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Effect of Copper Oxide (CuO) Nanoparticles on the Denitrifying Consortia Isolated from an Anaerobic Digester of a Sewage Treatment Plant

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ABSTRACT

Nanoparticles (NPs) are useful molecules with large surface area to volume ratios that confer unique properties and a wide variety of potential uses in medicine and biotechnology. The use of NPs has increased significantly in recent years resulting in accumulation in biological systems. While larger molecules made of the same constituents may be understood to have minimal effects on biological organisms, nanoparticles have been shown to have disruptive and even antimicrobial effects. Nanoparticle accumulation is a potential problem for many environmental systems. Sewage treatment plant functioning relies heavily on bacteria to remove excess carbon and nitrogen from the sewage before discharged to the environment. Increasing concentrations of nanoparticles in these plants can decrease its effectiveness if nanoparticles reach toxic levels for ammonia oxidizing or denitrifying bacteria. The purpose of this study was to determine the effect of copper oxide (CuO) nanoparticles on denitrification in wastewater treatment plant. A bacterial consortia developed from an anaerobic digester of a sewage treatment plant was exposed to various concentrations of CuO nanoparticles. Bacterial growth and COD removal were not affected at low concentration of NPs such as 5, 10, and 25 mg/L. At higher doses of NPs exposure including 100 and 250 mg/L, bacterial growth was severely inhibited along with denitrification process, and carbon removal. The consortia was stressed at higher doses above 25 mg/L of NP leading to excess production of extracellular polymeric substances (EPS).

Keywords: Nanoparticle; CuO; denitrification; ammonia oxidation; EPS

1. INTRODUCTION

Nanoparticles (NPs) are molecules ranging in size from 1-100 nanometers (nm). Their small size results in a large ratio of surface area to volume, contributing to these particles highly reactive and unique properties. These qualities are exploited by scientists, increasing the demand for NPs in the field of medicine and biotechnology (Sakla et al., 2016). The exceptionally small size of the particles allows them to interact with cells and biological processes. Nanoparticles can be conjugated to proteins for use in cancer cell detection and targeting, imaging, and drug delivery (Sinha and Khare, 2013). Other popular uses for nanoparticles are textile fabrication and cosmetics (Sakla et al., 2016). This increasingly widespread use of nanoparticles warrants the need for insight into where they end up and what impacts accumulation of NPs in the environment may have on biological systems.

While their bulk counterparts such as heavy metals may be well studied and known to have limited effects on biological organisms and systems, nanoparticle forms have adapted completely independent nature (Sinha et al.,

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2011). For this reason, it is risky to assume that these particles will have similar environmental effects without independent testing. Silver and copper nanoparticles have recently been highly attractive molecules to scientists due to their prospective applications. These have been shown to have antibacterial, antifungal, and antiviral activities making them popular to incorporate into apparel, footwear, paints, wound dressings, appliances, plastics, and cosmetics (Morsy, 2015; Sinha et al., 2011). As the demand for NPs has now far exceeded amounts that which can be currently naturally isolated, scientists have resorted to producing engineered nanoparticles (ENPs) through evaporation-condensation and laser ablation (Iravani et al., 2014). Production of NP is expected to increase to 58,000 tons by 2020 (Sinha and Khare, 2015). Accumulation of nanoparticles has been observed in plants and bacterial species where community and industrial wastes pass through. A prime model of a reservoir for community wastes is local sewage treatment plant.

Denitrification is the process by which nitrate is reduced to molecular nitrogen and other gaseous nitrous oxide intermediates. Denitrification is an anaerobic bacterial process carried out by bacteria called denitrifiers. The bacteria use a reduction pathway that transforms nitrate to nitrite to nitric oxide to nitrous oxide to dinitrogen. Denitrification is often carried out by bacteria in the genus *Pseudomonas,* such as *P. denitrificans* (Carlson and Ingraham, 1983). In sewage treatment plants, nitrification, denitrification, and assimilation play a key role in removing the excess ammonia from sewage. If this ammonia is left untreated, it can have detrimental effects on the environment and lead to eutrophication. The primary solids from primary settling tank and the secondary solids from the secondary settling tank in sewage treatment plant travels separately to the anaerobic digester, which allows the sludge to further settle out and also provides an opportunity for the denitrifiers to turn the nitrate in the sludge into nitrogen gas and any residual carbon to methane and CO_2.

The nanoparticles are of great concern due to their potential to disrupt the normal processes of carbon and nitrogen cycle and the performance of sewage treatment plant. It has been shown that NPs can accumulate in bacterial cell walls and disrupt the membrane functioning of the cells. Another potential way that these nanoparticles affect bacteria occurs after NPs are internally sequestered within the cells. Once inside the cell, studies have shown that the nanoparticles degrade to produce reactive oxygen species (ROS). ROS accumulation is extremely toxic to cells. For these reasons, it was expected that increasing the concentration levels of nanoparticles in the sewage would result in a decrease in efficiency of the ammonia oxidation and denitrification as well as carbon removal in the sewage treatment systems. Determining the effects of nanoparticles on the efficacy and viability of bacteria in the sewage treatment plant is the first step in identifying the potential future hazards these nanoparticles present in the treatment process. The NP chosen for this study was CuO because of its wider use in catalytic process, textiles, marine fouling, and in chemical sensors (Adam et al., 2015; Zhao et al., 2013). CuO NPs are released into industrial and municipal wastewater (Li et al., 2012).

Wastewater treatment plants (WWTP) receive wastewater from many sources including households, hospitals, pharmacies, veterinary clinics, and local industries. Sewage contains wide variety of chemicals including residual concentrations of antibiotics, heavy metals, and nanoparticles. Sewage also contains pathogenic and non-pathogenic microbes of all kinds including bacteria, viruses, and fungi. WWTPs are ideal habitat for the development of antibiotic and heavy metal resistance. If the heavy metal and nanoparticles

concentrations are increased in WWTP because of common use of many NP containing cosmetics and other household chemicals then the NP will have detrimental effect on bacteria that remove carbon and nitrogen in the sewage. Very few reports have been found in the literature on the effect of CuO NP in the sewage treatment process (Galabert et al., 2016; Ganzoury and Allam, 2015; Kang et al., 2013; Wang et al., 2017). There has been no report on the effect of NP on nitrogen cycle in a small rural sewage treatment plant. Therefore, this study was conducted from samples collected from the Thibodaux sewage treatment plant, which serves a population of 15,000 people in rural setting in the southeast Louisiana of USA. The main purpose of this study was to determine how the increasing use of nanoparticles may be impacting the denitrification process in removing nitrate.

2. MATERIALS AND METHODS

2.1 Sample collection

The sludge sample was collected from the anaerobic digester of Thibodaux sewage treatment plant in a 1 liter sterilized Nalgene collection bottle. The sample was transported to the lab and kept in a refrigerator until use.

2.2 Development of denitrification consortia

A 10 mL sample of the sludge sample was transferred to a 200 mL basic mineral salt medium (BMS) with nitrate as nitrogen source in an anaerobic culture bottle. The carbon to nitrogen ratio in the medium was 15:1. BMS was made with 3.5 g of KH_2PO_4, 1.5 g of K_2HPO_4, 0.1 g of $MnSO_4$, 0.1 g of NaCl, 0.1 g of KNO_3, 1.5 g of glucose, and 0.5 g of yeast extract in 1 liter of deionized water. The medium was incubated anaerobically at 37°C until the optical density reached 0.400 at 600 nm wavelength. The anaerobic condition was maintained with helium in the headspace of the anaerobic culture bottle as described by Boopathy (1997). This developed consortia removed nitrate effectively via production of nitrite and served as the inoculum for further studies.

2.3 Effect of CuO NP on denitrification and carbon removal

The CuO NP was obtained from Sigma Aldrich Chemical Company (St. Louis, MO) and the average size was 50 nm with 99% purity. The effect of various concentrations of CuO NP on denitrification and carbon removal was studied in culture bottles with 100 mL BMS. The concentrations used include 0, 5, 10, 25, 50, 100, and 250 mg/L CuO, A 5% inoculum was used to start the experiment. Cultures were incubated anaerobically in a shaker at 37°C. Triplicate cultures were maintained in each concentration. The experiment was conducted for ten days. Samples were periodically taken for bacterial growth, nitrate, nitrite, carbon, and extra cellular polymeric substances (EPS) analysis.

2.4 Analytical methods

The bacterial growth was monitored by total plate count method using trptic soy agar (TSA) medium. The plates were incubated anaerobically using anaerobic jar. Anaerobic conditions were created by placing plates in a Gaspak anaerobic jar with an oxygen scavenging gaspak (Oxoid, Fisher Scientific) kit before sealing the jar. Plates were incubated for 48 hours at 37°C and the bacterial colonies were counted and reported as colony forming unit (CFU)/mL of sample. The organic carbon in the sample was monitored using chemical oxygen demand (COD) analysis. The amount of chemically oxidizable carbon (COD) was measured using colorimetric Reactor Digestion Method using HACH LR COD following the

method given by Hach (1999). Nitrate and nitrite were analyzed by Hach method (Hach, 1999). EPS was analyzed as per the method described by Adav and Lee (2008). Heat extraction method was applied to extract EPS from the culture samples at the end of the experiment.

2.5 Statistical analysis

All data in this study were subjected to analysis of variance (ANOVA) for statistical significance.

3. RESULTS AND DISCUSSION

The new and emerging pollutants include antibiotics, nanoparticles, pharmaceuticals and personal care products, and micro plastic. There are many studies reported on the effect of antibiotics and other emerging pollutants, but there are not many reports available on the effect of NPs on nitrogen cycle in a small rural WWTP. Therefore, this study was conducted to study the effect of CuO NP on nitrate removal via denitrification process.

3.1 Development of denentirification consortia

A denitrification bacterial consortia was developed from the sludge sample of an anaerobic digester of Thibodaux sewage treatment plant, which is a small rural sewage plant serving a population of 15,000 in southeast Louisiana of USA. The consortia development method is given in the method section. The developed consortia efficiently removed nitrate within 7 days under anaerobic condition (data not shown). The initial concentration of nitrate of 100 mg/L was completely removed within 3 days, and the nitrate was converted to nitrite. The nitrite concentration slowly increased in the culture bottles and it was completely converted to nitrogen gas by the 7^{th} day of incubation via dentirification process.

3.2 Effect of CuO NP on bacterial growth

Table 1 shows the bacterial growth of the consortia exposed to various concentrations of CuO NP. The low concentration of NP of 5 and 10 mg/L showed the highest growth without any lag period. The growth in these low concentrations was one log order better than control. The 5 and 10 mg/L concentration showed a stimulating effect on bacterial growth. The concentration of 25, 50, and 100 mg/L showed lag phase of two or more days before bacteria started to grow. The lag phase in general was longer as the NP concentration was higher. The highest dose of 250 mg/L completely inhibited the growth of bacterial consortia, as there was no growth at this concentration. Previous studies on methanogens showed similar effect of CuO NP as concentration dependent and higher doses of NP was toxic to microbes (Gonzalez-Estrella et al., 2013; Luna-delrisco et al., 2011; Otero-Gonzalez et al., 2014). Yang et al. (2013) reported stimulating effect of low doses of CuO NP on bacteria and they reasoned that the Cu (II) is the component of some enzymes in microbes and this enhanced the enzyme activity by acting as a co-factor in bacteria and hence the growth of the bacteria was increased. At the highest dose of 250 mg/L, the NP becomes toxic and kills the bacteria as shown in this study. In a study by Kumari et al. (2017), Cadmium tellurium quantum dots (CdTe QD) were shown to not only destabilize the cell membranes of *Bacillus subtilis* within an hour of exposure, but also accumulate internally after only two hours of incubation, and reduce the metabolic capacity of the cell until lysis ultimately occurred at hour four of incubation. Minimal inhibitory concentration for 50% of reduction in cell metabolism (MIC_{50}) of CdTe was found to be 0.45 μmol/L. These results

were similar to the MIC ranges for CdSe QDs on *B. subtilis* as well as CdTe QDs on *E. coli* and *Pseudomonas sp*p. Once CdTe QDs were localized within *B. subtilis*, results pointed towards two factors contributing to the detrimental effect of QDs on the bacteria. At first the cell membrane protein no longer have room to function properly due to adsorption of quantum dots and the second that these quantum dots enhanced the production of reactive oxygen species (ROS). Evidence of these reactive oxygen species was found by quantifying the level of anti-oxidative enzymes such as superoxide dismutase and catalase present in the cell. Silver ions have also been shown to exhibit the ability to enhance ROS production (Sinha and Khare, 2013). Similar to these studies, the highest dose of CuO NP of 250 mg/L in the present study showed no bacterial growth and may be attributed to disruption of cell membrane transport protein and also may have increased the level of ROS in the cell lead to cell death.

3.3 Effect of NP on EPS production

When bacteria are under stress such as exposure to toxic substances like hazardous chemicals and high doses of NP, they produce a protective electronegative mixture of polysaccharides and proteins known as extra cellular polymeric substance (EPS) matrix (Wang et al., 2016). This protective layer has been shown to mitigate bactericidal effect of toxic substances. The EPS production started to increase from the CuO NP concentration of 25 mg/L. Very less EPS was observed in control and 5 and 10 mg/L of NP. At the highest dose of 250 mg/L, there was no bacterial growth and hence no production of EPS (Fig. 1). This result suggests that the EPS production is NP dose dependent. The increase of EPS in 25, 50, and 100 mg/L CuO NP might be attributed to bacterial self-protection mechanism caused by physical restraints (Cupi et al., 2015). The production of EPS was observed under environmental stress (Sheng et al., 2010) and the NP dose of above 25 mg/L may be considered as stress to the bacteria in this study. Although the more EPS production seemed to be an effective strategy for the mitigation of toxicity of NP, higher doses of NP might have exceeded the shielding capacity of EPS and the bacteria could not overcome the exposure to NP and succumbed to lysis as observed in this study at 250 mg/L CuO NP.

Table 1 Effect of CuO NP on Bacterial growth (CFU*/mL of sample)

Day	CuO NP concentration in mg/L					
	Control	5 mg/L	10 mg/L	25 mg/L	50 mg/L	100 mg/L
0	34×10^2	34×10^2	37×10^2	30×10^2	31×10^2	33×10^2
1	55×10^4	66×10^5	54×10^6	32×10^2	28×10^2	31×10^2
2	70×10^6	83×10^7	72×10^8	60×10^2	90×10^2	30×10^2
4	66×10^8	98×10^9	80×10^9	55×10^5	116×10^8	28×10^2
6	62×10^7	90×10^8	72×10^7	66×10^6	122×10^7	30×10^4
8	44×10^6	66×10^6	88×10^6	71×10^6	138×10^6	33×10^5
10	33×10^4	84×10^6	44×10^6	52×10^6	66×10^6	31×10^4

*Colony Forming Unit; Data value represents log average of triplicate plates at each sampling event. There was no growth in 250 m/L CuO NP culture bottles

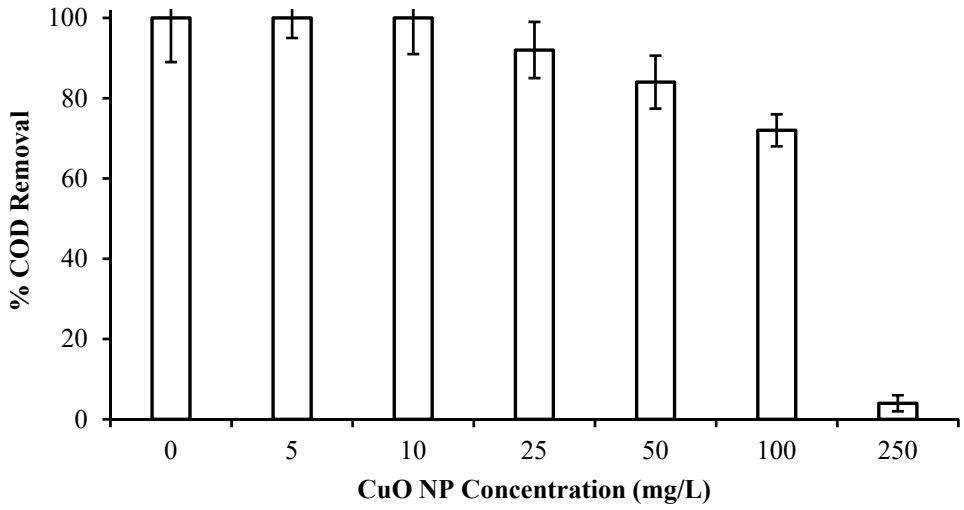

Figure 1 Effect of CuO NP on % COD Removal. The data represent after 10 days of incubation

3.4 Effect of NP on carbon removal

The effect of CuO NP on carbon removal was observed in the form of COD and the results are presented in Fig. 2. The COD removal mimicked the bacterial growth. In control and the low doses of NP (5 and 10 mg/L), COD was removed faster and almost 100% removal was noticed within four days of incubation. In 25, 50 and 100 mg/L the COD removal was delayed for 2 or more days reflecting the delay in bacterial growth and the removal of COD was progressively less from low to high concentration of NP. In the highest concentration, no COD was removed as there was no bacterial growth indicating the toxicity of CuO NP at 250 mg/L to the denitrifying consortia developed from the Thibodaux sewage treatment plant. Similar results were reported for inhibition of methanogenesis due to higher NP by Garcia et al. (2012) and Ma et al. (2013).

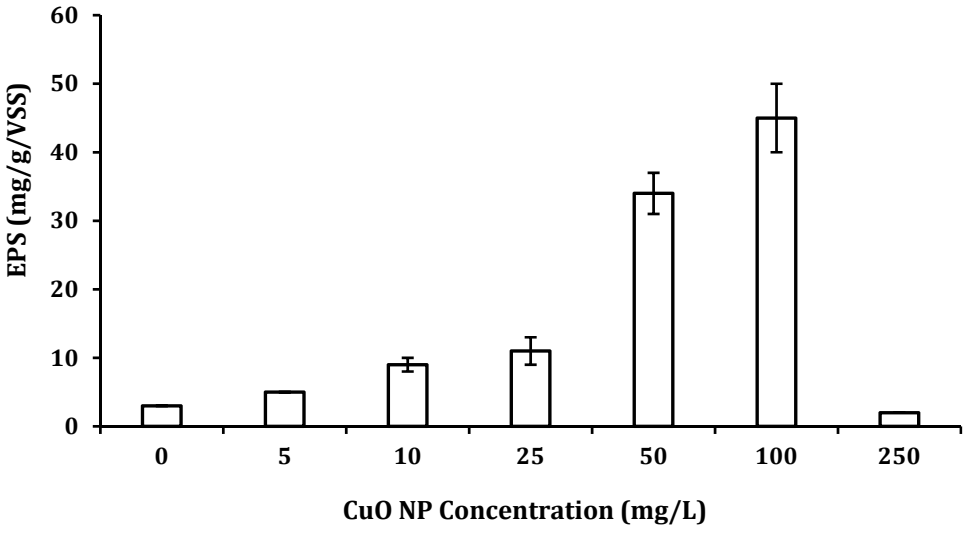

Figure 2 Effect of CuO NP on EPS production. The data represent after 10 days of incubation

3.5 Effect of NP on denitrification

Denitrification is the biochemical reduction of nitrate into nitrite and the reduction of nitrite into N_2 gas. The denitrifying bacteria use organic carbon as electron donors to reduce the nitrogenous compounds (Quan et al., 2005). Some examples of electron donors for denitrification are acetic acid (Her and Huang, 1995; Mohensi-Bandpi et al., 2004), glucose (Chou et al., 2003), methanol (Louzeiro et al., 2016), and ethanol (Sauthier et al., 1998). If adequate carbon is not available for the denitrifying bacteria, carbon limitation can cause the process to stall due to poor reducing conditions (Sauthier et al., 1998). Denitrification is often carried out by bacteria in the genus *Pseudomonas,* such as *P. denitrificans* (Carlson and Ingraham, 1983). Denitrification reaction and energy consumption is shown below as per Thauer et al. (1977):

$$4NO_3^- + C_6H_{12}O_6 \rightarrow 2N_2 + 6H_2O + 6CO_2O^- - 268 \text{ kcal}$$

The effect of CuO NP on denitrification is given in Table 2 and it shows the concentration of nitrate, which served as electron acceptor for the first reaction shown above. In control and in low concentrations of NP (5 and 10 mg/L) nitrate was rapidly reduced within four days of incubation resulting in 100% removal of nitrate from the culture media, whereas in 25, 50 and 100 mg/L, the nitrate reduction was delayed and the nitrate was not completely removed. In 250 mg/L, there was no removal of nitrate, which is consistent with cell death and no carbon removal in this treatment.

Table 2 shows the concentration of nitrite in the culture media indicating the second part of the reaction shown above in which nitrate is reduced to nitrite. A typical denitrification reaction was observed in control and in low doses of NP (5 and 10 mg/L) in which nitrate was reduced to nitrite within four days of incubation and there was a steady increase in nitrite during the first four days and then the nitrite concentration was completely reduced within ten days. A moderate and delayed production of nitrite was observed in 25, 50, and 100 mg/L NP. In 250 mg/L, no production of nitrite was observed. These results showed that the CuO NP at low doses did not have any effect on denitrification and at moderate doses there were some negative effects of nitrate reduction and nitrite loss. In the high dose of 250 mg/L denitrification was totally inhibited as there was no loss of nitrate in the culture medium.

The denitrification process is affected by many factors in the wastewater such as the presence of heavy metals, antibiotics, salinity, sulfides, and NP (Wang et al., 2015; Zhang et al., 2017). Wide use of NP has inevitably released NPs into the environment and most of these NP are discharged into sewage treatment plant (Ganesh et al., 2010; Hou et al., 2015; Keller and Lazareva, 2014). This increase in the concentration of NP especially CuO will have very negative effect on the performance of wastewater treatment as indicated here in this study. The carbon removal as well as nitrogen removal via denitrification was significantly affected by the high dose of CuO NP. At moderate dose, the bacteria produced EPS to protect against the toxic effect of NP and at low dose the NP has a stimulating effect as it serves as a co-factor in enzyme function. Even though the higher concentration tested in this study may not be realistic in the wastewater treatment system, this study shows the effect of various doses of CuO NP on bacterial growth, carbon removal, and nitrogen removal. The results presented in this work may provide some valuable guidelines for the operators of wastewater treatment plants.

Table 2 Effect of CuO NP on Nitrate removal via denitirification by the bacterial consortia isolated from the anaerobic digester

Day	CuO NP concentration						
	Control	5 mg/L	10 mg/L	25 mg/L	50 mg/L	100 mg/L	250 mg/L
NO_3 Concentration in mg/L							
0	100	100	100	98	98	101	102
1	77	62	41	89	91	96	100
2	32	21	11	71	80	89	101
4	0.5	0	0	53	72	82	98
6	0	0	0	22	66	72	101
8	0	0	0	5	41	61	100
10	0	0	0	2	22	44	100
NO_2 Concentration in mg/L							
0	0	0	0	0	0	0	0
1	21	28	44	12	11	9	0
2	54	43	65	21	23	17	0
4	78	81	21	70	67	56	0
6	45	55	8	61	60	50	0
8	10	11	2	54	51	44	0
10	0	0	0	41	32	40	0

Note: Standard deviation was consistently less than 5% in all treatments

CONCLUSIONS

This study showed a basic understanding of copper oxide nanoparticle interaction in the microbial community of wastewater treatment plant. The CuO NP has enhanced bacterial growth, carbon removal and nitrate removal at low doses of 5 and 10 mg/L. The bacterial growth was inhibited when the NP concentration was increased to 50 mg/L or more and also the carbon removal efficiency and denitrification process were reduced significantly. The bacterial consortia produced EPS to overcome the toxicity, however, the high concentration of 250 mg/L of CuO NP completely killed the bacteria and as a result carbon and nitrogen losses were not observed. As long as the CuO NP were 10 mg/L or less in the sewage treatment plant, the dentirication process will not be inhibited and in fact may be enhanced.

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