunscreens, dye-sensitized solar cells, paint, textile, electronics, and pharmaceuticals (Tyner et al., 2009; Borm and Berube, 2008; Adams et al., 2006; Oskam, 2006). The eventual disposition of consumer products will be the environment via a variety of potential pathways including wastewater treatment residuals, landfill application, and air emissions. One significant area of concern is the impact of metal oxide nanoparticles on bacteria since microorganisms perform many critical roles, which are required for healthy ecosystem function including nutrient cycling and litter decomposition. Previous research has indicated that contaminants can impact microbial structure and function in soil and sediments (Oliveira and Goulder, 2006; Seghers et al., 2005).

Considerable data indicate that nanoparticles exhibit antibacterial activity toward pure cultures including Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, and Streptococcus aureus (Huang et al., 2008; Adams et al., 2006; Morones et al., 2005; Sondi and Salopek-Sondi, 2004). Specifically, metal oxide NPs, including zinc oxide, silicone dioxide, titanium dioxide, and magnesium oxide, were proven to be toxic to
microorganisms (e.g., bacteria, protozoa, and algae) in laboratory-based studies at concentrations ranging from 10 to 5000 mg/L (Aruoja et al., 2009; Heinlaan et al., 2008; Adams et al., 2006; Gelover et al., 2006; Fu et al., 2005; Zang et al., 2006). Some previous studies suggest that the toxicity of metal oxides to bacteria is primarily attributed to the disruption of cell membrane activity (Neal, 2008; Kubo et al., 2005; Lovric et al., 2005). Some NPs (e.g., titanium dioxide) exhibit photocatalytic effects resulting in the generation of reactive oxygen species (ROS) in the presence of UV irradiation, which may destroy the lipid bilayer constituting the cell membrane allowing nanoparticles to enter the cytoplasm (Neal, 2008; Tsuang et al., 2008; Choi et al., 2006; Lovric et al., 2005). Toxicity has also been observed with metal oxide nanoparticles in the absence of light, which suggests that toxicity may not be solely attributed to photocatalysis induced ROS formation (Adams et al., 2006; Daoud et al., 2005).

Dissolved metal ions have also been reported as contributing factor to toxicity NPs (Aruoja et al., 2009; Jiang et al., 2009).

In addition, research suggests that nanoparticle precipitation, aggregation, and interaction with natural organic matter (NOM) may be important indicators of nanoparticle fate and subsequent toxicity (Kim, et al., 2009, Neal, 2008). For example, their predisposition to aggregate or their tendency to attach to the bacterial surface might play a role in their toxicity (Jiang et al., 2009). Previous research suggests that NOM can stabilize nanomaterials, reducing the likelihood of aggregation or adherence to bacteria (Hyung et al., 2007; Neal, 2008). Extracellular proteins and polypeptides produced by bacteria may also contribute to the attachment of the metal nanoparticles to bacteria (Moreau et al., 2007). However, previous studies provide conflicting findings as to
whether metal oxide nanoparticle characteristics such as size, surface charge, morphology, and chemical properties contribute to their toxicity (Aruoja et al., 2009; Heinlaan et al., 2008; Huang et al., 2008; Zhang et al., 2007; Adams et al., 2006).

This research focused on zinc oxide (ZnO) and titanium dioxide (TiO2) nanoparticles due to their growing presence in consumer products. The objective of this research was to determine if metal oxide nanoparticles impact cell membranes. Our hypothesis was that metal oxide nanoparticles disrupt cell membranes and that outcome could be measured using fluorescence microscopy. Gram-negative (Escherichia coli) and Gram-positive (Enterococcus faecium) bacteria were used to determine the toxicity of metal oxide nanoparticles. Toxicity to bacteria was determined by evaluating the cell membrane integrity using fluorescence microscopy since cell membrane damage has frequently been reported in nanoparticle toxicity studies (Li et al., 2008; Pal et al., 2007; Adams et al., 2006; Fu et al., 2005; Morones et al., 2005; Stoimenov et al., 2002). Many previous microbial toxicity studies have been based on plating techniques, which are labor intensive and rely on cell culturability rather than cell membrane integrity (Liu et al., 2007; Pal et al., 2007; Rampaul et al., 2007; Adams et al., 2006; Daoud et al., 2005; Fu et al., 2005; Stoimenov et al., 2002). This research employs direct detection techniques through fluorescent labeling of biological components which are preferred because they have favorable comparisons with slower, more laborious culture-based methodologies and can be employed in environmental samples containing bacteria that are not readily cultured (DeBoer and Beumer, 1999; Ivnitski et al., 1999).
4.3 Methods and Materials

Experimental Design. The toxicity assays performed were modified from Adams and co-workers (2006). Nanoparticles were weighed and placed in 50mL Falcon™ tubes. Ultrapure water (Type I) was added to 50mL to achieve nanoparticle concentrations of 0, 10, 100, 500 mg/L samples were shaken by hand and subsequently vortexed (Vortex-Genie-2, Model No. G-560, Scientific Industries, Inc., Bohemia, N.Y.) for 10 seconds for even distribution of nanoparticles. Bacteria were added to the samples to obtain cell concentration in range 10^5 – 10^6 bacteria per milliliter (mL) of the sample. Sample tubes were inverted 4 – 5 times prior to incubation. Samples were incubated at 24±2°C in the dark for up to 6 hours on a platform shaker (Model Classic C1, New Brunswick Scientific Co., Inc., Edison, NJ) at 45 rpm. The incubation time selected for these experiments was based on previous research (Adams et al., 2006; Daoud et al., 2005). Preliminary experiments, which included incubations up to 24 hours, indicated that toxicity could be effectively assessed following 1 hour of incubation since there was no statistically significant change in response.

STEM was used to visualize the samples processed for the nanoparticle toxicity assays. The samples were processed to determine if there was an interaction between bacteria cells (Gram-negative bacteria E. coli JM 109 and Gram-positive bacteria E. faecium ATCC #19434) and nanoparticles. STEM allows direct visualization of morphological changes to cells and/or evidence of the presence of the cell protein and DNA following the nanoparticle toxicity assays (Brayner et al., 2006; Stoimenov et al., 2002). Prior to STEM analysis, a 1 mL subsample of solution from the nanoparticle toxicity assay was amended with formaldehyde (2% v/v) and incubated in the dark at
37°C while stirring at 45rpm for one hour. Following cell fixation, a drop of the solution was placed on the TEM grid (400-mesh carbon type-B support film grid, TED PELLA, Inc., Redding, CA). Samples were then air dried and analyzed on the TEM. Control samples included bacteria cells only without NPs and NPs without bacteria. The samples processed for this study were those including the metal oxide NPs (either ZnO or TiO₂) at a final concentration of 100 mg/L.

**Toxicity Assays with natural organic matter.** In an effort to determine if the presence of NOM might affect the toxicity of nanoparticles to the bacteria tested, NOM solution was prepared in ultrapure water using Suwannee River humic and fulvic acids (International Humic Substances Society, St. Paul MN). NOM included 2 ml humic acid (Stock 1 g/L, Standard HA I Cat. No. 1S101H), 2 mL standard fulvic acid (Stock 1 g/L, Standard FA I Cat. No. 1S101F), and 0.0147 g/L of calcium chloride dihydrate (CaCl₂•2H₂O) (Cat. No. C79-500, Fisher Scientific). Final concentration of NOM in experiments was 2mg/L as dissolved organic carbon (DOC) to be in the range of natural waters (Cho et al., 1999; Jung and Son, 2008; Zhang et al., 2009).

**Cultures.** Prior to the experiment, Gram-negative bacteria (*E. coli* JM 109) and Gram-positive bacteria (*E. faecium* ATCC #19434) were grown overnight at 37°C in LB (Luria-Bertani) broth (Ref. No. 244620, BD Difco™, Becton, Dickinson and Company, Sparks, MD, USA).

**Nanoparticles.** Titanium dioxide (TiO₂) (nominal size 5-10 nm) and zinc oxide (ZnO) (nominal size 20nm) nanoparticles (Cat. No. SN 3401 and Cat. No. SN 3301; Sun Innovations Inc., CA) were acquired for this research. Nanoparticles stock solutions were not included in our protocols in an effort to minimize changes in the reactivity of the
nanoparticles. Instead, nanoparticles (dry powder) were added by mass (w:V) to the samples at the beginning of each experiment to achieve desired concentrations (Aruoja et al., 2009; Tsuang et al., 2008).

**Particle Charge.** The zeta potential of nanoparticles suspended in ultrapure water was determined based on electrophoretic mobility using the Smoluchowski equation. Electrophoretic mobility measurements were conducted in 10 runs of 10 cycles each at 25 °C (ZetaPALS, Brookhaven Instrument Corp., Holtsville, NY) with 660 nm excitation line. Bacteria, including the species tested in this research (*E. coli, E. faecalis*), are consistently reported to have a negative surface charge (Pembrey et al., 1999; van Merode et al., 2006; Soni et al., 2008).

**Transmission Electron Microscopy.** The morphology of zinc oxide and titanium dioxide nano-particles (NPs) was determined using transmission electron microscopy (TEM) following methods modified from previous studies (Xia et al., 2008; Brayner et al., 2006; Cheng et al., 2006; Fu et al., 2005). The shape and size of the nanoparticles, ZnO and TiO₂, were analyzed using scanning transmission electron microscopy (STEM) (Hitachi HD2300A, Hitachi High Technologies America, Schaumburg, IL) with a magnification range of (1x10² - 5x10⁶). The instrument operates at 200 keV with electron optics of a Schottky emission electron source that has a built-in anode heater. For these two metal oxide NPs, a stock solution (100 mg/L) was prepared in ultrapure water and vortexed for 10 seconds. Then the solution was re-suspended in the dark for one hour at 45 rpm using a platform shaker. Replicate samples were prepared for each metal oxide by placing a drop of the respective NP suspension onto a 400-mesh carbon type-B TEM support film grid. As specified by the manufacturer (TED PELLA, Inc., Redding, CA),
the copper grid (approx. grid size 42 μm) has a formvar support film coated with a heavy layer of carbon that is stable for TEM conditions including high magnification and high beam intensity. Finally, the samples were dried at room temperature for 24 hours in the dark and transferred to the TEM for imaging. To obtain a representative image, 20 to 25 fields were scanned and a minimum of five images were collected for each sample.

**Microbiological Characterization.** Microbiological characterization included a determination of the total number of bacteria and the number of bacteria with intact cell membranes (viable cells) in a given sample. For toxicity assays including Gram-negative bacteria and TiO₂ NPs, cells with intact cell membranes (viable) and total numbers of cells were compared to the culturable numbers of cells, since culturability has commonly been used to determine nanoparticle toxicity to bacteria (Heinlaan *et al*., 2008; Tsuang *et al*., 2008; Adams *et al*., 2006; Brayner *et al*., 2006; Daoud *et al*., 2005).

**Plating.** TiO₂ toxicity assay samples (0, 100, and 500 mg/L) were serially diluted (to 10⁻³ and 10⁻⁴). Two Petri dishes were plated for each replicate, thus six dishes for each tested concentration. Tryptic Soy Agar (Soybean-Casein Digest Agar, USP, REF 236950, Becton, Dickinson and Company, Sparks, MD, USA) was the media used to determine culturability (Druso *et al*., 2004; Hara-Kudo *et al*., 2000). Plates were inoculated with 100 μL diluted samples and incubated at 37 °C for 24 hours in the absence of light. Plates containing a number of colonies that was too numerous to count (TNNTC) were disregarded. Colony forming units (CFU) were counted on the remaining plates, which totaled six plates (at 10⁻⁴ dilution) for each sample. Average data was reported.
**Cell Membrane Integrity Staining.** Propidium iodide (Cat. No. 11348639001, Roche Diagnostics GmbH, Mannheim, Germany), a red indicator stain ($\lambda = 617$nm maximum), was used to evaluate cells based on their membrane integrity often referred to as a viability assay (Foley et al., 2008; Gruden et al., 2003; Lopez-Amoros et al., 1997). Two 1mL aliquots were transferred from each sample to microcentrifuge tubes. Samples were stained with PI (10 $\mu$g/mL commercial solution) and counterstained with Picogreen (PG; P-7859; Molecular Probes, Inc., Eugene OR) ($4 \times 10^{-3}$ of stock) for cell membrane integrity assessment. Bacteria that maintained their membrane integrity produced green fluorescence while organisms with permeable and damaged membranes fluoresced red and orange, respectively.

**Fluorescence Microscopy.** Samples were filtered using 0.22 $\mu$m black polycarbonate filters (Cat. No. K02BP02500, Osmonics Inc., Minnetonka, MN) supported with glass fiber prefilter (Cat. No. APFA 025 00, Millipore, Billerica, MA). Filters were slide mounted on glass microscope slides (Cat No. 12-544-1, Fisher Scientific, Pittsburgh, PA) with a drop of immersion oil and covered with a cover slip (Cat. No. 12-548-C, Fisher Scientific, Pittsburgh, PA). Samples were analyzed with an upright fluorescent microscope with a reflected fluorescent system (Olympus BX-51; Olympus optical CO. LTD, Melville, NY).

**Statistics.** All experiments based on 3 replicates were repeated 2 or more times. Data were analyzed in Excel utilizing the One-Tailed $t$-Test: Two-Sample Assuming Equal Variances. Where, the null hypothesis was that the control sample and sample with nanoparticles have equal numbers of viable cells, total cells, and percent viability. The alternative hypothesis is that the control sample had a statistically higher number of
viable cells, total cells, and percent viability than samples exposed to nanoparticles. The significance level tested was 95% ($p < 0.05$).

### 4.4 Results and Discussion

TiO$_2$ and ZnO nanoparticles were characterized using TEM. Both solutions of NPs were found to be polydispersed and in the size range of 10-20nm (Figure 4-1). Previous research suggests that nano-sized and bulk metal oxides both have the potential to confer toxicity on bacteria (Aruoja et al., 2009; Heinlaan et al., 2008; Zhang et al., 2007). However, in some cases nano-sized metal NPs have demonstrated a higher level of toxicity as compared to bulk materials, presumably due to increased metal oxide surface area (Aruoja et al., 2009; Jiang et al., 2009; Zhang et al., 2007).

Toxicity assay results indicated that an increased concentration of TiO$_2$ NPs resulted in a decrease in *E. coli* cells with intact cell membranes in comparison with control samples (Figure 4-2). The decrease in *E. coli* with intact cell membranes (viable percent) was statistically significant at 100 mg/L TiO$_2$ NPs ($p=0.0355$). Notably, the same result was achieved in the presence of NOM, which was added to increase sample complexity (Figure 4-2). Previous research suggests that nanomaterials, specifically carbon nanotubes and zero valent iron nanoparticles, were stabilized in the presence of NOM, preventing aggregation (Hyung et al., 2007; Klupinski et al., 2004). At this NP concentration (100 mg/L), the *E. coli* cell may be impacted resulting in damage to the cell membrane due to contact with the NPs. These findings correspond to previous research which indicated a decrease in the culturability of *E. coli* introduced to TiO$_2$ NPs in the absence of light (Adams et al., 2006).
Figure 4-1: (A) Transmission electron microscopy (TEM) image (200 keV; magnification, 800k) of titanium dioxide (TiO$_2$). (B) TEM image (200 keV; magnification, 800k) of zinc oxide (ZnO) nanoparticles (NPs).
Figure 4-2: Percent of *Escherichia coli* with damaged cell membrane as a function of TiO$_2$ NP concentration (0–500mg/L in ultrapure water). Cell membrane integrity assays were completed in the absence and presence of natural organic matter (2mg/L dissolved organic carbon). Cells were incubated for 1 h in the dark at 20±2°C. Error bars are equivalent to ±one standard deviation of the mean where n=3.

No statistically significant (α= 0.05) decrease in the percent of cells with intact membranes was observed with the Gram-positive bacteria (*E. faecium*) in the presence of either ZnO or TiO$_2$ NPs (Table 1). This outcome is likely attributable to well-established differences between Gram-negative and Gram-positive bacteria. Gram-negative bacteria have a cell wall and an outer membrane composed of complex macromolecules, whereas Gram-positive bacteria have a thicker cell wall composed of peptidoglycan and lack the outer cell membrane (Huang *et al.*, 2008; Mitoraj *et al.*, 2007). Some studies, which have focused on the toxicity of TiO$_2$ to Gram-positive as compared to Gram-negative bacteria,
have reported conflicting findings (Adams et al., 2006; Fu et al., 2005; Rincon and Pulgarin, 2005). These results suggest that nanoparticle toxicity can not only vary considerably between bacterial types (Gram-positive and Gram-negative bacteria) but can vary, in some cases by organism (Jiang et al., 2009).

Table 4.1: Percent of bacterial cells with damaged cell membrane following incubation with metal oxide nanoparticles for one hour in the dark at 20±2°C.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Nanoparticles</th>
<th>Concentration of the nanoparticles, mg/L</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Gram negative</td>
<td>TiO₂</td>
<td>22.4 ± 6.8</td>
</tr>
<tr>
<td>(Escherichia coli)</td>
<td>ZnO</td>
<td>16.7 ± 1.5</td>
</tr>
<tr>
<td>Gram positive</td>
<td>TiO₂</td>
<td>10.1 ± 3.3</td>
</tr>
<tr>
<td>(Enterococcus faecium)</td>
<td>ZnO</td>
<td>8.8 ± 2.9</td>
</tr>
</tbody>
</table>

a These data are statistically higher (p < 0.05) when compared to the control sample without nanoparticles. Other data are not statistically significant. ± One standard deviation of the mean where n=3.

Although some previous studies suggest photocatalysis as a primary mechanism the impact of metal oxide NPs on bacteria (Alrousan et al., 2009; Tsuang et al., 2008; Gelover et al., 2006), others have reported toxicity in the absence of light (Jiang et al., 2009; Zhang et al., 2007; Adams et al., 2006). In the absence of light, non oxidant toxicity may occur due to nanoparticle binding on cells, which may result in adverse biological effects including protein unfolding and loss of enzymatic activity (Neal, 2008; Xia et al., 2008). This hypothesis is supported by the TEM micrograph of *E. coli* in the presence of TiO₂ NPs which showed agglomeration of the NPs on the bacteria cell wall.
(Figure 3). TiO$_2$ NPs were bound to the cell wall despite the negative zeta potential (-29 (± 1.1) mV) measured, suggesting that electrostatic attraction was not a factor. Previous research suggests that other factors, specifically NP concentration, have a more significant influence on NP-cell interactions than electrostatic attraction (Zhang et al., 2007). Indeed, cell binding seems to be a common mechanism for toxicity to cells (Jiang et al., 2009; Heinlaan et al., 2008). After processing several samples on the TEM, none of the samples including ZnO NPs or Gram-positive bacteria indicated NP-cell interaction. This may be explained by the fact that Gram-positive cells lack the outer membrane composed of complex macromolecules present in Gram-negative organisms. In addition, the ZnO NPs tended to aggregate and were destabilized at concentrations at or above 100 mg/L (Neal, 2008; Stoimenov et al., 2002).

Figure 4-3: TEM image (200 kV; magnification, 300k) of *E. coli* cell and TiO$_2$ NPs (100mg/L) in ultrapure water.
Our results suggest that the cell membrane integrity assay utilized in this research may provide a more sensitive and applicable approach to evaluating NP toxicity to bacterial cells as compared to studies based on cell culturability. Results from TiO₂ NP toxicity assay using cell membrane as a measure of NP toxicity were directly compared to a culturability study under the same conditions (Figure 4). The total CFU determined using plating techniques corresponded with total cell numbers determined using a non-specific DNA stain and fluorescence microscopy.

![Graph](image)

**Figure 4-4**: Total cells, cells with intact cell membrane, and culturable cells (*E. coli*) in ultrapure water as a function of TiO₂ NP concentration (0–500mg/L in ultrapure water). Samples were analyzed using direct microscopic count methods including nonspecific DNA stain (total) and cell membrane integrity stain (viable) (average ± standard deviation, n=3) as well as plate counts (colony-forming units, CFU) (average ± standard deviation, n=6).
However, CFU data did not suggest any measurable impact of TiO$_2$ NPs on bacterial cells. The same samples analyzed with the cell membrane integrity stain indicated a statistically significant decrease in bacteria with an intact cell membrane for 100 and 500 mg/L TiO$_2$ NPs. This result may explain conflicting findings regarding the potential impact of TiO$_2$ NPs on bacteria since culturability will not necessarily reveal presence of the damaged cells. In addition, plating techniques are prone to more variability, and they tend to favor organisms that are easily cultured, which will limit their effective application in environmental samples (Coutard et al., 2007, Heidelberg et al., 1997).

4.5 Summary

In this study, a cell membrane integrity assay was used to establish the impact of selected metal oxide nanoparticles on bacteria. This assay was chosen since many previous studies suggest that nanoparticles cause toxicity to bacteria by disrupting their cell membrane (Neal, 2008; Kubo et al., 2005; Lovric et al., 2005). Most nanoparticle toxicity studies have investigated cell culturability rather than cell membrane integrity (Liu et al., 2007; Pal et al., 2007; Rampaul et al., 2007; Adams et al., 2006; Daoud et al., 2005; Fu et al., 2005; Stoimenov et al., 2002). Titanium dioxide nanoparticles impacted the membrane integrity of Gram-negative (E. coli) cells. TEM imaging supported the hypothesis developed from the literature that nanoparticle toxicity to bacteria can be attributed to the agglomeration of nanoparticles on their cell walls. Results from this research suggest that cell membrane integrity assays may be a more sensitive measure of nanoparticle impact on bacteria since culturability assays do not necessarily indicate cell
membrane damage. In addition, cell membrane integrity assays will likely find broader
application in environmental matrices where traditional growth-based approaches are
limited due to their bias toward easily cultured organisms.

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STEM Education: An Environmental Science Learning Community at the Land-Lake
Ecosystem Interface” through the Lake Erie Research Center.”

Author Disclosure Statement

There are no competing financial interests.

4.6 References


"Photocatalytic inactivation of $E. coli$ in surface water using immobilised

nanoparticles of CuO, ZnO and TiO$_2$ to microalgae Pseudokirchneriella


Chapter 5

5 Objective 2: Sludge. Investigation into the impact of titanium dioxide and zinc oxide nanoparticles on the microbiological activity of anaerobic digester bacteria

5.1 Abstract

The goal of this research was to assess the impact of titanium dioxide (TiO$_2$) and zinc oxide (ZnO) nanoparticles (NPs) on the activity of microorganisms in anaerobic digester sludge. The impact of NP on microorganisms was evaluated using the fluorescent microscopy (membrane integrity stain) and anaerobic biological activity (biogas production and composition). Samples were amended with TiO$_2$ and ZnO NPs (0 – 500 mg/L (0.31 – 15.43 μg TiO$_2$/mg total solids (TS)), or as titanium (Ti) in TiO$_2$ calculated by weight: 375, 750, and 3752 μg /L or 0.01, 0.023, and 0.12 μg/mg TS) and incubated for 144 h at 35°C. Results revealed a significant reduction of biogas production at 500 mg/L of ZnO (15.43 μg NPs/(mg TS)). A significant increase in percent of damaged cells was detected after 1 h of incubation in diluted sludge samples amended with 100 and 500 mg/L of TiO$_2$ NPs. Total cell numbers in sludge samples diluted 10$^4$ times were in range 1.74 x 10$^5$ to 2.1 x 10$^6$ cells/mL. Undiluted sludge samples and samples amended with ZnO NPs did not demonstrate an increase of
damaged cells. Data suggest that ZnO NPs at concentration 500 mg/L might affect the methanogenic microorganisms in the sludge, while TiO_2 NPs did not demonstrate a toxic effect on methanogenic microorganisms in undiluted sludge at tested concentrations. However, an increased level of damaged cells in diluted sludge samples amended with TiO_2 NPs indicates the possibility of a toxic affect at the ratio 4.9 × 10^{-5} of mg/# of cells.

**Key words:** nanoparticles, sludge, zinc oxide, titanium dioxide.

### 5.2 Introduction

Nanotechnology enables the production of numerous consumer products that are constantly increasing in variety and numbers. The Consumer Products Inventory (The Project on Emerging Nanotechnologies) listed 540 products incorporating nanomaterials that were manufactured in the US in 2009, while the worldwide number of the consumer products with nanomaterials reached 1,015 as of August 25, 2009. Properties of the materials in nanoscale are different from the properties of materials in micro and macro scale. Nanomaterials have a high surface to volume ratio and are considered to have more atoms on their surface, therefore being more reactive than bigger particles (Christian et al., 2008; Farré et al., 2009). The increasing number of consumer products incorporating NPs is contributing to the growing quantities of NPs released into the environment when these products are naturally aging, get washed or flushed into the sewage. Consequently, quantities of released NPs entering water sources and wastewater treatment plants would continue to increase. The possible negative impact of engineered
NPs on populations of microorganisms inhabiting surface water might affect the nutrients cycle processes carried on by these microorganisms and thus affect the quality of the water sources. The toxic impact of NPs on microorganisms involved in wastewater treatment processes might affect the treatment process in several different stages, and as a result, decrease the quality of effluent discharged into surface water sources.

Anaerobic digestion is one of the steps of wastewater treatment that take place in the absence of light and it is essential to evaluate possibilities and extent of the toxic impact of NPs on the microorganisms in the dark conditions. During anaerobic digestion bacteria produce biogas as a byproduct that contains 65 – 70% of methane (Metcalf & Eddy. et al., 2003). Methane is utilized as an energy source, and any disruption in the digestion process might affect the biogas production, which will therefore have an impact on the methane production. The photocatalytic bactericidal effect of TiO₂ and ZnO NPs has attracted the attention of many researchers (Rajagopal et al., 2006; Mitoraj et al., 2007; Tsuang et al., 2008; Alrousan et al., 2009), though currently, an impact of these NPs on bacteria in the absence of light has not been well studied.

Currently there is very limited information regarding quantities of NPs in raw sewage. In one study, raw sewage entering a wastewater treatment plant (WWTP) contained 100 – 3000 μg/L of Ti, part of which was presumed to be TiO₂, the effluent contained up to 15 μg/L of Ti, and settled solids contained 1 – 6 μg of Ti/mg (Kiser et al., 2009). Researchers hypothesized that the significant accumulation of NPs in biosolids may lead to the release of NPs into the environment when biosolids are utilized in agricultural or landfill applications. While some studies revealed the toxic impact of TiO₂ and ZnO NPs on microorganisms in the absence of light (Daoud et al., 2005;
Adams et al., 2006; Zhang et al., 2007; Battin et al., 2009; Simon-Deckers et al., 2009; Mileyeva-Biebesheimer et al., 2010), other studies did not establish the toxic effect of TiO2 NPs on microorganisms in the absence of UV radiation (Tsuang et al., 2008; Jiang et al., 2009). Recent study revealed morphological changes and damage to *Nitrosomonas europaea* cell wall after 4 h exposure to TiO2 and ZnO NPs in the dark (Fang et al., 2010). A recent study evaluated the toxic effect of silver NPs on activated sludge. The study revealed the inhibition effect of the nitrification process by silver NPs (41.4 ± 13%) that was greater than the inhibition effect from silver ions (13.5 ± 6.7%) at a total silver concentration of 1 mg/L (Liang et al., 2010). It was also reported that in aerobic sludge copper NPs aggregated and demonstrated lesser toxic effect on the respiration process than copper ions (Ganesh et al., 2010). Researchers proposed that the dissolution of specific ions is a contributing factor to toxicity (Jiang et al., 2009; Fang et al., 2010; Wu et al., 2010). However, it was observed that direct contact of ZnO NPs and microorganisms resulted in a greater rate of antimicrobial effect of ZnO NPs (Wu et al., 2010). In another study, dissolved Zn$^{2+}$ ions did not exhibit a toxic effect towards bacteria, while ZnO NPs demonstrated a toxic effect (Feris et al., 2009).

The goal of this research was to evaluate the impact of TiO2 and ZnO NPs on the anaerobic digestion process. At the present time, the lack of information regarding the impact of TiO2 and ZnO NPs on anaerobic digester microorganisms in sludge raises questions about potential susceptibility of these microorganisms to growing and accumulating quantities of engineered NPs. This study focused on concentrations of NPs (0.31 – 15.43 μg NPs /mg TS. The concentrations of Ti in solids of WWTP reported by Kiser and coworkers (2009) as total Ti were as follows: in secondary effluent 35 μg/L,
primary solids 803 µg/L, in secondary solids 8464 µg/L. In the current study, the concentrations of titanium in TiO₂ calculated by weight were as follows: 375, 750, and 3752 µg/L or 0.01, 0.023, and 0.12 µg/mg TS. Sludge is a complex matrix that contains various organic and inorganic compounds, including surfactants that might interact with NPs and therefore change their surface chemistry and bioavailability. More information regarding the impact of TiO₂ and ZnO NPs on anaerobic digester microorganisms, would provide data for a reliable assessment and the prediction of environmental impact from engineered NPs.

5.3 Methods and materials

Materials

Nanoparticles. Titanium dioxide (TiO₂), anatase phase (Cat. No. SN 3301) and zinc oxide (Cat. No. SN 3401) were ordered from Sun Innovations Inc., CA. Characteristics of the nanoparticles are provided in the Table 5.1. The zeta potential of NPs was analyzed and reported in previous work (Mileyeva-Biebesheimer et al., 2010). Electrophoretic mobility measurements were conducted in 10 runs of 10 cycles each at 25°C (ZetaPALS, Brookhaven Instrument Corp., Holtsville, NY) with 660 nm excitation line. The pH values of the samples dispersed before the test in purified laboratory grade water were as follows: TiO₂ pH=7.13 and ZnO pH=7.4. The zeta potential of NPs was evaluated using Smoluchowski equation (Kim et al., 2009). Nanoparticles in dry form were added to the samples at the beginning of each experiment (Tsuang et al., 2008; Aruojja et al., 2009).
Table 5.1: Characteristics of the nanoparticles.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Nominal size, nm</th>
<th>Purity, %</th>
<th>Zeta potential, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO₂</td>
<td>5 – 10</td>
<td>&gt; 98</td>
<td>-29 ± 1.1</td>
</tr>
<tr>
<td>ZnO</td>
<td>20</td>
<td>&gt; 99.8</td>
<td>-11 ± 0.94</td>
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</tbody>
</table>

**Sludge source.** Anaerobic sludge was collected at the Toledo Bay View Wastewater Treatment Plant (WWTP) on January 14, 2009 (Batch 1) and March 24, 2009 (Batch 2). The daily capacity of the Toledo Bay WWTP is 72 million gallons/day. The operating temperature of anaerobic digesters is 37°C (98°F). Solids retention time is 25 days. Sludge samples were taken from a feeding line to the sludge dewatering belt. The sludge was stored in an airtight container in the refrigerator at 4°C prior to the experiments.

**Sludge chemical and physical characterization**

**Oxidation reduction potential (ORP).** Sludge (50mL) was placed into a beaker, and covered with parafilm. Sludge was brought to the room temperature (25°C) prior to the test. Samples were analyzed for ORP and pH (Thermo Scientific Orion 2 Star bench-top pH meter with ORP Triode).

**Solids.** Prior to the experiments, samples were stored in the refrigerator at 4°C for 4–5 days. Samples were prepared in three replicas (50 g each) and analyzed for total, volatile and fixed solids according to the Standard Methods (Greenberg *et al.*, 1992), sections 2540 A, G. Samples were evaporated in a water bath for 2 hours at 80°C before they were placed in the drying oven.
**Total organic carbon (TOC).** Sludge was stored in a tightly closed container at 4°C prior to the test. Supernatant collected from the sludge was filtered with 0.45 μm syringe filter (Cat No 09-719-B, Fisher Scientific, PA). Sludge samples were analyzed in two different concentrations to assure that at least one of the samples would fit in the detection range of the TOC analyzer. The filtered sample was diluted with laboratory grade water 0 and 10⁻¹ times. Each sample (30 mL) was analyzed twice with TOC-VCSH Total Organic Carbon Analyzer, SHIMADZU. Results were reported as average value of the two measurements for each sample.

**Ionic strength.** The ionic strength of all types of media utilized in the experiments was determined from the relationship of the ionic strength (I) and the electrical conductivity (EC) utilizing a formula proposed by Russell (1976):

\[ I \text{ (mol/L)} = 1.6 \times 10^{-5} \times EC \text{ (dS/m)} \]

Conductivity was measured with a portable conductivity meter accumet® AP75 (Cat. No. 13636 AP75A, Fisher Scientific, PA). Sludge samples were analyzed in concentrations corresponding to samples used for sludge toxicity assays: undiluted and diluted (10⁻¹). Samples of several butches of purified laboratory grade water were tested and the average value was reported. Prior analysis, samples with TiO₂ and ZnO NPs (10, 100, and 500 mg/L) were re-suspended in purified laboratory grade water, and incubated in the dark in still condition at room temperature for 24h. Functional characteristics of anaerobic sludge are summarized in the Table 5.2.
Table 5.2: Functional characteristics of the digester and anaerobic sludge.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Units</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time of digester</td>
<td>Days</td>
<td>25</td>
</tr>
<tr>
<td>Temperature</td>
<td>°C</td>
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</tr>
<tr>
<td>pH</td>
<td>pH</td>
<td>7.68</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>as CaCO₃, mg/L</td>
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</tr>
<tr>
<td>Oxidation reduction potential (ORP)</td>
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</tr>
<tr>
<td>Total organic carbon (TOC)</td>
<td>mg/L</td>
<td>235</td>
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<tr>
<td>Ionic Strength of undiluted sludge</td>
<td>mol/L</td>
<td>2.54x10⁻⁴</td>
</tr>
<tr>
<td>Ionic Strength of sludge diluted 10⁻⁴ times</td>
<td>mol/L</td>
<td>2.51x10⁻⁷</td>
</tr>
<tr>
<td>Total solids</td>
<td>%</td>
<td>3.24</td>
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<tr>
<td>Volatile solids</td>
<td>%</td>
<td>27.02</td>
</tr>
</tbody>
</table>

**Sludge toxicity assays**

**Incubated sludge.** Experiments were conducted with four replicas for each sample. Sludge (50g) was placed in 100 mL glass vials. Nanoparticles of TiO₂ in quantities 0, 50 and 500 mg/L were added to key labeled samples. The same procedure was performed for samples with ZnO NPs. Vials were sealed with aluminum caps with robber septas lined with Teflon®. Sludge samples were incubated in the dark for 6 days at 35°C. Biogas was collected in each vial with a 10 mL syringe inserted through the robber septa during first 4 h and later with a 60 mL syringe. Cumulative biogas production was measured at 4, 24, 48, 120 and 144 h.

**Biogas components.** Biogas was analyzed with Gas Chromatograph (Thermal conductivity detector) GOW-MAC® Series 400 G/C (GOW-MAC® Instrument Co., Bethlehem, PA). Helium was used as carrier gas with a flow rate 30 mL/min. HayeSep
DB column C4212A10002 (10’X1/8” ST. ST., 400/A 80/100 mesh was ordered from GOW-MAC® Instrument Co., (Bethlehem, PA). Column, detector and injector temperature settings were 45, 125, and 110°C respectively. Current detector was adjusted to 175mA. Injected volumes for this test were 3 ml for all samples. Biogas collected in 60mL syringes was forced back into the vials. Sampling syringe 3mL was used to withdraw a sample for the test. Each replica for each sample was tested 4 – 6 times.

Data were analyzed with DACS chromatography software LabVIEW™ Run-Time Version 6.0 (National Instruments™). Software provided results as areas under the curves. Results occurring out of range (2 or 3 times lower than in other tests for the same replica) were not used for final calculations. As a result, each replica for each sample was tested 3 to 5 times. Data were averaged for each replica and then averaged for the sample. A standard deviation for the sample was calculated using average values for each replica. Further analysis was conducted with a priori calculated “calibration curves” for methane and carbon dioxide. Calibration curves were calculated by analyzing various (0.5 - 3.0 mL) volumes of the pure standard gases. Gases were supplied by Matheson Trigas and ordered from Fisher Scientific: Carbon Dioxide BDRY (Cat. No. S43691) and methane (Cat.No S43695).

**Sludge toxicity assay.** Prior to the experiments, sludge was incubated overnight at 35°C to mimic the digester environment and to revitalize microorganisms. Sludge (50 mL) was placed in a 100 mL glass vial and, sealed with an aluminum cap with a robber septa with Teflon® liner. A syringe was inserted through the robber septa to monitor the activity of the sludge prior to the experiment via biogas production. Samples were protected from light. Purified water (800 mL) was amended with laboratory grade
nitrogen gas for 10 min to remove oxygen and create anoxic dilution media for anaerobic microorganisms. Purified water was stored in the incubator overnight at 35°C to reduce any temperature stress for anaerobic microorganisms. Nanoparticles were weighed and placed in a 50 mL FalconTM tubes.

Sludge was diluted with purified water to obtain final cell concentration in the range of $10^5$ – $10^6$ bacteria per milliliter (mL) of the sample. One half of the required purified water was added directly to the sludge. The other half of the purified water was added directly to the tubes with nanoparticles to obtain 50 mL of sample and to achieve nanoparticle concentrations of 0, 10, 100, 500 mg/L when the sample was combined with diluted sludge. The final sludge dilution rate was $10^{-4}$ times. After the final dilution, preparation of the diluted sludge samples, all experimental procedures were identical for both diluted and undiluted sludge samples (Figure 5-1).

![Flow chart of the toxicity assay preparation.](image)

**Figure 5-1:** Flow chart of the toxicity assay preparation.
Tubes with nanoparticles were shaken by hand and subsequently vortexed (Vortex-Genie-2, Model No. G-560, Scientific Industries, Inc., Bohemia, N.Y.) for 10 seconds for even distribution of nanoparticles. Diluted sludge was added to the samples with dispersed nanoparticles and inverted 4 – 6 times by hand to homogenize the samples. Samples were then incubated at 35 °C in the dark for 1 hr on a platform-shaker (Model Classic C1, New Brunswick Scientific Co., Inc., Edison, NJ) at 45 rpm. NP toxicity to diluted sludge was estimated utilizing fluorescence microscopy. Samples were stained with Propidium iodide (4 μL/mL) and counterstained with PicoGreen® (3 μL/mL) for 10 and 5 minutes respectively. During the staining procedure samples were protected from light with aluminum foil. Samples were filtered on black polycarbonate filters (0.22 μm, Cat. No. K02BP02500, OSMONICS Inc., Minnetonka, MN) supported with glass fiber prefilters (Cat. No. APFA 02500, Millipore, Billerica, MA). Filters were slide mounted on glass microscope slides (Cat No. 12-544-1, Fisher Scientific) with a drop of immersion oil and covered with a cover slip (Cat. No. 12-548-C; Fisher Scientific). Samples were analyzed with an upright fluorescent microscope with a reflected fluorescent system.

Statistics. All experiments, except biogas components, were repeated two or more times. Average data were reported for each experiment. Replicate samples were processed for all integrity assays reported. Data were analyzed in Excel utilizing the One-Tailed $t$-Test: Two-Sample Assuming Equal Variances. The null hypothesis stated that the control sample and sample with nanoparticles have equal numbers of intact cells, total cells, and percent integrity. The alternative hypothesis is that the control sample had
a statistically higher number of intact cells, total cells, and percent integrity than samples exposed to nanoparticles.

5.4 Results and Discussion

Sludge toxicity assays

Biogas production. Sludge samples were incubated with 0, 50, and 500 mg/L of TiO$_2$ NPs (0.31, 1.54 and 15.43 μg NP/mg TS). Each sample produced 25 – 31 mL of biogas cumulatively after 144 h of incubation. Samples with 50 and 500 mg/L of TiO$_2$ did not indicate a statistically significant decrease (α = 0.05) in the volume of biogas produced in comparison to the control sludge samples with 0 mg/L of TiO$_2$ NPs (Figure 5-2).

![Figure 5-2: Cumulative biogas production by sludge samples incubated in the dark with TiO$_2$ NPs (0 – 500 mg/L) for 144 h at 35°C (average ±1 standard deviation, n =4).](image-url)
Sludge samples incubated for 144 h with 0 and 500 mg/L of ZnO NP cumulatively produced 30 and 15.7 mL of biogas. Sample with 50 mg/L of ZnO produced 32.3 mL of biogas, which was similar to the sample with 0 mg/L of ZnO (Figure 5-3). All samples incubated with 500 mg/L of ZnO NPs indicated a statistically significant reduction in biogas production ($p < 0.05$). A slight increase in the volume of the produced biogas was noticeable after 100 h of incubation and it was extended to the end of incubation period (144 h). This increase in the volume of the produced biogas might be attributed to several factors. The depletion of Zn ions might be linked to a reduction of the toxicity level from the toxicity level that was originally applied to the samples. The bioavailability of ZnO NPs might also have been reduced due to the interactions with organic and inorganic compounds that were present in the sludge. As a result, the population of microorganisms had increased their activity and a biogas production.
Figure 5-3: Cumulative biogas production by sludge samples incubated in the dark with ZnO NPs (0 – 500 mg/L) for 144 h at 35°C (average ±1 standard deviation, n =4).

Analysis of the cumulatively produced biogas utilizing Gas Chromatograph revealed that there is no statistically significant difference in the produced volume of methane (CH₄) and carbon dioxide (CO₂) among the samples incubated with TiO₂ NPs (Figure 5-4).
Figure 5-4: Cumulative production of CH₄ and CO₂ by 50 mL of sludge as a function of TiO₂ NPs concentration (0-500 mg/L) (average ±1 standard deviation, n=4).

This might be attributed to the complex nature of sludge, which might reduce bioavailability of NPs. A low ratio of NP concentration compared to the cell density needs to be considered as well. The clumping nature of TiO₂ NPs might also reduce the bioavailability of NPs. Observed insignificant increase in biogas production in samples amended with TiO₂ NPs might imply that in sludge TiO₂ NPs might adsorb some toxic organic and/or inorganic compounds and thus alter sludge chemistry and components to more favorable for methanogens. It was reported that TiO₂ NPs adsorb organic matter and were utilized for remediation (Bang et al., 2005; Pena et al., 2006; Kim and Shon, 2007; Luo et al., 2010). Since interactions of TiO₂ NPs with surfactants and other
organic and inorganic compounds of the sludge were out of the scope of this study, further investigation of the observed phenomenon might identify processes causing this trend. Since toxic effect of NPs is species specific, it is possible that TiO$_2$ NPs did not affect methanogens at tested conditions and concentrations. Samples incubated with 50 and 500 mg/L ZnO NPs produced less CH$_4$ ($p=4.29 \times 10^{-5}$ and $p=1.78 \times 10^{-9}$ respectively) in comparison with the control samples (0 mg/L ZnO NPs). Samples with 500 mg/L of ZnO NPs produced less CO$_2$ ($p=0.03$) in comparison to the control samples with 0 mg/L of ZnO NPs (Figure 5-5).

Figure 5-5: Cumulative production of CH$_4$ and CO$_2$ by 50 mL of sludge as a function of ZnO NPs concentration (0-500 mg/L) (average ±1 standard deviation, n=4).
**Cell integrity.** Experiments with undiluted sludge did not reveal any reduction in the percent of intact cells after incubation for 1h with either TiO$_2$ or ZnO NPs. Sludge contains a higher concentration of cells ($10^9$ cells/mL), in comparison to the previously reported experiments with pure culture (Mileyeva-Biebesheimer et al., 2010) where total cell numbers were in range $10^6$ cells/mL, thus the actual concentration of NPs per cell number were most likely below the toxicity level. Serial dilution provided comparable cell numbers to pure culture samples ($10^6$ cells/mL) and analogous concentrations of NPs. Experiments with diluted sludge exposing microorganisms to 0, 10, 100, and 500 mg/L TiO$_2$ and ZnO NPs exhibited response similar to the pure culture experiments. At concentrations 100 and 500 mg/L of TiO$_2$ NPs experiments revealed statistically significant reduction in percent of the cell integrity ($p=0.0009$ and $p=7.77\times10^{-5}$ respectively) in comparison to the control sample 0 mg/L TiO$_2$ NPs. Experiments with 0, 10, 100, and 500 mg/L ZnO NPs did not indicated any statistically significant reduction in the percent integrity for microorganisms in sludge diluted $10^4$ times (Figure 5 – 6). These data are in line with the previously conducted experiments with the pure culture planktonic cells (Mileyeva-Biebesheimer et al., 2010). Results suggest that at tested concentrations and conditions ZnO NPs do not exhibit acute toxic impact on microorganisms.
However, the reduction in biogas production observed in the undiluted sludge toxicity assay, indicates that the toxic impact on methanogenic microorganisms in anaerobic sludge develops over longer period of time and might be noticed after 24h of incubation period. The average volumes of biogas produced by sludge samples with 0, 50, and 500 mg/L of ZnO after 24 h of incubation were 17, 11.5, and 8.8 mL respectively. These data are in agreement with reported inhibition of biogas production (methanogenesis) after the exposure of the anaerobic sludge to 500 mg/L of zinc sulfate heptahydrate (ZnSO₄ • 7H₂O) (Leighton and Forster, 1998). Inhibition of the methanogenesis and the high level of the toxic effect was also reported after incubation of
the anaerobic sludge with zinc chloride (ZnCl₂) at concentrations below 500 mg/L (Codina et al., 1998). Concentrations of toxic metals, such as Zn, in proportion to the cell mass might determine various responses of microorganisms. It was reported that low concentrations of zinc (1 mg/L) promoted growth of microorganisms in activated sludge, while at higher concentrations (10 and 20 mg/L) biomass production was reduced by 15% (Cabrero et al., 1998). It was proposed that low concentrations of Zn serve as micronutrients for microorganisms, while higher concentration of Zn exhibit a toxic effect (Cabrero et al., 1998; Jones et al., 2008; Padmavathy and Vijayaraghavan, 2008).

Toxicity as a function of cell numbers was analyzed in experiments conducted with a pure culture of E. coli (Figure 5-6.A) and diluted sludge (Figure 5-6.B). Samples with pure culture of E. coli contained 10⁶ cells/mL and were amended with 0, 10, 50, 100, and 500 mg/L of TiO₂ NPs. Samples with diluted sludge were incubated with 0, 10, 100, and 500 mg/L of TiO₂ NPs. Sludge samples that were diluted 10⁻⁴ times contained 10⁵ – 10⁶ cells/mL. The percent of cell integrity was reduced significantly (α=0.05) at concentration 100 mg/L of TiO₂ NPs in both, pure culture and diluted 10⁻⁴ times sludge samples (Figures 5-6. A and B), in comparison to the control samples with 0 mg/L TiO₂ NPs. Reduction in cell integrity for sludge samples that were diluted 10⁻² and 10⁻³ times (10⁷ – 10⁸ and 10⁶ – 10⁷ cells/mL respectively) was not statistically significant.
Figure 5-7: Percent of cell integrity as a function of TiO$_2$ NPs concentration (mg/L) and total cell numbers (Average ± 1 standard deviation, n=3). (A) Pure cell culture. (B) Diluted sludge.
No reduction in the produced volume of biogas occurred in samples incubated with TiO$_2$ NPs, the absence of the toxic effect might be attributed to several factors. Undiluted sludge has $10^9 - 10^{10}$ cells/mL of sludge and thus actual concentration of TiO$_2$ NPs per number of cells in the representative sample were $4.04 \times 10^{-8}$ to $4.49 \times 10^{-9}$. This ratio was significantly lower than the toxicity threshold $4.88 \times 10^{-5}$. Sludge is a complex media that contains multiple organic and inorganic compounds, and dispersants that might alter surface characteristics of the NPs, promote their aggregation, and affect their bioavailability and therefore toxicity. It was observed that the size of aggregated TiO$_2$ particles and their cell adsorption rate varied between the types of media used (Gogniat et al., 2006; Ji et al., 2010). It was hypothesized that the toxicity of NPs might be species specific and this fact also has to be taken to a consideration (Jiang et al., 2009).

Statistically significant damage to bacteria observed in the diluted sludge samples at concentration 100 mg/L of TiO$_2$ indicated the possibility of a harmful impact on bacteria found in sludge, or in the effluent, that the wastewater treatment plants release to the environment. It was reported that at relevant environmental concentrations, Suwannee River fulvic acid stabilized TiO$_2$ NPs and reduced their aggregation thus supporting the dispersion and transport of NPs (Neal, 2008; Domingos et al., 2009). In addition, the potential for toxicity will likely depend on the concentration of NPs introduced to the environment and their ability to interact with biota.

5.5 Summary

This study evaluated the impact of metal oxide NPs (TiO$_2$ and ZnO) on bacteria in anaerobic sludge. Samples incubated with TiO$_2$ NPs did not demonstrate reduction in
biogas production, while samples amended with 500 mg/L of ZnO NPs indicated reduction in biogas production in 24 h. Percent of the damaged cells increased to statistically significant level in diluted (10000X) samples amended with 100 mg/L of TiO$_2$, while undiluted samples or samples amended with ZnO NPs did not indicate an increase in percent of damaged cells after 1 h of incubation in the dark. Results suggest that ZnO NPs might exhibit a toxic effect toward methanogenic microorganisms in anaerobic sludge during 24 h contact time. The negative effect of TiO$_2$ NPs demonstrated in the diluted sludge samples might indicate that toxic effects might depend on the ratio of (NPs, mg)/(Total cell number) and composition of the organic and inorganic compounds in the sludge. The complex nature of sludge, that contains variable organic compounds including surfactants, might coat NPs or interact with NPs and thus alter their surface chemistry. Presence of inorganic compounds, such as heavy metals (Cu, Cr, etc.) might, in some cases, increase the toxic effect of investigated metal oxides, while some other compounds might diminish this toxic impact (Leighton and Forster, 1998; Martin-Gonzalez et al., 2006). Since TiO$_2$ NPs have tendency to agglomerate at higher ionic strength (Ji et al., 2010), TiO$_2$ NPs might create aggregates of NPs making them less bioavailable to microorganisms. Agglomerated NPs might also have lesser surface area available for interaction with microorganisms in comparison to dispersed NPs. Interaction of TiO$_2$ NPs with microorganisms in anaerobic sludge requires further study that would assess aforementioned factors that might influence experimental outcome.
Acknowledgements

This work was supported with the grant #0833213 CBET SGER: Nanoparticles in Personal Care Products and their Impact on Digester Energy Production and a fellowship from the NSF GK-12 program #08-DGE-0742395 “Graduate Fellows in High School STEM Education: An Environmental Science Learning Community at the Land-Lake Ecosystem Interface” through the Lake Erie Research Center.”

We are thankful to the administration and employees of the Toledo Bay View Wastewater Treatment Plant for cooperation and assistance in samples collection.

5.6 References


Chapter 6

6 Objective 3: Biofilm. Evaluation of the impact of titanium dioxide and zinc oxide nanoparticles on Escherichia coli biofilm in the absence of light

6.1 Abstract

In general, biofilm cells are more resistant to environmental stress and bactericidal agents than planktonic cells; hence control of biofilm growth is problematic. At the present time, the information on the toxic properties of NPs towards bacteria in a biofilm matrix in the absence of light is extremely limited. The goal of this research was to investigate the impact of TiO$_2$ and ZnO NPs at concentrations of 10 and 100 mg/L on Escherichia coli biofilm (grown for 24h prior the experiment) after 24 h of incubation with nanoparticles in the dark. Fluorescent microscopy was used to evaluate damaged and intact cells in biofilm that was stained with cell membrane integrity stain propidium iodide (PI) and then counterstained with dsDNA specific stain PicoGreen® (PG). Under experimental conditions TiO$_2$ NPs did not increase the number or percent of damaged cells in biofilm and damage to the cells was confined to the outer layer of biofilm. Data suggested that the toxicity mechanism of TiO$_2$ NPs is linked to the ratio of (NP, mg/Cell
No), and involves direct contact of TiO$_2$ NPs with the cells. Samples with 100 mg/L of ZnO NPs indicated a statistically significant increase in the number of damaged cells in the biofilm. Current data suggested that ZnO NPs might exhibit a negative impact towards bacteria in a biofilm matrix (by inhibiting the growth rate) even in the absence of light. Data suggested that the toxicity mechanism might be attributed to the interaction of dissolved Zn ions with the cell wall. However, attachment of the ZnO NPs to the cell walls cannot be excluded as additional mechanism of toxic effect. Cell membrane integrity stain PI and upright fluorescence microscopy might be a useful tool for assessment of NP impact on the biofilm, since it is more accessible than confocal microscopy.

KEY WORDS: nanoparticles, zinc oxide, titanium dioxide, bacteria, *E. coli*. 

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6.2 Introduction

6.2.1 The impact of nanoparticles on bacteria

The introduction of metal oxide NPs in constantly increasing quantities to the environment might be harmful to the indigenous environmental bacteria, including planktonic and biofilm cells, involved in nutrient cycling. In the environment, cells are known to colonize various surfaces (e.g., organic debris, rocks and sediments) in the creeks, rivers, and lakes. Bactericidal properties of metal oxide nanoparticles (NPs) have previously been proposed for control of undesired bacterial colonization, particularly in engineered processes and medical devices to prevent infectious diseases.

Intensive studies have been devoted to the bactericidal properties of metal oxide NPs linked to photocatalytic reactions in the presence of light (Adams et al., 2006; Rajagopal et al., 2006; Liu et al., 2007; Mitoraj et al., 2007; Tsuang et al., 2008; Alrousan et al., 2009). Additionally, there is a need to determine NPs effect on bacteria (planktonic cells and biofilm) in the absence of illumination since many environmental compartments are not exposed to light. At the present time, there is very scarce and conflicting information regarding impact of metal oxide NPs on planktonic cells and biofilm in the absence of light. Bactericidal effect was reported for both titanium dioxide (TiO₂) and zinc oxide (ZnO) NPs in the absence of light (Daoud et al., 2005; Adams et al., 2006; Zhang et al., 2007; Battin et al., 2009; Simon-Deckers et al., 2009; Mileyeva-Biebesheimer et al., 2010). It was also observed that cell attachment to ceramic ultrafiltration membranes coated with TiO₂ NPs was significantly reduced under both illuminated and dark conditions (Ciston et al., 2008). Other researchers reported that without illumination there was no reduction in bacterial numbers after the exposure of
bacteria to TiO$_2$ NPs (Tsuang et al., 2008; Jiang et al., 2009), while ZnO NPs exhibited bactericidal effect, even in the dark, towards *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas fluorescens* (Zhang et al., 2007; Jiang et al., 2009). Battin and coworkers (2009) reported that TiO$_2$ NPs at concentrations of 5.3 mg/L damaged planktonic cells and, to a lesser extent, a biofilm, under both light and dark conditions. They hypothesized that the complex biofilm matrix protected biofilm from the toxic impact of TiO$_2$ NPs.

### 6.2.2 Bacterial resistance to the toxic impact of NPs

It was observed that planktonic organisms are more susceptible to environmental stress and bactericidal agents in comparison to biofilms (Dunne, 2002; Teitzel and Parsek, 2003; Battin et al., 2009). In the environment, bacteria can attach to various surfaces and live as an organized biological system, referred as a biofilm, in a secreted glycocalyx matrix (Tortora et al., 2004). Biofilm is mainly composed of extracellular polymeric substances (EPS) produced by bacteria (Harrison et al., 2005; Zhang et al., 2008). The biofilm provides additional protection for bacteria from environmental stress, toxins, and bactericidal agents. Costerton (1999) hypothesized that the ionic bonds between the bactericidal agent and the glycocalyx matrix caused the depletion of bactericidal agents with the depth of the biofilm matrix. It was reported that bacteria in biofilm produce more EPS under the environmental stress or exposure to toxic compounds (Fang et al., 2002). The chemical and physical properties of EPS may vary among bacterial types and strains (Sutherland, 2001; Zhang and Fang, 2001).
There are conflicting reports regarding proposed mechanism related to NP toxicity. Some attribute the toxicity to the availability of dissolved metal ions. (Navarro et al., 2008; Aruoja et al., 2009; Jiang et al., 2009; Fang et al., 2010; Wu et al., 2010).

Other researchers observed no bactericidal effect in samples with concentrations of dissolved Zn$^{2+}$ ions in the form of ZnCl$_2$ that were significantly higher than concentrations of ZnO in nano (Feris et al., 2009) or micro (Sawai, 2003) scale. It was proposed that not only are dissolved metal ions causing toxic effect, but the attachment of NPs to the cells caused NP toxicity as well (Sawai, 2003; Jiang et al., 2009). NPs have also been observed as internal inclusions in bacterial cells (Morones et al., 2005; Brayner et al., 2006; Zhang et al., 2007; Huang et al., 2008; Simon-Deckers et al., 2009; Fang et al., 2010). Morones and coworkers (2005) pointed out that internalized NPs had a similar size to NPs attached to cell membrane, and that most likely, modifications of cell membrane by NPs increased membrane permeability. Electrostatic attraction between bacteria and NPs has also been implicated as a possible toxicity mechanism (Stoimenov et al., 2002; Morones et al., 2005; Neal, 2008). Zhang and coworkers (2007) hypothesized that the toxicity of ZnO NPs might be linked to a penetration mechanism of bacterial cell wall since smaller NPs demonstrated a higher bactericidal effect in comparison to larger ZnO NPs and that reactive oxygen species might be linked to observed toxic effect in the absence of light.

The decrease of the bacterial population in the absence of UV light indicates that the toxicity of NPs is more complex than it was presumed to be. It was hypothesized that there might exist a toxicity mechanism that has yet to be determined (Jiang et al., 2009; Simon-Deckers et al., 2009). Currently, information regarding the toxic effect of
nanoparticles in the absence of light is deficient. This information may be crucial in the assessment of the toxic effect of NPs on bacterial communities developing in the environment where biofilm has no naturally occurring exposure to light (trickling filters, pipelines, sediments, etc.). The purpose of this study was to determine whether or not TiO$_2$ and ZnO NPs damage *E. coli* cells in biofilm matrix in the absence of light.

### 6.3 Methods and Materials

**Materials**

**Nanoparticles.** Titanium dioxide (anatase phase) and zinc oxide NPs were purchased from Sun Innovations Inc., CA. The zeta potential of NPs was analyzed and reported in previous work (Mileyeva-Biebesheimer *et al.*, 2010). Nanoparticles in dry form were added to ultrapure water at the beginning of each experiment. Electrophoretic mobility measurements were conducted in 10 runs of 10 cycles each at 25 °C (ZetaPALS, Brookhaven Instrument Corp., Holtsville, NY) with 660 nm excitation line. The zeta potential of NPs was evaluated using Smoluchowski equation (Kim *et al.*, 2009). The pH values of the samples dispersed a priori the test in purified laboratory grade water were as follows: TiO$_2$ pH=7.13 and ZnO pH=7.4. Description of the nanoparticles provided in the Table 6.1.

<table>
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<th>Cat. No</th>
<th>Nominal size, nm</th>
<th>Purity, %</th>
<th>Zeta potential, mV</th>
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<td>5-10</td>
<td>&gt;98</td>
<td>-29±1.1</td>
</tr>
<tr>
<td>ZnO</td>
<td>SN 3301</td>
<td>20</td>
<td>&gt;99.8</td>
<td>-11±0.94</td>
</tr>
</tbody>
</table>

Table 6.1: Description of nanoparticles.
**Bacterial culture.** Gram-negative bacteria *Escherichia coli* K12 JM109 was grown overnight at 37°C in Luria-Bertani (LB) broth (Ref. No. 244620; BD Difco™; Becton, Dickinson and Company, Sparks, MD).

**Methods**

**Ionic strength of media**

Ionic strength of all media types utilized in these experiments was determined from the relationship of the ionic strength (\(I\)) and electrical conductivity (EC) utilizing formula proposed by Russell (1976).

\[
I \ (\text{mol/L}) = 1.6 \times 10^{-5} \times \text{EC} \ (\text{dS/m})
\]

Conductivity was measured with a portable conductivity meter accumet® AP75 (Cat. No. 13636 AP75A, Fisher Scientific, PA). Ultrapure water was amended with 10% of LB broth and analyzed using two different batches of LB broth. Data are reported as the average of two tests. Prior analysis, samples with TiO\(_2\) and ZnO NPs (10, 100, and 500 mg/L) were re-suspended in ultrapure water, vortexed for 10 seconds (Vortex-Genie-2, Model No. G-560; Scientific Industries, Inc., Bohemia, NY) for even distribution of NPs. Samples were incubated at room temperature for 24h in the dark without agitation. Ionic strength for all tested media and compounds are given in the Table 6.2.

Actual size and distribution of the dispersed NPs in the media, as a function of ionic strength, were determined with Dynamic Light Scattering (DLS) method using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd, United Kingdom). Samples were tested in clear disposable polystyrene cells (DTS 1060 C, Malvern Instruments Ltd, United Kingdom) at 25°C.
Cell membrane integrity staining

Propidium iodide (Cat. No. 11348639001; Roche Diagnostics GmbH, Mannheim, Germany), a red fluorescent integrity stain (\(\lambda=620\) nm maximum) indicating cells with damaged membranes that would emit red or orange color (Lopez-Amoros et al., 1997; Gogniat et al., 2006; Foley et al., 2008). Samples were counterstained with PicoGreen® (PG; P-7859; Molecular Probes, Inc., Eugene, OR) dsDNA specific stain (\(\lambda=522\) nm) for assessment cells with intact membranes that would emit green color.

Fluorescence microscopy

Samples were filtered on with 0.22 mm black polycarbonate filters (Cat. No. K02BP02500; Osmonics Inc., Minnetonka, MN) supported with glass fiber prefilters (Cat. No. APFA 02500; Millipore, Billerica, MA). Filters were placed with a drop of immersion oil on the top of glass microscope slides (Cat No. 12-544-1; Fisher Scientific) and covered with a cover slip (Cat. No. 12-548-C; Fisher Scientific).

Microplate reader

The impact of NPs on the outermost top surface of biofilm was analyzed via microplate reader utilizing integrity stain Propidium iodide (PI) and dsDNA stain PicoGreen® (PG). Microtiter plates with stained samples (staining procedure described in the section “Biofilm development verification”) were scanned with a microplate reader GEMINI XPS (Molecular Devices, Inc., CA) in dual fluorescence wavelength mode, excitation (485 and 536 nm) and emission (522 and 620 nm), corresponding to excitation
and emission of fluorescent stains PG and PI. Results, in relative fluorescent units (RFU), were analyzed in Excel, and average values were reported.

**Biofilm development**

Protocol was modified from protocols described elsewhere (Pratt and Kolter, 1998; Colon-Gonzalez et al., 2004; Kadouri and O'toole, 2005; Raulio et al., 2006; Burton et al., 2007; Li et al., 2007). Prior to the experiment, Gram-negative bacteria (*E. coli* JM109) was grown overnight at 37°C in Luria-Bertani (LB) broth (Ref. No. 244620; BD Difco™, Becton, Dickinson and Company, Sparks, MD). Biofilm was grown statically in the dark at 20°C for 24 h in 96-well polystyrene black U-shaped microtitre plate (Cat. No, 14-245-176, Thermo Scientific Nunc, NY). Media (LB broth) was added to each well and inoculated with 5% (V/V) of the *E. coli* stock. Each sample (180mL) was replicated in eight wells of the microtitre plate. To reduce evaporation of the media during incubation, the plate was covered with parafilm.

**Biofilm development verification**

After 24 h of incubation, one of the designated control samples (8 wells) was used to obtain a representative sample of the developing biofilm. Old media was removed from the wells and wells were washed with purified ultrapure water to remove detached cells. Wells were filled with purified water second time and samples were stained with cell integrity stain Propidium iodide (12 μL/mL) for 10 minutes then counterstained with PicoGreen® (9 μL/mL) for 5 minutes. During staining samples were protected from light with aluminum foil. After the staining procedure was completed, water and stains were
removed from the wells. Wells were washed again with purified water to remove any detached cells and stain residuals. To remove excessive water droplets from the wells, the microtitre plate was inverted and placed on the absorbent paper and gently tapped on the bottom of the plate. The plate was then scanned with a microplate reader GEMINI XPS (Molecular Devices, Inc., CA) in dual wavelength mode. Verification of biofilm development was applied only to one representative sample to minimize biofilm disturbance and reduce the cost of the experiments.

**Biofilm toxicity assay**

Nanoparticles 0, 10 and 100 mg/L were weighed and placed in 50 mL Falcon™ tubes. Ultrapure water (90% of required volume) was added to the nanoparticles at the beginning of each toxicity test. Tubes with NPs were shaken by hand 4 – 5 times and subsequently vortexed for 10 seconds for even distribution of nanoparticles. After biofilm formation was confirmed, old LB media was removed from the rest of biofilm samples. All samples with 24 h old biofilm were washed with water one time to remove detached cells. All samples were supplied with fresh media (10% LB V/V) and 0, 10 and 100 mg/L of NPs in purified water. Plate was covered with parafilm to reduce evaporation of the media, protected from light and incubated for 24 h in the dark at 20°C.

**Assessment of NP impact**

Toxicity of TiO₂ and ZnO NPs to *E. coli* biofilm was evaluated utilizing fluorescence microscopy and microplate reader. Three tests were conducted and
evaluated with both, fluorescent microscope and microplate reader. Several additional tests with each set of NPs were conducted with microplate reader only.

Naturally detached biofilm cells have more surface area exposed to the media and therefore to the NPs in comparison to the permanently attached biofilm cells, that have lesser surface area exposed to the media and therefore to NPs (Figure 6-1). However, naturally detached biofilm cells have lesser area exposed to the media than free-floating planktonic cells. Availability of the cell surface for NP attachment and interaction with the cell might be one of the factors in NP toxicity. In this study NP toxicity was evaluated and compared for biofilm and for naturally detached biofilm cells.

Figure 6-1: Permanently attached biofilm (BF) and naturally detached biofilm cells (DT) in the well of the microplate (not to a scale).
After incubation with NPs biofilm growth media with naturally detached biofilm cells and 0, 10, 100 mg/L of NPs was collected in correspondingly labeled centrifuge tubes for microscopic analysis. The content of two wells was combined into one centrifuge tube to create four pooled replicas for each sample. Three of four replicas were processed for microscopic examination of the samples (details in section below “Microscopic examination”). After old media and naturally detached biofilm cells were removed, all wells with attached biofilm samples were washed and stained as described in above section “Verification of biofilm development”. The plate was analyzed with plate reader GEMINI XPS (Molecular Devices, Inc., CA).

**Biofilm removal from the well surface**

Biofilm removal and dispersion protocol was modified from the protocols described elsewhere (Harrison *et al.*, 2006; Thein *et al.*, 2007; Mariscal *et al.*, 2009). Samples were incubated with Tween80® 0.1% (V/V) for 10 minutes. After incubation, the plate was sonicated for 2 minutes in Ultrasonic Cleanser (70 W, 42kHz ± 6%, Branson®, Model 1510 R-MTH; Branson Ultrasonic Corporation, Danbury, CT). To enhance biofilm removal, media was mixed with pipette 10 times in each well. Walls of the wells were gently scraped with pipette tips 4 – 6 times during mixing. Media from each well (200 μL) was collected in correspondingly labeled centrifuge tubes for microscopic examination. The content of two wells was combined in one centrifuge tube to create three pooled replicas for each sample for subsequent microscopic examination. After biofilm was removed from wells, plate was subsequently scanned with the plate reader to verify biofilm removal from the surface of the wells.
**Microscopic examination**

NP toxicity to naturally detached biofilm cells in growth media and biofilm itself was estimated by utilizing fluorescence microscopy. Naturally detached biofilm cells from growth media samples were diluted $10^{-3}$, and biofilm samples were diluted $10^{-5}$ times. Samples were stained with Propidium iodide (4 μL/mL) and counterstained with PicoGreen® (3 μL/mL) for 10 and 5 minutes respectively. Samples were protected from light with aluminum foil during staining procedure. All samples, detached biofilm cells and biofilm, were filtered using 0.22 μm black polycarbonate filters (Cat. No. K02BP02500; Osmonics Inc., Minnetonka, MN) supported with glass fiber prefilters (Cat. No. APFA 02500; Millipore, Billerica, MA). Filters were mounted on glass microscope slides (Cat No. 12-544-1; Fisher Scientific) on a drop of immersion oil and covered with a cover slip (Cat. No. 12-548-C; Fisher Scientific). Samples were analyzed with an upright fluorescent microscope with a reflected system (Olympus BX-51; Olympus optical Co. Ltd., Melville, NY).

**Statistics**

All experiments were repeated three or more times. Average data were reported for each experiment. Replicate samples were processed for all integrity assays. Data were analyzed in Excel utilizing the One-Tailed $t$-Test: Two-Sample Assuming Equal Variances. The significance level tested was 95% ($p < 0.05$). The null hypothesis stated that the control sample and sample with NPs have equal numbers of viable cells, total cells, and percent viability. The alternative hypothesis stated that the control sample had a
statistically higher number of viable cells, total cells, and percent viability than samples exposed to NPs.

6.4 Results and Discussion

Biofilm toxicity tests

Under microscopic examination, permanently attached biofilm incubated with 10 mg/L of TiO₂ NPs did not indicate a statistically significant \((p < 0.05)\) increase in numbers or percent of damaged cells in comparison with the control samples, with 0 mg/L of TiO₂ NPs. Naturally detached biofilm cells from corresponding biofilm growth media, indicated a statistically significant \((p < 0.05)\) increase in number of damaged cells, but not in the percent of damaged cells.

Total cell numbers did not demonstrate any statistically significant changes for either permanently attached biofilm or naturally detached biofilm samples for 10 or 100 mg/L of TiO₂ NPs. Microscopic examination of the permanently attached biofilm did not demonstrate a statistically significant increase in the number of damaged cells, while increase in percent of damaged cells was statistically significant. Naturally detached biofilm cells from corresponding biofilm growth media, indicated a statistically significant \((p < 0.05)\) increase in number of damaged cells, but not in the percent of damaged cells (Figures 6-2 and 6-3). Pooled mean values, used for all graphics, represent data for three replicas in three experiments. Error bars represent ± standard error of the pooled mean in all graphics.
Figure 6-2: Percent of damaged cells of *E. coli* biofilm as a function of TiO$_2$ NP concentration (0 – 100 mg/L) in ultrapure water amended with 10% of LB broth. Samples were incubated in the dark for 24h at 20°C. Error bars represent ± 1 standard error of the pooled mean, $n$=9 (replicas = 3, experiments = 3). BF – permanently attached biofilm samples, DT – naturally detached biofilm cells from biofilm growth media.
Figure 6-3: Damaged and total cells of *E. coli* as a function of TiO$_2$ NP concentration (0 – 100 mg/L) in ultrapure water amended with 10% of LB broth. Samples were incubated in the dark for 24h at 20°C. Error bars represent ± 1 standard error of the pooled mean, *n*=9. BF – permanently attached biofilm samples, DT – naturally detached biofilm cells from biofilm growth media.
In previous experiments with planktonic cells the toxic effect of TiO$_2$ NPs was detected at concentration 100 mg/L for samples contained $10^5 - 10^6$ cells per milliliter (Mileyeva-Biebesheimer et al., 2010). In this study, cell numbers of permanently attached biofilm removed from the well surface were in the range of $10^{10} - 10^{12}$ per milliliter. Therefore, the ratio of (NPs, mg/Cell number) was significantly lower (2 orders of magnitude) than the toxicity level established in previous tests with planktonic cells. These data suggest that the toxicity of TiO$_2$ NPs may depend on the ratio of (NP, mg/Cell No) and the toxicity mechanism based on direct contact of TiO$_2$ NPs with the cells. These findings are consistent with previous studies. For example, Costerton (1999) stated that significantly higher doses of bactericidal agents (in this case $10^4$ times higher) and increased contact time with bactericidal agent are required to achieve bactericidal effect on cells in biofilm communities in comparison with planktonic cells.

The media used for biofilm toxicity tests might impact the outcome of experiments by altering the surface characteristics of NPs, thus promoting their aggregation, and reducing their bioavailability and toxicity. It was observed that the size of aggregated TiO$_2$ particles and cell adsorption rate varied between the types of media used (Gogniat et al., 2006). The addition of 10% of LB media provided nutrients for bacteria during 24 h of incubation. The presence of LB might change NP surface chemistry, affecting the bioavailability of NPs, and increasing the rate of NP aggregation and the size of clumped NPs due to the increased ionic strength of the media.

Ionic strength of ultrapure water was equal $2.49 \times 10^{-8}$ mol/L. Purified water amended with TiO$_2$ NPs (10 and 100 mg/L) demonstrated lowest ionic strength among amended samples $3.52 \times 10^{-8}$ and $9.34 \times 10^{-8}$ mol/L respectively. Purified water amended
with ZnO NPs (10 and 100 mg/L) had ionic strength $1.82 \times 10^{-7}$ and $1.24 \times 10^{-6}$ mol/L, which was approximately one magnitude higher, in comparison to ionic strength of TiO$_2$ NP. Purified water amended with 10% of LB broth indicated ionic strength $9.18 \times 10^{-5}$ mol/L, which was one to three magnitudes higher than ionic strength of NPs in purified water (Table 6.2).

### Table 6.2: Ionic strength of the media.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ionic strength, mol/L</th>
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<tbody>
<tr>
<td></td>
<td>Nanoparticles concentration, mg/L</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Water, purified</td>
<td>$2.49 \times 10^{-8}$</td>
</tr>
<tr>
<td>Water, purified 90% and LB broth 10%</td>
<td>$9.18 \times 10^{-5}$</td>
</tr>
<tr>
<td>TiO$_2$</td>
<td>$2.49 \times 10^{-8}$</td>
</tr>
<tr>
<td>ZnO</td>
<td>$2.49 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

Agglomeration rate of uncoated TiO$_2$ nanoparticles was directly linked to ionic strength of the media, where higher agglomeration rates corresponded to higher ionic strength (Domingos et al., 2009). All media used in current experiments contained 10% of LB broth, which increased ionic strength of the media and therefore might contribute to NP agglomeration. Ji and coworkers (2010) analyzed agglomeration of TiO$_2$ P25 in deionized water and various cell culture media. For suspended in water, TiO$_2$ NPs at concentrations 2 – 100 $\mu$g/mL, they did not notice significant changes in the size of TiO$_2$ NPs (209 nm), while in a cell culture media, the size of aggregated TiO$_2$ NPs reached 700 – 1052 nm. Ji and coworkers (2010) hypothesize that the high ionic strength of cell culture media caused NP agglomeration. However, in the current study using the DLS
test there was no difference detected in the size distribution of the aggregated NPs between samples incubated for 1h in purified water and samples incubated in 10% of LB broth. The distribution of TiO₂ and ZnO NPs, as analyzed with DLS test, was determined as polydispersed for all samples at 0 h and after 1 h of incubation.

Battin and coworkers (2009) reported that TiO₂ NPs at concentrations of 5.3 mg/L damaged planktonic cells and, to a lesser extent, a biofilm, under both light and dark conditions. They hypothesized that the complex biofilm matrix protected biofilm from the toxic impact of TiO₂ NPs. In this current study, the microplate was incubated in still condition and NPs might have been agglomerating and precipitating, therefore their bioavailability might be reduced during the test. Since the toxic impact of TiO₂ NP was linked to their attachment to the cells (Zhang et al., 2007; Huang et al., 2008; Simon-Deckers et al., 2009), precipitated and agglomerated NPs would not be available for floating in the media naturally detached biofilm cells or for permanently attached to the well surface biofilm above the precipitation level. Area of the cell surface available for NPs attachment varies for planktonic, naturally detached biofilm cells and permanently attached biofilm cells. Therefore, a reduced area for attachment of NPs might explain reduced toxicity levels. While planktonic cell surfaces are readily available for NP attachment, biofilm cells have only part of the cell exposed to the surrounding media and NPs; therefore NPs attachment sites are limited. The results suggest that under experimental conditions at a concentration 100 mg/L TiO₂ NPs demonstrated a statistically significant damage to the permanently attached E. coli biofilm, but not to the naturally detached biofilm cells, since only the number of damaged naturally detached cells increased, but not the percentage of damaged naturally detached cells. Microplate
reader data suggest that the damage to the permanently attached biofilm caused by TiO$_2$ NPs might be confined to the outer layers of the biofilm, where NPs were able to come into contact with cell surfaces.

Microscopic examination of permanently attached biofilm samples and naturally detached biofilm cells did not indicate a statistically significant increase in the number or in the percent of damaged cells incubated with 10 mg/L of ZnO NPs in comparison to the control samples with 0 mg/L of ZnO NPs. Biofilm and naturally detached biofilm cells incubated with 100 mg/L of ZnO NPs demonstrated a statistically significant ($p<0.05$) increase in the percent of damaged cells in comparison to the control samples with 0 mg/L ZnO NPs (Figure 6-4), while damaged cells numbers did not increase in amended samples (Figure 6-5). Total cell numbers were lower in permanently attached biofilm and in naturally detached cell samples exposed to 100 mg/L of ZnO NPs in comparison to corresponding total cell numbers in control samples with 0 mg/L of NPs (Figures 6-4 and 6-5).
Figure 6-4: Percent of damaged cells of *E. coli* biofilm as a function of ZnO NP concentration (0 – 100 mg/L) in ultrapure water amended with 10% of LB broth. Samples were incubated in the dark for 24h at 20°C. Error bars represent ± 1 standard error of the pooled mean, *n*=9 (replicas = 3, experiments = 3). BF – permanently attached biofilm samples, DT – naturally detached biofilm cells from biofilm growth media.
Figure 6-5: Damaged and total cells of *E. coli* as a function of ZnO NP concentration (0 – 100 mg/L) in ultrapure water amended with 10% of LB broth. Samples were incubated in the dark for 24h at 20°C. Error bars represent ± 1 standard error of the pooled mean, n=9. BF – permanently attached biofilm samples, DT – naturally detached biofilm cells from biofilm growth media.
Figure 6-6: Total cells of *E. coli* as a function of ZnO NP concentration (0 – 100 mg/L) in ultrapure water amended with 10% of LB broth. Samples were incubated in the dark for 24h at 20°C. Error bars represent ± 1 standard error of the pooled mean, \( n=9 \). BF – permanently attached biofilm samples, DT – naturally detached biofilm cells from biofilm growth media.

The microplate reader data, represented as a ratio of relative fluorescent units (RFU) of (Propidium iodide) / (PicoGreen®), did not indicate a statistically significant increase of damaged cells in a biofilm outer surface at concentration 10 mg/L TiO₂ NPs. However, the microplate reader indicated a statistically significant increase of damaged cells in a biofilm outer surface for samples incubated with 100 mg/L of TiO₂ NPs (Figure 6-7). The microplate reader data did not indicate a statically significant increase of damaged cells for either 10 or 100 mg/L of ZnO (Figure 6-7).
Figure 6-7: Microplate reader data are represented as a ratio (PI RFU)/ (PG RFU). Intact and damaged cells of *E. coli* biofilm as a function of TiO$_2$ and ZnO NPs concentration (0 – 100 mg/L) in ultrapure water amended with 10% of LB broth. Samples were incubated in the dark for 24h at 20°C. Pooled averaged data for 3 experiments. Error bars represent ± 1 standard error of the pooled mean, $n = 24$. RFU – relative fluorescent units, PI – Propidium iodide, PG – PicoGreen®.

Disagreement between microscopic assessment and microplate reader evaluation can be linked to the mode of sample analyses. The microplate reader detects a signal from the external surface layer of the permanently attached biofilm. In addition, some of the biofilm surface may be masked due to aggregated and precipitated NPs, which was observed. Removal and dispersion of permanently attached biofilm creates a representative sample of the biofilm cells from various depths of the biofilm structure.
Thus, a fluorescent microscope was used to examine not only the outer biofilm layer, but also more representative biofilm sample that includes cells from various layers of the permanently attached biofilm structure. The microplate reader assessment might be useful for rapid screening of the impact of low concentrations of NPs on the outer biofilm layer where cells are readily exposed to the NPs. However, this method does not reflect cell condition in the deeper layers of the biofilm, so observed toxic effect of the NPs would likely be reduced due to a number of various factors including physical proximity to NPs.

At higher concentrations NPs (500 mg/L) not only precipitated during incubation, but formed a film at the bottom part of the wells that masked biofilm cells. This film was dense and detached in small flakes from the wells. These samples were not processed further. Thus, the microplate reader data were considered as a supplementary assessment reflecting the toxic impact on the outermost biofilm structure at relatively low concentrations of NPs.

The Ionic strength of media was previously linked to aggregation of TiO$_2$ NPs (Domingos et al., 2009; Ji et al., 2010). It was reported that TiO$_2$ NPs at concentrations 2-100 mg/L demonstrated insignificant agglomeration in the water, while in the cell culture media, which has higher ionic strength, the agglomeration rate and size increased with concentration (Ji et al., 2010). Since precipitation of the NPs at the bottom of the wells or test tubes was noticeable after 1 h of incubation, further aggregation and precipitation of increasing in size aggregates during 24h of incubation would cause the accumulation of aggregated NPs at the bottom of the well. As a result, precipitated and
enlarged NPs were less available for interactions with floating naturally detached biofilm cells.

Data of the current experiments indicate that ZnO NPs were toxic to the biofilm of *E. coli* JM109 after an exposure time of 24 h in the absence of light. These results suggest that in the absence of light, the toxicity mechanism of ZnO NP towards *E. coli* cells depends on the contact time of the NPs and bacterial cells. Microplate reader data suggest that damage to biofilm was not limited to the outer surface of biofilm. Damage to the naturally detached biofilm cells exposed to ZnO nanoparticles suggests that presence of the dissolved Zn ions might be involved in toxicity mechanism along with direct contact between cell surface and NPs.

Zeta potential values, negative for both nanoparticles, suggest that under experimental conditions electrostatic forces did not govern the interaction of NPs and negatively charged bacterial surfaces. Decline in total cell numbers was observed only in samples exposed to 100 mg/L of ZnO NPs, but not in samples exposed to 100 mg/L of TiO₂ NPs. Previously conducted experiments with planktonic *E. coli* cells did not demonstrate a statistically significant increase of damaged cells numbers or a percentage of damaged cells after 1 h of incubation in the dark with 100 mg/L of ZnO NPs (Mileyeva-Biebesheimer et al., 2010). Since ZnO NPs inhibit the growth rate of the cells, changes in the numbers of population might not be obvious after 1h of incubation with NPs in experiments evaluating the acute toxic impact of NPs on bacteria. During that time microorganisms could still be in the adaptation stage to the changed environment – lag phase, therefore changes in total cell numbers would not be evident at that time.
Control experiments (3 replicas) with planktonic cells incubated for 24 h in the dark at 20°C with ZnO NPs (100 mg/L) and ZnOSO₄•7H₂O as 10 and 100 mg/L of ZnO NPs demonstrated trend similar to experiments with permanently attached biofilm and naturally detached biofilm cells (data are not shown). The growth rate of *E. coli* was also suppressed in the samples that were exposed to ZnOSO₄•7H₂O and did not contain NPs. Current data suggest that in the absence of light ZnO NPs do not exhibit acute toxicity to *E. coli* cells at tested ratio of NPs (mg/L) per (cell No/mL), but rather impact cells after a long period of contact. Internalization of ZnO NPs reported elsewhere (Zhang *et al.*, 2007; Huang *et al.*, 2008) and consequent damage to the cells might explain delayed NP impact on the cells. Direct contact of NPs with cell wall cannot be excluded as one of steps of NP toxicity mechanism.

Bioavailability of NPs might be linked to NP delivery and dispersion mechanism. It was demonstrated by Wu and coworkers (2010) that NPs administered as an aerosol directly on bacterial surfaces contacted directly with bacterial cell wall and resulted in a higher rate of the bactericidal effect, in comparison to bactericidal effect of NPs introduced to bacteria in an aqueous media. Researchers concluded that dissolved ions are a key factor to NPs toxicity in aqueous media, and pointed out that NPs administered in form of aerosol might have different toxicity mechanism. Reported data imply that in aqueous media aggregated NPs exhibit reduced bactericidal effect (Wu *et al.*, 2010). However, if the toxicity mechanism governed by dissolved ions, aggregation of the soluble NPs would dissolve over an extended period of time and released ions would be available to the cells. Aggregated NPs are less likely to attach to bacterial surface, sense the numbers of dispersed particles get reduced; therefore the toxic effect of NPs might be
reduced. Wu and coworkers (2010) reported that in aqueous environment ZnO NPs affected *Mycobacterium smegmatis* that produced limited quantities of EPS, while *Escherichia coli* BL21 that produced more EPS was not affected. However, ZnO NPs delivered in an aerosol form affected *E. coli* BL21 (Wu et al., 2010). Reported results correlate to findings of current research indicating toxic effect of ZnO NPs on biofilm formed by *E. coli* JM109.

Bacteria, such as *E. coli* JM109, that do not produce extracellular polymers, use for adhesion lipopolysaccharides (LPS) that are components of an outer membrane (Li-Chong and Logan, 2005). It was reported that *E. coli* JM109 develops biofilm with flatter structures where cells are packed more tightly in comparison to other *E. coli* strains (Van Houdt and Michiels, 2005; Wood et al., 2006). The *E. coli* JM109 used in this experiments has a full-length lipopolysaccharides (LPS) structure: keto-deoxy-octulonate (KDO), the inner and outer core polysaccharide, and 1 – 50 repetitive units of O-antigen (Velegol and Logan, 2002; Burks et al., 2003; Li and Logan, 2004). Repeating polysaccharide units of O-antigen protruding up to 30 nm away from the cell surface might promote the adhesion of bacteria to surfaces (Jucker et al., 1997; Camesano and Abu-Lail, 2002). It was hypothesized that the O-antigen in the LPS structure exhibit high levels of adsorption to TiO$_2$ surfaces (Jucker et al., 1997).

### 6.5 Summary

Fluorescence microscopy was utilized to assess the toxic impact of TiO$_2$ and ZnO NPs on single culture *E. coli* JM109 biofilm grown in microtiter plates. This genotype
was previously reported as one that does not form EPS. The biofilm was stained with cell membrane integrity stain propidium iodide to distinguish cells with damaged membranes and counterstained with dsDNA specific stain PicoGreen® to evaluate intact cells. Data suggested that impact of TiO₂ NPs was confined to the outermost layer of biofilm. Toxicity mechanism of TiO₂ NPs was likely based on the direct contact of NPs and the cell wall and depended on the dispersion and the ratio of (NPs, mg) per number of cells. Data suggested that at concentration 100 mg/L and ZnO NPs were toxic to E. coli JM109 biofilm after 24 h exposure time even in the absence of the light. While results suggest that the toxicity of ZnO NPs might be attributed to dissolved Zn ions, the combined effect from the dissolved ions and direct contact of the cell and ZnO NPs also has to be taken to the consideration.

**Acknowledgements**

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6.6 **References**


Chapter 7

7 Contributions of the Study

It was reported that bacteria with hydrophilic cell surfaces, such as *E. coli*, tend to attach to hydrophobic surfaces at a higher rate than to hydrophilic surfaces (Reid *et al.*, 1993; Li and Logan, 2004; Kochkodan *et al.*, 2008). Kochkodan and coworkers (2008) concluded that hydrophobic interactions govern the attachment of microorganisms to surfaces. However, it was also reported that the properties of materials that demonstrate hydrophilicity or hydrophobicity did not affect the cell attachment rate, but rather it was the pH levels that determined the attachment process (Mafu *et al.*, 2011). This study demonstrated that *E. coli* cells were attached to TiO$_2$ NPs (7.13 pH), while no cell attachment was detected with ZnO NPs (7.4 pH) (Chapter 4 of this document).

Interactions between bacteria and metal oxides were attributed to interactions of bacterial O-antigen and dextrans with hydroxyl group of water molecules adsorbed by the metal oxide surface (Jucker *et al.*, 1997). Jucker and coworkers (1997) also reported that the attachment of *E. coli* to the surface of TiO$_2$ was irreversible.

In another study, it was proposed that the electrostatic interactions between the cell wall and the surface of NPs are more important than hydrophobic interactions for attachment and bactericidal effect (Feris *et al.*, 2009). Since the surface charge of
bacteria is negative and the zeta potentials of TiO₂ and ZnO NPs also were negative, (–29 and –11 mV respectively) interactions between NPs and bacteria might experience an electrostatic repulsion force, instead of the electrostatic attraction that might be expected for NPs with positive zeta potential. Nevertheless, other factors such as gravity or the Brownian motion of NPs and the motility of bacteria might also facilitate interactions between NPs and microorganisms.

The results of this study suggest that the toxicity mechanism of TiO₂ NPs might be attributed to the direct contact of TiO₂ NPs with the cell wall of microorganisms (outer cell membrane in case of Gram-negative bacteria). Data suggest that the toxicity of TiO₂ NPs depends on the ratio (NP, mg/Cell No), since, at the same concentration of NPs, the toxic impact was detected only in samples with cell numbers in range of 10⁶, while the toxic impact was not noticeable in samples with higher cell content (10⁸ – 10¹⁰). Toxicity might also depend on the size of aggregated NPs, their precipitation rate and interactions with organic and inorganic compounds in media that might reduce the bioavailability of NPs. It was reported that aggregated TiO₂ NPs demonstrated a toxic effect towards microorganisms (Battin et al., 2009). However, the increase in size of aggregated NPs would increase the precipitation rate of these aggregated NPs and, therefore makes precipitated NPs less available for floating microorganisms.

The data suggest that the toxicity mechanism of ZnO NPs targets cell growth and requires extended exposure of microorganisms to ZnO NPs. Toxicity might be attributed to dissolved Zn ions. However, considering the high numbers of cells (10⁹ – 10¹⁰) in the experiments with biofilm, and considering the quite low ionic strength of the media (Table 6.2), involvement of some other toxicity mechanisms cannot be disregarded. Wu
and coworkers (2010) demonstrated that ZnO NPs delivered as an aerosol on bacterial surfaces exhibited a higher toxic impact towards the bacteria in comparison to ZnO NPs in an aqueous media. Therefore, direct contact between bacteria and NPs might be a key factor for the observed NP toxicity. Since in the absence of UV radiation TiO₂ and ZnO NPs are considered to be very stable, the toxicity of the studied NPs was not linked to the differences in temperatures applied for the incubation of biofilm or anaerobic sludge samples. The stability of NPs in experimental conditions might also be demonstrated by the observation that at the end of incubation the pH levels of the sludge samples amended with NPs were only slightly reduced in comparison to the pH value of the original sludge sample (pH=7.68) measured prior to the test (Tables 5.3 and 7.1).

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Concentration of NPs, mg/L</th>
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>TiO₂</td>
<td>7.54 ± 0.04</td>
</tr>
<tr>
<td>ZnO</td>
<td>7.54 ± 0.04</td>
</tr>
</tbody>
</table>

This study demonstrated that ZnO NPs might exhibit toxic impact on methanogens at concentration 50 mg/L. The extent of the impact at this concentration varied among batches from an insignificant to a significant level (data are not shown). Introduction of 500 mg/L of ZnO NPs significantly suppressed biogas production in all tested batches and replicas of sludge samples. A decrease in biogas production was
registered after 24h of incubation. Therefore, increased volumes of ZnO NPs discarded in wastewater and accumulation of NPs in the sludge might potentially disrupt biological processes in anaerobic digester.

The results of this research demonstrated that the toxic effect of TiO₂ and ZnO NPs may vary. The observed toxic effect of TiO₂ NPs towards microorganisms might be attributed to an acute toxic impact, while ZnO NPs demonstrated a chronic toxic impact. The extent of the impact also varies among microorganisms. Even an acute toxic impact might cause immediate damage to microorganisms, whereas chronic exposure to accumulating volumes and concentrations of NPs might cause greater damage to the environmental and engineered biological processes such as biogeochemical cycles, decomposition of organic and xenobiotic compounds. Since TiO₂ NPs have demonstrated an acute toxic impact towards the planktonic cells of *E. coli* in the absence of light, the introduction of these NPs to the environment at increasing concentrations might potentially impact free floating microorganisms in the surface waters. It was demonstrated that in surface waters the presence of NOM stabilized TiO₂ and ZnO NPs and reduced their aggregation (Keller *et al.*, 2010). Therefore, there is a possibility that NPs introduced to surface water might have a negative impact on free-floating microorganisms. The data suggested that the chronic exposure of microorganisms to ZnO NPs is detrimental to microorganisms at concentrations exceeding the micronutrient level. Since in the anaerobic digester, solids are retained for 25 days, exposure of anaerobic microorganisms would be chronic and with increasing concentrations of deposited and accumulated ZnO NPs there is a possibility that the increasing
concentrations of ZnO NPs might reach a toxic level that would be harmful to methanogens, and therefore the treatment process of the sludge might be disrupted.
Chapter 8

8 Summary

This study evaluated the impact of metal oxide NPs (TiO$_2$ and ZnO) on planktonic Gram-negative bacteria *E. coli* and Gram-positive bacteria *E. faecium*, microorganisms in anaerobic digester sludge, and *E. coli* biofilm. Assessment of the impact utilized fluorescent microscopy and was based on direct cell count method. Fluorescence integrity stain propidium iodide was applied to distinguish microorganisms with damaged cell walls/membranes from intact cells counterstained with dsDNA specific stain PicoGreen®. Results of these experiments demonstrated a statistically significant increase in the percent of damaged cells at a concentration 100 mg/L of TiO$_2$ NPs in pure culture samples of *E. coli* and in diluted sludge samples. However, cell membrane damage was not statistically significant at this concentration for planktonic samples with *E. faecium*. Zinc oxide NPs did not demonstrate an acute toxic effect on pure cultures of *E. coli* or *E. faecium*.

The impact of NPs on microorganisms in anaerobic sludge was also monitored via biogas production. Biogas production was not reduced to statistically significant level in the sludge samples incubated with TiO$_2$ NPs. Sludge samples amended with 500 mg/L of ZnO NPs (15.43 µg NPs /mg total solids) significantly reduced biogas production after 24 h and biogas production remain significantly lower during 144 h of incubation.
Microscopic examination of biofilm samples amended with 100 mg/L of ZnO NPs indicated a statistically significant increase in the percentage of damaged cells. Samples amended with TiO$_2$ NPs did not reveal a statistically significant increase in the number of damaged cells under microscopic examination. However, under examination with a microplate reader, biofilm samples amended with TiO$_2$ NPs demonstrated a statistically significant increase of the damaged cells in the outermost layer of biofilm exposed to the media and NPs. Current data suggest that studied NPs might affect microorganisms in the absence of light. However, toxic affect and toxicity levels would vary among microorganisms, and would depend on the ratio of (NPs, mg) per (Cell No), exposure time, presence of other organic and inorganic compounds in the media. Data obtained in this research are in agreement with proposed elsewhere hypothesis (Aruoja et al., 2009; Jiang et al., 2009) that the toxic impact of NP on microorganisms varies among investigated NPs, microorganisms and their species.
Chapter 9

9 Future considerations

Modification of the experiment involving incorporation of the toxic level ratio (NPs mg/L) per (Cell number) might provide more information on biofilm susceptibility or resistance to TiO$_2$ NPs. Utilization of additional techniques for future biofilm experiments such as growing biofilm in a flowcell that will provide sufficient nutrient levels for cells and reduce the starvation impact on biofilm and enables investigation of the long term impact. Flowcell will enable to utilize confocal microscopy that will analyze biofilm structure layer by layer and provide information on the distribution of NPs within a three dimensional biofilm structure. Flowcell utilization will allow the adjustment of the concentration of NPs by enabling media circulation and reduction of NP precipitation rate. These modifications might provide more information on the toxicity of TiO$_2$ NPs to *E. coli* biofilm. Since LPS and EPS of *E. coli* vary among strains, further experiments should include *E. coli* strains with distinctively different LPS and EPS characteristics.

Incorporation of the ratio (NPs, mg/Cell number) would be also useful for a detailed study of the NP toxicity levels in complex sludge media. Controlled amendment of the sludge samples with selected inorganic (for example Cu) and organic compounds.
(most commonly found in sludge surfactants) might provide valuable information on possible synergistic effect of the NPs with some other compounds and might provide more information what compounds might increase or diminish toxic impact. Since TiO$_2$ and ZnO NPs are commonly found in personal care products and household items, it might be beneficial to investigate effect, on microorganisms in anaerobic sludge, of both of these NPs combined in various proportions.
Chapter 10

10 Implications of the Study

This study provides a relatively quick technique for the evaluation of the environmental impact of metal oxide NPs on microorganisms in pure culture, anaerobic sludge, and in biofilm. Evaluation of the damaged cells in biofilm might be conducted with fluorescent microscopy that is less expensive and more accessible for researchers in comparison to a confocal microscopy that is often utilized for evaluation of biofilm samples. Cell integrity stain propidium iodide provides a rapid assessment of the impact of environmental pollutants, such as NPs, on cell membranes. Application of the fluorescent integrity stain eliminates culturability issues common for environmental assessments. Reduction of the experimental time, via elimination of the plating steps, provides information comparatively close to the acute contact point of the cell with NPs.

Results of this study imply that the planktonic culture of Gram-positive bacteria *E. faecium* was not affected after 1 h of incubation with either TiO$_2$ or ZnO NPs. Statistically significant damage to the cell membranes of planktonic *E. coli* culture and microorganisms in diluted sludge samples was determined after 1 h of exposure to TiO$_2$ NPs. This finding indicates that TiO$_2$ NPs might have negative impact on some
microorganisms in environmental compartments even in the absence of illumination. Results of this research suggest that the toxicity of TiO$_2$ NPs is strongly dependent on the ratio of NP concentrations to the cell numbers and might be linked to direct contact of NPs with the cell walls of microorganisms. Since LPS structure varies among species and genotypes, interactions of TiO$_2$ NPs with the cell wall might depend on presence or absence of some structural components of LPS.

Numerous factors, such as variations in the ionic strength of surface water sources and wastewater, or the occurrence of various natural and xenobiotic organic and inorganic compounds, might alter the surface chemistry and toxicity of TiO$_2$ and ZnO NPs. This study revealed a reduction in the production of biogas by anaerobic sludge microorganisms indicating the toxic impact of ZnO NPs on methanogenic microorganisms at concentration 500 mg/L (15.43 $\mu$g/(mg total suspended solids). A decrease in the total cell numbers, demonstrated in biofilm samples amended with 100 mg/L of ZnO NPs, indicates the impact of ZnO NPs on bacterial growth rate. The synergistic toxic effect of some metal oxides might be expected on microorganisms in wastewater and particularly in sludge due to solids accumulation that would result in the accumulation of NPs. All these factors have to be considered in future design of the models for the evaluation and prediction of the possible environmental impact of these NPs.
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