The effects of engineered nanoparticles on nitrification during biological wastewater treatment

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Abstract

Technological advancements in the past few decades have made it possible to manufacture nanomaterials at large scale and ENPs are increasingly found in consumer products such as cosmetics, sports products and LED displays. A large amount of these ENPs are in wastewater and potentially impact the performance of wastewater treatment plants (WWTPs). One important function of the WWTP is nitrification, which is carried out by the actions of two groups of bacteria, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Since most ENPs are found to have or are designed to have antimicrobial activities, it is a legitimate concern that ENPs entering WWTPs may have negative impacts on nitrification. In this paper, the effects of ENPs on nitrification is discussed, focusing mainly on autotrophic nitrification by AOBs and NOBs. This review also covers ENPs effects on ANAMMOX. Generally, nitrifiers in pure and mixed culture can be inhibited by a variety of ENPs, but stress response mechanisms may attenuate toxicity. Long-term studies demonstrated that a wide range of NPs can cause severe deterioration of AOBs and/or NOBs when the influent concentration exceeded an inhibition threshold. Proposed mechanisms include the generation of reactive oxygen species, dissolved metals, physical disruption of cell membranes, bacterial engulfment, and intracellular accumulation of ENPs. Future research needs are also discussed.

1. Introduction

1.1 The widespread use of nanomaterials. The enhanced strength, durability, flexibility, and performance associated with nanomaterials have been exploited in a multitude of applications. Engineered nanomaterials (ENPs) are already being used in sporting goods, tires, stain-resistant clothing, sun-screens, cosmetics, and electronics and are being increasingly utilized in medical devices (Nel et al., 2006). By some estimates, the production of ENPs is expected to increase to 58,000 tons by 2020 (Maynard et al., 2006). The impacts of engineered nanomaterials on the environment are two-sided: on one hand, technological advances in nanotechnology have undoubtedly brought great potential for innovative environmental remediation and monitoring applications (Gao & Wang, 2014). On the other hand, the potential harmful effects of engineered nanomaterials on humans and the environment, particularly the natural aquatic environment are of great interest. A recent study estimated that 10-30%, 3-17% and 4-19% NPs are discarded into water bodies in Asia, Europe and North America respectively (Keller et al., 2013). Wastewater treatment plants (WWTP) are among the last barriers prior to their release to the environment and the presence of nanomaterials in wastewater effluent has already been reported (Kunhikrishnan et al., 2015).

1.2 Objectives. The focus of this review is on the effects of various ENPs on nitrification and it is arranged as follows: 1). a brief introduction to nitrification, 2). a review of important issues that impact NP dynamics at full scale operations of wastewater treatment plants 3). effects of NPs on nitrification including observations from pure and enriched cultures, activated sludge and annamox processes; 4) proposed toxicity mechanisms, and 5) concluding remarks. Nitrification is a critical, microbially-driven process needed as part of most modern municipal water pollution control facilities, and this article discusses its susceptibility to NP-induced inhibition.

2. Nitrification

2.1. Introduction. Nitrification is the chemical conversion of oxidation of ammonia into nitrate. Ammonia must be removed from wastewater in order to help prevent eutrophication of receiving waters, and regulatory agencies have responded to this need by promulgating strict (e.g. < 0.5 mg NH_3-N/L) effluent NH_3-N limits. Such limits can be achieved by incorporating nitrification into the wastewater treatment process. Typically, wastewater treatment plants operate in a manner that supports the microbial communities that carry out nitrification, and there are now a wide range of suspended growth or fixed film configurations available for successful implementation. Nitrification is also, in many cases, coupled with denitrification to convert NO_3-N to nitrogen gas and achieve complete removal of soluble nitrogen. Nitrification is widely-practiced, and one of the most important components of modern water quality infrastructure.

2.2. Microbiology and Biochemistry. Nitrification in wastewater treatment is primarily mediated by the cooperative action of two distinct groups of chemoautotrophic bacteria: ammonia-oxidizing bacteria (AOB) for oxidation of ammonia (NH_3) to nitrite (NO_2^-) and nitrite-oxidizing bacteria (NOB) for oxidation of NO_2^- to nitrate (NO_3^-) (Dionisi et al., 2002). AOB are distinguished by their gram-negative multilayered cell walls and they are motile by means of flagella. There are five genera of AOBs that belong to two phylogenetically distinct groups, β- and γ-subclass of*Proteobacteria* . The β-subclass consists of four genera, including*Nitrosomonas* , *Nitrosospira* , *Nitrosovibrio* and*Nitrosolobus* ; the γ-subglass contains *Nitrosococcus*(Madigan et al, 2000). AOBs are obligate chemolithotrophs that derive energy from the oxidation of NH_3 to nitrite (Arp et al, 2002). NOBs belong to the *alpha-* , *beta* -, and*gamma-proteobacteria* , including *Nitrobacteria sp.*(*alpha*), *Nitrotoga sp.* (*beta*), *Nitrococcus sp.* (*gamma*). *Nitrospina sp.* and *Candidatus Nitromaritima* belong to the *Nitrospinae sp.* phyla and*Nitrospira sp.* belong to the *Nitrospirae* phyla (Daims et al., 2016). *Nitrotoga sp.* and *Nitrobacteria sp* are commonly detected at WWTPs (Ge et al, 2015).

Ammonia oxidation is the first and rate-limiting step of the nitrification process. Ammonia oxidation in AOB takes places in three steps: ammonia is first oxidized to hydroxylamine by ammonia monooxygenase (AMO), hydroxylamine is then oxidized to nitric oxide (NO) by hydroxylamine oxidoreductase (HAO), and NO is further oxidized to nitrite by as-yet-unidentified nitric oxide oxidoreductase (NOO), likely NcyA (Stein 2019). AMO is an integral membrane metalloenzyme that uses Cu as a cofactor whereas HAO is located in the periplasm and is a α-trimer of 60-kDa polypeptide each containing eight hemes (Arp & Stein, 2003). In the reaction carried out by AMO:

$NH_3 + O_2 + 2e^- + 2H^+$ $NH_2OH + H_2O$ (Eq.1)

Two exogenous electrons must be supplied to AMO to reduce one atom of O_2 to water; these electrons are provided from the oxidation of hydroxylamine by HAO:

$2NH_2OH + 1.5O_2$ $2NO + 3H_2O + 3e^-$ (Eq.2)

The oxidization of NO to nitrite is carried out by NOO/NcyA:

$NO + 0.5O_2 + e^-$$NO_2^-$ (Eq.3)

The subsequent oxidation of nitrite to nitrate is carried out by NOB. The key enzyme involved in this one-step oxidation process is nitrite oxidoreductase (NXR).

$NO_2^- + 1.5$ $O_2$$NO_3^-$ (Eq.4)

The membrane-bound NXR consists of α-, β-, and γ-subunits (Pester et al., 2014).

2.3. Inhibition. AOB and NOB activities are susceptible to inhibition through direct effects on the cell wall, essential enzymes, or on components involved in electron transport. Both AOB and NOB are slow-growing bacteria and sensitive to environmental factors (e.g., pH, dissolved oxygen, temperature) (Gu et al., 2012, Zhang et al., 2014, Fitzgerald et al., 2015). They are also inhibited by a large number of common wastewater constituents, including:

- Heavy metals such as copper, zinc, nickel, cadmium, and hexavalent chromium (Hu et al., 2004, Kim et al., 2016)
- Salt (Dincer et al., 2001)
- Soluble organic chemicals including 2,6-dichlorophenol (2,6-DCP), N-methylaniline, 3,4-dimethylpyrazolephosphate (DMPP), phenol, methanol, pyrazol, indole, (Pagga et al., 2006, Zhang et al., 2013)
- Un-ionized ammonia (Svehla et al., 2014).

Since many nanoparticles are designed to inhibit or prevent biological activity, nanoparticles retained in the activated sludge flocs are expected to alter the interactions among activated sludge bacterial populations and impact the effectiveness of ammonia oxidation. This review is focused on the effects of ENPs on nitrification because of the unique and well-documented history of nitrifier inhibition. For convenience, summaries of ENP-related inhibition studies are provided in tabular form in Supplemental Materials (Tables S1 – S4).

3. The effects of ENPs on nitrification

3.1. Observations from pure and enriched cultures

Studies with pure and enriched cultures can guide activated sludge research by identifying relevant inhibition mechanisms. There are few pure culture ENP studies with AOB and there are no relevant pure culture NOB studies in published literature. Pure culture studies with *N. europaea* demonstrated that ZnO NP can reduce cell growth and ammonia removal by causing severe damage to the cell membrane and by interfering with AMO and HAO gene expression (Wu et al., 2018). The threshold ZnO NP concentration responsible for nitrification inhibition appears to be between 1 – 20 mg ZnO NP/L (Wu et al., 2018), and likely depends on the population density and the availability of stress response mechanisms. In the case of AgNPs, inhibition may be caused by the dissolution of Ag+, which in turn leads to ROS production and membrane cell membrane damage (Arnaout & Gunsch, 2012). The threshold inhibition AgNP concentration appears to be not more than 2 mg/L, and depends on how the NP is coated (Arnaout & Gunsch, 2012). None of these pure culture studies demonstrated that AOB can recover following NP-induced inhibition.

Enriched AOB cultures contain relatively small populations of non-AOB, and such cultures have been exposed to AgNP (Michels et al., 2017, Alito & Gunsch, 2013), TiO_2 NP (Luo et al 2015), and ZnO NP (Luo et al., 2015). In each case nitrification was reduced in the presence of NP concentrations that exceeded the inhibition threshold. Additionally, phylogenetic data revealed that inhibition coincided with reductions in both the diversity and abundance of AOBs (Luo et al, 2015). Alito and Gunsch (2013) demonstrated that enriched nitrifying bioreactors can recover within 3 to 5 days following inhibition caused by exposure to influent pulses containing 0.2 mg Ag NP/L. This result implied that stress response mechanisms may attenuate toxicity. Michels et al (2017) also demonstrated that specific nitrite production of an enriched AOB culture can recover following inhibition caused by a shock load of magnetic NPs.

3.2. Observations with activated sludge: The effect of ENP injections

In activated sludge bioreactors, nitrifying microbial communities co-exist with large populations of the heterotrophic bacteria that are responsible for the removal of soluble organic compounds. Numerous studies using different types of ENPs have examined the short-term impact of ENP injection on nitrification rates in such bioreactors (Giao et al., 2012; Gu et al., 2014; Zheng et al., 2011). Injection of AgNPs may cause intracellular damage and ROS production (Giao et al., 2012; Gu et al., 2014). Dong et al. (2017) reported that a shock load of graphite ENPs can damage the AOB cell wall for *Nitrosomonas* and*Nitrosospira* . Hou et al. (2013) reported that ZnO NP injections (at 5 mg/L) inhibited nitrifier respiration in activated sludge. Yang et al. (2014) injected 40 mg/L of 35 nm diameter AgNPs, inhibited nitrification, and reduced the relative abundance of *Nitrospira sp.* from 10 – 12% to <5% during batch tests. Similar results have been reported for SiO2 NPs (Li et al., 2017). Influent pulses of some types of ENPs can damage AOB in mixed culture.

3.3. Observations with activated sludge: The effects of long-term ENP exposure

Longer-term impacts have been assessed using continuously operated bioreactors, usually sequencing batch reactors. These studies offered a dynamic assessment of reactor performance, microbial activity, and phylogenetic structure. When the threshold inhibition concentration is exceeded, a wide range of NPs can cause severe deterioration of AOBs and/or NOBs (Li et al., 2014; Ma et al., 2015; Li et al., 2017; Yazdanbakhsh et al., 2019). Reductions in species populations have been reported. The relative abundance of Nitrospira species was reduced from 20.1% to 6.9% in the presence of 50 mg/L of bimetallic Ag-Fe NPs (Yazdanbakhsh et al., 2019). Ma et al., 2015 reported that 10 mg/L Ag NPs decreased the normalized amoA gene abundances (e.g. amoA gene/16S rRNA gene) by two orders of magnitude. Gradual reductions in the abundances of *Nitrosomonas* and *Nitrospira* were observed in the presence of 10 mg/L of MWCNT NPs (Gao et al., 2019). No effects were observed during continuous loading of CeO2 NP (at up to 20 mg/L) and zero-valent Fe NP (at up to 20 mg/L) (Wu et al., 2013, Ma et al., 2015). Interestingly, the literature appears conflicted on whether heterotrophic bacteria have higher (Jeong et al., 2014; Hou et al., 2013) or lower (Xu et al., 2017) AgNP tolerance, compared to that of autotrophic nitrifiers. The differences in the sludge composition, reactor operation, and NP properties may contribute to the apparent conflict.

Recovery of nitrification is of interest during long term ENP exposure studies. Ni et al. (2013) documented the recovery of TN removal as well as AMO and NOR activity during long-term exposure (i.e. 50 days) to 50 mg/L magnetic NPs. They also found that NAR and NIR activities were not affected after short-term exposure but increased by 23% and 27%, respectively after long-term exposure. A 10-day study showed recovery of ammonia removal 8 days after the start of long term exposure to< 5 mg/L graphene oxide (GO) and graphene nanomaterials (Nguyen et al., 2018). They also observed the recovery of nitrification in spite of the loss of several genera associated with nitrification.

Membrane bioreactors (MBRs) include micro- or ultrafiltration modules which are directly submerged into the activated sludge basins, and they are generally operated to maintain higher MLVSS concentrations than those found in conventional activated process. These systems are now possible due to advances in membrane materials, and they were developed to reduce the facility footprint (by replacing the secondary clarifiers) and improve the quality of the treated wastewater. MBRs appear to provide more operational resilience, relative to the effects of ENPs. Zhang et al (2014) reported that long-term (i.e. 60 days) continuous loading with 0.1 mg/L AgNPs did not affect nitrification in the MBR. Yuan et al. (2015) also found that 0.1–5 mg/L AgNPs caused no adverse effects on nitrification (or denitrification) in an anaerobic-anoxic-oxic membrane bioreactor system. The higher resilience of MBR-based nitrification to NP stress may be attributed to the higher mixed liquor suspended solids concentrations in these systems.

3.4. Effects of ENPs on nitrification during anaerobic ammonium oxidation

Anaerobic ammonium oxidation (anammox) process is a novel biological nitrogen removal technology that is gaining popularity for nitrogen removal in wastewater streams. In this process, ammonium is directly converted to dinitrogen gas using nitrite as the electron acceptor in the absence of oxygen (Eq. 5) for nitrogen removal from wastewater streams.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_5N_{0.15} + 2.03H_2O \quad (Eq.5)$$

No addition of organic carbon source is required since CO_2 is utilized as the only carbon source. In addition, it significantly reduces oxygen demand since ammonium is only required to be partially nitrified to NO_2-instead of NO_3-; thus leading to considerable saving in operational cost. Due to these and other advantages (low CO_2 emission and low biomass yield), anammox processes have been widely regarded as an innovative and sustainable alternative to the classical activated sludge process (Zhang ZZ et al., 2016). There are more than one hundred full-scale anammox installations worldwide that are being applied for the treatment of side-stream wastewater (reject water).

A few studies have studied addressing the of ENPs effects on nitrogen removal by annamox processes (Table S4). Zhang et al (2017) studied the short-term (24 hrs) effects of CuNPs, CuONPs, ZnONPs and AgNPs

in a batch study using anammox sludge. Their results showed that CuONPs, ZnONPs and AgNPs up to 50 mg/g suspended solid did not affect anammox activity, ROS generation or LDH release. By contrast, CuNPs at 1.25 and 2.5 mg/g SS resulted in severe inhibition of anammox activity, without inducing an increase in LDH release. Higher loads of CuNPs caused significant inhibition of anammox activity and increased LDH release. The toxicity was primarily attributed to dissolved Cu^{2+} ions. Another batch study (Zhang et al. 2017) demonstrated that the addition of EDTA or S^{2-} could attenuate the adverse effects of CuNPs, presumably due to the chelation or sulfidation of Cu^{2+} ions. Later, the long-term effect of CuNPs was studied by adding CuNPs to an up-flow anaerobic sludge blanket (UFAB) reactor at 0.5 mg/L for 15 days, 1.0 mg/L for 15 days, and 5 mg/L for 30 days. Results showed that 5 mg/L of CuNP caused near complete inhibition of nitrogen removal and significant a decrease of the abundance of anammox bacteria. Withdrawing CuNPs from the influent permitted the recovery of nitrogen removal.

The long-term effects of ZnO NPs on annammox sludge was also studied using UFAB reactor (Zhang et al. 2018). ZnO NPs (˜30 nm) were added to the bioreactor at 1.0 mg/L on day 31, increased to 5.0 mg/L on day 46 and 10 mg/L on day 61. Results showed that shock-load of 10 mg/L ZnONPs resulted in the deprivation of 90% of the nitrogen removal capacity within 3 days. Anammox activity was significantly inhibited without any significant increase in LDH release or intracellular ROS production. These effects were attributed to dissolved Zn^{2+} ions and complete recovery was observed within 40 days after withdrawing the NPs from the influent. Another study by the same group investigated the effects of other metal oxides NPs on the performance of anammox process (Zhang et al. 2018). SiO2 NPs (˜30 nm), TiO2 NPs (˜60 nm, hydrophilic), CeO_2 NPs and Al_2O_3 NPs (30 nm, hydrophilic) on granular anammox sludge in lab-scale UFAB bioreactors. NPs were added to the bioreactors at 1, 50 and 200 mg/L in a step-wise fashion with a 30-day interval and lasted for an entire duration of 90 days. No adverse effects on nitrogen removal were observed, and this resilience was attributed to adaptation of the microorganisms through community shift and enhanced EPS production. Most recently, Li et al (Li et al. 2019) reported that exposure to graphene oxide (1 and 10 mg/L) resulted in acute toxicity and inhibition of annamox nitrogen removal. The effects disappeared by day 19 and reversed by the end of the study at day 61, with a TN removal efficiency higher than control. The same doses of AgNP caused long-term inhibition on TN removal, which did not recover. The long-term enhancement of TN removal by GO was accompanied by the relative high abundance of anammox bacteria *C.Anammoxoglobus* ; while the TN removal inhibition by AgNP was accompanied by the disappearance of some species with anammox ability. This observation seems to contradict with the findings by Zhang et al (2018), in which they reported no long-term adverse effects of AgNPs on anammox activity at concentrations of 1, 10 and 50 mg/L. This discrepancy may be related to differences in the type of sludge, bioreactor and particles used in these studies.

The effects of iron NPs seemed to be beneficial to anammox nitrogen removal. Li et al (2018) reported that adding Fe3O4 NPs (1, 10 mg/L) to an unplanted anammox subsurface flow constructed wetlands produced concentration-dependent acute toxic effects on ammonia removal; these effects disappeared overtime and by day 61, nitrogen removal rate were actually enhanced. Nano scale zero valent iron (nZVI) have also been proved to be beneficial for anammox bacteria growth and nitrogen removal (Erdim et al. 2019). In summary, these early studies have shown that in an anammox process, ENP toxicity was mainly caused by dissolved ions; the role of ROS generation was less significant than in the conventional activated sludge process, likely due to the lack of oxygen supply.

4. Mechanisms of ENP toxicity

Several mechanisms for ENP toxicity have been proposed based on experimental observations (Figure 1). Metal based ENPs are believed to exert their toxicity mainly through dissolved ions, in combination with the effects from nanoparticles. Metal ions bind with the negatively charged compounds in the bacteria cell wall, resulting in cell wall destabilization or collapse. Metal ions have high affinity to molecules containing –SH groups, such as cysteine; this binding can break S-S bond bridges that are necessary to maintain the integrity of folded proteins or directly disrupt the function of certain enzymes (Slavin et al. 2017). For example, the activity of most AMOs in *N. Europaea*are inhibited by Co^{2+}, Zn^{2+}, Ni^{2+}, and Fe^{2+} in a

concentration-dependent manner (Ensign et al. 1993). The dissolution of metal-based NPs also generates reactive oxygen species, which could cause cell membrane damage via oxidation of membrane components such as lipids. The internalized metal ion can also react with mitochondrial H_2O_2 and produce intracellular ROS or affect DNA repair and cause mutation (Huangfu et al., 2019). Intracellularly-produced ROS may also damage the cell membrane via lipid oxidation or damage cellular DNA without visible cell membrane damage. Non-metal based ENPs such as carbon nanomaterials also produce ROS upon light illumination, a property that is shared by TiO2 and ZnO NPs.

ENPs may enter cells through direct penetration or endocytosis. Direct penetration is caused by non-specific binding forces (electrostatic, hydrophobic, van der Waals) between the particle and the cell membrane; while endocytosis involves specific receptor-ligand interactions. Once inside the cell, ENPs can bind with intracellular biomolecules, interact with mitochondria, induce ROS production, or damage cellular functions (Yang et al, 2019). Increased ROS production leads to enzyme inactivation and DNA damage, likely the reason for the observed inhibition of key enzymes involved with nitrification.

ENPs can cause physical damage to bacteria in various ways. Adsorption of ENPs onto cell wall/membrane leads to depolarization of the cell wall/membrane, which changes the negativity of the membrane and makes it more permeable. Carbon nanotubes can puncture the cell membrane like needles. Graphene nanosheets can both cut through cell membrane and also disruptively extract phospholipids from the membrane due to strong van der Waals interactions and hydrophobic effects. These types of physical damage disrupt and weaken the cell membrane, resulting in release of biomolecules such as LDH. NP aggregation onto the cell surface may also facilitate NP dissolution, releasing metal ions that can easily enter the cell (Slavin et al, 2017). Large graphene nanosheets and aggregates of smaller NPs can entrap bacteria and prevent them from taking up nutrients. Long CNTs can wrap around the bacteria and induce osmotic cell lysis.

EPS generally acts as a protective layer for the microorganisms by absorbing ENPs and the dissolved ions. On the other hand, the EPS may promote ENP dissolution after the absorption capacity has been reached. Under some circumstances, strong interactions between ENPs and the EPS may result in stripping of the protective EPS layer off sludge microorganisms, thus making the microorganisms more vulnerable. As mentioned earlier, the ENP-microorganism interactions also depend heavily on the properties of the latter including type (gram-negative vs. gram-positive) (Mocan et al, 2017), shape (rod-shaped vs. spherical) (Al-Jumaili et al, 2017), hardness (Liu et al, 2009), cell wall structure (lipopolysaccharides, phospholipids defects) (Hsu et al, 2016), and enzyme and metabolism activities (Krishnamoorthy et al. 2012). Therefore, it is expected that different microorganisms in the activated sludge will respond differently to the same ENP stress, resulting in microbial community shifts as observed by several studies cited in this review. These shifts however, may not always cause inhibition.

5. Issues and challenges

There are a number of issues and challenges associated with assessing the effects engineered nanomaterials on nitrification in activated sludge.

First, even though NP physical-chemical properties are critical factors causing microbial toxicity, current literature is frequently missing critical data regarding NP physical-chemical properties, including hydrodynamic size, shape, surface charge, hydrophobicity, surface roughness, deformability (soft vs hard NPs), surface chemistry, electronic structure and coating. The most frequently provided data is particle size, many of them determined with TEM, but the hydrodynamic size is more appropriate for NPs in an aqueous environment. Some papers reported the size information provided by the manufacturer, which is not always correct and requires verification. A limited number of papers provided zeta potential measurements, even fewer have included hydrophobicity, surface roughness, deformability (soft vs hard NPs), surface chemistry, or coating (Tables S1-S3). Lack of comprehensive physical-chemical characterization makes it very difficult to draw meaningful comparisons between studies, because NP toxicity and interactions with the microorganisms and the EPS are governed by these properties (Huangfu et al., 2019, Slavin et al., 2017).

Second, it is also necessary to understand fate and transformation of these particles post entry into the

wastewater. Majority of these particles, especially those which are not stabilized with coatings, will undergo changes and take on a new physio-chemical identity. These changes could include biodegradation, dissolution, precipitation, aggregation, adsorption of naturally occurring substances and chemical transformation, depending on both particle-specific properties & particle state (free or matrix incorporated), and on the chemistry of the surrounding solution (pH, ionic strength, ionic composition) (Petosa et al., 2010). As a result, NPs identity in the wastewater could be vastly different from the original NPs. Clar et al (Clar et al., 2016) showed that aggregated CuO NPs in wastewater were about 1600 nm in diameter, about 40 times larger than the original NP (46 nm). Since it is these transformed particles that interact directly with the microorganisms, a thorough characterization of these transformed NPs is vital.

NP transformations are particularly relevant in the pipes that carry sewage to wastewater treatment plants. This underground network is anaerobic and contains soluble and insoluble constituents that may react with a wide range of ENPs (Metcalf and Eddy, 2014). The principle compound of interest is sulfide, which is biologically produced by a consortia of sulfate-reducing microorganisms. Sulfide forms complexes with Ag NPs (Kaegi et al., 2013), Cu NPs (Hatamie et al., 2014), and ZnO NPs (Lupitskyy et al., 2018). There are also coarse particles and colloids that can heteroaggregate with ENPs during transit in the sewer (Zhang et al., 2016). These processes tend to reduce the bioavailability and ecotoxicity of ENPs, however, the extent of NP transformation may be limited in sewer systems with short residence times.

There are other important, unresolved issues. The synergetic effects of multiple species of NPs are particularly relevant for WWTPs due to the presence of a vast variety of NPs found in sewage. The purity of the nanomaterials and variations among different preparations must also be considered; this is important because the toxicity may be affected by the impurities during the manufacturing process. For instance, CuO NPs that contains Cu NPs contaminants are expected to have a toxicity profile different from that of pure CuO NPs, since the release of dissolved Cu^{2+}ions from CuO NPs is significantly slower than from CuNPs (Zhang et al., 2017). The majority of literature so far has used unfunctionalized or minimally functionalized ENPs in their studies, while in reality, many applications use functionalized ENPs because of the improved efficacy, usability or added functionality. Functionalized ENPs will have different surface structure, chemistry, and aggregation properties; all of which will result in a completely different toxicity profiles.

Lastly, there is considerable uncertainty about the values of ENP concentrations that are present in wastewater. There are a small number of published studies that present such data and the range and temporal variability of ENP concentrations in domestic wastewater are not yet understood. There is a major information gap because it is difficult to relate published findings to realistic operational scenarios. There is a need to determine environmentally relevant concentrations of ENPs using field sampling campaigns. Such work should be done with a well-organized series of grab samples, taken together with flow and water quality data.

6. Conclusions

There is an urgent need to understand the effects of ENPs on wastewater treatment because of the growing use of nanomaterials. Nitrification is a critical wastewater treatment process that may be disrupted under certain conditions. Studies have confirmed that short-term, environmentally-relevant concentrations generally did not inhibit nitrification in conventional activated sludge systems. Long-term exposure to relatively high concentrations of ENPs inhibited nitrogen removal and shifted the microbiological community structure in activated sludge. Some studies have shown resiliency of activated sludge systems. Physical & chemical properties of the ENP, properties of the microorganisms and their environment are all believed to contribute to the variabilities in toxicity results observed in literature. Several mechanisms may contribute to the ENP-induced toxicity, including physical disruption of the cell membrane, generation of ROS, inhibition of enzymes and metabolic processes, and intracellular accumulation of ENPs. The effects of non-metal and composites ENPs have not been well-studied and need to be thoroughly investigated in future studies; as these materials are gaining increasing popularity in real applications. Aggregation and transformation of ENPs in wastewater are common and thus the observed toxic effects are in fact caused by aggregates or transformed NPs, thorough characterization of these "transformed NPs" will help to better interpret the results and

explain the variabilities among different studies. Early studies on the emerging anammox technology provided evidences of ENP-induced nitrification inhibition in these processes, however, the mechanisms are expected to different from that in activated sludge due to the lack of oxygen and differences in the nitrification microorganisms. Future research should also include the even more recent development of biological nitrogen removal processes that combine partial nitrification with anammox.

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Table S1: Metal Nanoparticles and their effects on the nitrification process in activated sludge

Particle	Size (nm)	Surface charge	concentration	duration	Major findings	References
AgNP	25.2 (citrate coated), 27 (GA coated), 21 (PVP coated)	n/a	0.2,2, 20 mg/L	4 hrs	Gum Arabic and citrate coated AgNPs were more toxic to cultured *N. Europaea*	Arnaout et al, 2012
AgNP	15 nm (TEM)	n/a	0.5 mg Ag/L	3 months	Freshly prepared AgNPs are beneficial for microbial diversity and biomass concentration	Sheng at al,2018
AgNP	32.3 (GA) TEM 15.5 (Citrate) TEM	n/a	0.2 mg/L, 2 mg/L		1) Coated AgNPs were less toxic than AgNO3; 2)2 mg/L dose caused significant reduction in N-removal;	Alito & Gunsch, 2014

Particle	Size (nm)	Surface charge	concentration	duration	Major findings	References
AgNP	14 nm	n/a (PVA coated)	1 mg/L	800 s test	1) 1 mg/L of Ag inhibited nitrifying bacteria growth, AgNP > Ag+; 2) No evidence of membrane damage	Choi et al, 2008
AgNP	21 (TEM), 29 (DLS)	n/a, (PVA coated)	1 mg/L shock loading	Short term batch test and bioreactor test (25 days)	1mg/L AgNP inhibited nitrification by 41.4%; decreased abundance of AOBs and NOBs	Liang et al,2010
AgNP	6.0±2.0 (STEM) 10.6±0.3	-26.2±1.0 mV	0.1 mg/L, continuous loading	60 days	No impacts on activated sludge performance	Zhang et al,, 2014
AgNP	118.5±1.2 (DLS, in Milli-Q water)		1.0 mg/L, 5.0 mg/L	27 days (1 mg/L) 36 days (5mg/L)	No effects on nitrification, denitrification or COD removal. Sludge settling abilities affected.	Qiu et al, 2016
AgNP	14 (TEM)	n/a	0-10 mg/L	1.5 hr	AgNPs of 0.25 to 10 mg/L inhibited ammonia oxidation by 4-50%	Giao et al, 2012

Particle	Size (nm)	Surface charge	concentration	duration	Major findings	References
AgNP	13.0±3.3 (as synthesized) 63.5±15.2 (glucose feed) 79.2±18.1 (ammonia feed)	-24.0±2.8 mV (as synthesized) 15.5±1.7 mV (glucose feed) -11.5±1.4 mV(ammonia feed)	0.1-50 mg/L	24 hrs; 50 days	1).Concentration-depedent inhibition of N-removal and COD removal; effects started at concentrations below 1 mg/L; 2. Nitrification was more severely inhibited; 3) nitrifying bacteria more sensitive to AgNP than heterotrophic bacteria	Jeong et al, 2014
AgNP	7±3; 40±14 (TEM)	n/a (Na_2ATP coated, PVA-coated)	0-10 mg/L	3 hrs (*N. Europaea* cell culture)	1).Size and coating-dependent ammonia oxidation inhibition; 2. Toxicity due to release Ag ion and AgNP dispersity; 3. Cell membrane damage	Yuan et al, 2013
AgNP	7±3 (TEM)	n/a (PVP coated)	0.1, 1 and 5 mg/L	125 days (0.1 mg/L) 40 days (1 mg/L) 30 days (5 mg/L)	No impact on nitrification & denitrification; significantly inhibited P-removal; toxicity attributed to dissolved silver	Yuan et al, 2015
AgNP	Synthesized NPs: < 10 nm (TEM) 38±10 (DLS) Commercial NPs: < 100	Synthesized:-25±0.88 mV Commercial: n/a	0-15 mg/L	21 hr (respirometry)	Commercial AgNPs showed no effects on sludge respiration; synthesized AgNPs has an IC50 value of 3.2-22.1 mg/L	Geyil and Cecen, 2016

Particle	Size (nm)	Surface charge	concentration	duration	Major findings	References
AgNP	n/a	n/a	0-30 mg/L	290 days	1. > 5 mg/L Ag NPs inhibited COD and P removal, not N removal; 2) Decreased enzymatic activities (AMO, NOR, NIR, NR, PPX and PPK); 3) dose-dependent ROS production and LDH release	Xu et al, 2017
AgNP	10 ±5 (DLS) 20 (TEM)	-36.1 mV	0.1, 1, 10	50 days	1.0 and 10 mg/L AgNP decreased COD, N and SOP removal; 2. AgNP inhibited EPS production; reduced the SOUR and increased LDH release, reduced nitrifyer abundance	Zhang et al, 2016
AgNP	44.61 (TEM)	n/a	30 mg/L	14 hrs	1).30 mg/L reduced nitrite production by 90%; 2. IC50 value of 10.5 mg/L for AOB activity inhibition	Michel et al, 2017

Particle	Size (nm)	Surface charge	concentration	duration	Major findings	References
AgNP	20 (DLS)	n/a	1-100 mg/L	12 hr, 22 days	1).short-term exposure led to 21-24.9% reduction in ammonia oxidation in flocculent sludge; 2)long-term exposure significantly inhibited ammonia oxidation, denitrification and oxygen uptake rate; 3) no effect on granular sludge	Gu et al, 2014
CuNP	336 (wastewater) $<$ 100 (TEM)	-24.6 mV	1 mg/L	180 days	1 mg/L led to: 1)inhibited N & P removal; 2) decreased AMO and NOR activity; 3)no effects on denitrification or its enzymes; 4)microbial shift and decreased ecological network interactions	Wang et al, 2017

Particle	Size (nm)	Surface charge	concentration	duration	Major findings	References
CuNP	220±25 (DLS)	n/a	0-10 mg/L	92 days	5 mg/L or high led to 1) enhanced N removal; 2) enhanced denitrification enzyme activity, 3) increased number of denitrifiers; 4) decreased glycogen metabolism	Chen et al, 2012
NZVI	n/a	Zero charge	20, 50 and 200 mg/L	10 hrs	200 mg/L inhibited ammonia removal and increased LDH release; no significant effects at 50 mg/L and below	Wu et al, 2013
NZVI & AgNP mixture	35 (nominal) 60 (DLS)	n/a	5, 50 and 100 mg/L	60 days	Nitrification inhibition at 100 mg/L, slight decrease in AMO and NOR activity	Yazdanbakhsha et al, 2019

Table S2: Metal Oxide Nanoparticles and their effects on nitrification in activated sludge

Particle	Size (nm)	Surface charge	concentration	Duration	Major Findings	Reference
SiO2	80-100 (DI water)	n/a	1, 50 mg/L	1 day, 50 days	50 mg/L NP led to: 1) inhibited N removal; 2) decreased NAR and NIR activities; 3) shift in microbial communities	Zheng et al, 2012

Particle	Size (nm)	Surface charge	concentration	Duration	Major Findings	Reference
SiO2	12, 151, 442, 682 (TEM)	n/a	5-500 mg/L	20 minutes	1) Smaller NPs (< 151) are stronger inhibitors than larger NPs on oxygen uptake; 2)cell membrane damages	Sibag et al, 2015
SiO2	30 (TEM)	n/a	0-30 mg/L	300 days	1) Slight inhibition on COD & N removal; 2) significant inhibition on P removal; 3) increased ROS production and LDH release	Li et al, 2017

Particle	Size (nm)	Surface charge	concentration	Duration	Major Findings	Reference
MWCNT	40-60 nm (D), 5-15 mm (L)	n/a	1, 20 mg/L	1 day, 180 days	1) No adverse effects from short-term exposure; 2) 20 mg/L long term led to decreased N removal, decreased AMO & NOR activities; and decreased abundance of PAOs; 3) 1 and 20 mg/L long-term led to decreased P removal, enzyme activities (PPX, PPK) and decreased abundance of AOBs, 4) 1 mg/L long term led to increased abundance of GAOs	Hai, et al, 2014
MWCNT	2-6 nm (ID), 10-15 nm (OD), 0.1-10 um (L)	n/a	0.64, 1.44, 2.16 and 3.24 g/L	3 hr	1) Dose-dependent inhibited respiration; 2) stronger effects on sheared sludge liquor	Luongo et al, 2010

Particle	Size (nm)	Surface charge	concentration	Duration	Major Findings	Reference
MWCNT-COOH	2-5 (ID), 2-8 (OD), 10-30 μm (L)	n/a	10, 30 mg/L	150 days	10 mg/L had no adverse effects, 30 mg/L inhibited ammonia removal, increased ROS production	Ma et al, 2019
MWCNT-COOH SiO2 TiO2	30-50 (OD) 0.5-2 μm (L) 80 10-30	n/a n/a n/a	0.5, 1, 2.5, 5 mg/L	overnight	1) MWCNT caused slight inhibition in oxygen uptake; & strong inhibition of BOD removal; 2) SiO2 caused slight change in BOD removal and oxygen uptake; 3)TiO2 caused significant inhibition on both BOD removal and oxygen uptake	Ergön-can et al, 2016
SWNT	n/a	n/a	270 mg/L	Shock load, 18 days	1) No negative impact on sludge performance; 2) improved sludge setttleability and dewaterability	Yin & Zhang, 2008
SWNT	1-2 nm (OD), 5.0-30 μm (L)	n/a	250 mg/L	3 hr	1)Inhibition of respiration and release of biomacromolecules; 2) stronger effects of sheared sludge liquor	Thakor et al, 2015

Particle	Size (nm)	Surface charge	concentration	Duration	Major Findings	Reference
SWNT	1-2 (diameter), 5-15 μm (L)	n/a	219 mg/L	5 hr	SWNTs (not the impurities) caused significant change to microbal structures	Goyal et al, 2010
GO	n/a	n/a	10-300 mg/L	5 days	1) Inhibition of micriobial community metabolic activity; 2) decreased organic carbon removal; 3) 200 mg/L higher affected P removal; 4) enhanced ROS production	Ahmed et al, 2013
Graphite	n/a	n/a	0.5 mg/L	3 hr	1) Inhibited nitrification; 2) damage to nitrifying bacteria; 3) dispersion of EPS	Dong et al, 2017
GO			0-1 mg/mL		1) GO inhibited P. Putida growth; 2) cells lost membrane integrity but preserved metabolic activity	Combarrous & Diaz, 2016

Particle	Size (nm)	Surface charge	concentration	Duration	Major Findings	Reference
Graphene	n/a	n/a	0-300 mg/L	10 hrs	10 mg/L and higher led to 1) Decreased COD, N and P removal; 2) decreased abundance of AOB, AMO and PAOs	Nguyen et al, 2017
GO & G	n/a	n/a	1, 5 mg/L	10 days	5 mg/L led to 1) reduction in COD, N and P removal after day 3; steady state reached after day 8; 2) shift in microbial community	Nguyen et al, 2018
GO	n/a	n/a	60 mg/L	72 min, 235 min	1) No significant effects of denitrifying bacteria growth; 2) significantly improved the activities of nitrifying bacteria; 3) no significant effects on bacterial community change	Guo et al, 2018
GO & Ag-GO	0.5-2 um (GO, TEM) 45.4 nm (AgNP, TEM)	n/a	10-100 mg/L	48 hr	1) Decreased nitrification; 2) LDH release, 3) ROS production	Ko et al, 2019

Particle	Size (nm)	Surface charge	concentration	Duration	Major Findings	Reference
QDots		PEI-coated; PMAO coated	Pure culture study	1-10 nM	1) Nitrifiers more susceptible than nitrogen fixing bacteria and denitrifying bacteria; 2) toxicity came from the NP instead of the coating or released ion;	Yang et al, 2012

Table S3. Non-metal NPs and their effects on the nitrification process in activated sludge

Table S4: Effects of ENPs on Anammox

Particle	Particle	Size (nm)	Size (nm)	Surface charge	Surface charge	concentration	concentration	Duration	Duration	Major Findings	Major Findings	F
CuNP, CuONP, ZnONP, AgNP	CuNP, CuONP, ZnONP, AgNP	10-30 (CuNP); 40 (CuONP) 30±10 (ZnONP), 60-120 (AgNP)	10-30 (CuNP); 40 (CuONP) 30±10 (ZnONP), 60-120 (AgNP)	n/a	n/a	0.25, 1.25,2.5, 12.5, 25, 50 mg/g SS	0.25, 1.25,2.5, 12.5, 25, 50 mg/g SS	24 hr	24 hr	no toxic effects observed for up to 50 mg/g SS of CuONP, ZnONP or AgNP; 1.25 mg/g SS of CuNP significantly inhibited nitrogen removal; Anammox granule showed high tolerance to CuNP than flocs; CuNPs caused LDH release; EPS play an important role in CuNP toxicity	no toxic effects observed for up to 50 mg/g SS of CuONP, ZnONP or AgNP; 1.25 mg/g SS of CuNP significantly inhibited nitrogen removal; Anammox granule showed high tolerance to CuNP than flocs; CuNPs caused LDH release; EPS play an important role in CuNP toxicity	Z e 2

Particle	Particle	Size (nm)	Size (nm)	Surface charge	Surface charge	concentration	concentration	Duration	Duration	Major Findings	Major Findings	F
CuNP, CuONP, ZnONP	CuNP, CuONP, ZnONP	10-30 (CuNP); 40 (CuONP); 30±10 (ZnONP)	10-30 (CuNP); 40 (CuONP); 30±10 (ZnONP)	n/a	n/a	5 mg/g SS	5 mg/g SS	12 hr	12 hr	CuNP toxicity not affected by coexistence of CuONP or ZnO NP CuNP toxicity attenuated by EDTA and S^{2-}	CuNP toxicity not affected by coexistence of CuONP or ZnO NP CuNP toxicity attenuated by EDTA and S^{2-}	Z e 2

Particle	Particle	Size (nm)	Size (nm)	Surface charge	Surface charge	concentration	concentration	Duration	Duration	Major Findings	Major Findings	F
CuNP	CuNP	10-30	10-30	n/a	n/a	0.5 mg/L for 15 days, 1.0 mg/L for 15 days, 5.0 mg/L for 30 days	0.5 mg/L for 15 days, 1.0 mg/L for 15 days, 5.0 mg/L for 30 days	60 days	60 days	No significant effects for low CuNP concentrations (0.5, 1.0 mg/L); Significant nitrogen removal inhibition and decrease of anammox bacteria observed at 5 mg/L; Inhibition effects can be recovered by CuNP withdrew from the influent	No significant effects for low CuNP concentrations (0.5, 1.0 mg/L); Significant nitrogen removal inhibition and decrease of anammox bacteria observed at 5 mg/L; Inhibition effects can be recovered by CuNP withdrew from the influent	Z e 2

Particle	Particle	Size (nm)	Size (nm)	Surface charge	Surface charge	concentration	concentration	Duration	Duration	Major Findings	Major Findings	F
ZnO NP	ZnO NP	30±10	30±10	n/a	n/a	1.0 mg/L on day 31; 5.0 mg/L on day 46 and 10 mg/L on day 61	1.0 mg/L on day 31; 5.0 mg/L on day 46 and 10 mg/L on day 61	days	days	No significant effects at low concentrations (1, 5 mg/L); Acute inhibition at 10 mg/L; no effect on ROS production or LDH release; Recovery after NP withdraw from the effluent	No significant effects at low concentrations (1, 5 mg/L); Acute inhibition at 10 mg/L; no effect on ROS production or LDH release; Recovery after NP withdraw from the effluent	Z e 2
Si2O NPs TiO2 NP CeO2 NP α-Al2O3 NP	Si2O NPs TiO2 NP CeO2 NP α-Al2O3 NP	30±5 (Si2O); 60 (TiO2); 20-50 (CeO2) 30 (α-Al2O3)	30±5 (Si2O); 60 (TiO2); 20-50 (CeO2) 30 (α-Al2O3)	n/a (siO2) hydrophilic (TiO2) n/a (CeO2) hydrophilic (Al2O3)	n/a (siO2) hydrophilic (TiO2) n/a (CeO2) hydrophilic (Al2O3)	1 mg/L (day 1-30); 50 mg/L (day 31-60) 200 mg/L (day 61-90)	1 mg/L (day 1-30); 50 mg/L (day 31-60) 200 mg/L (day 61-90)	See "concentration"	See "concentration"	No significant effects on nitrogen removal; Microbial shift Enhanced EPS production	No significant effects on nitrogen removal; Microbial shift Enhanced EPS production	Z e 2

Particle	Particle	Size (nm)	Size (nm)	Surface charge	Surface charge	concentration	concentration	Duration	Duration	Major Findings	Major Findings	F
Graphene oxide, AgNP	Graphene oxide, AgNP	n/a (GO); 50 (AgNP)	n/a (GO); 50 (AgNP)	n/a	n/a	1, 10 mg/L (GO, AgNP)	1, 10 mg/L (GO, AgNP)	61 days	61 days	Acute inhibition by GO (1 & 10 mg/L), recovered by day 20 and reversed by day 61 with enhanced TN removal; No acute effect of AgNP, long-term inhibition effect of AgNP, no signs of recovery till end of study	Acute inhibition by GO (1 & 10 mg/L), recovered by day 20 and reversed by day 61 with enhanced TN removal; No acute effect of AgNP, long-term inhibition effect of AgNP, no signs of recovery till end of study	I a 2

Particle	Particle	Size (nm)	Size (nm)	Surface charge	Surface charge	concentration	concentration	Duration	Duration	Major Findings	Major Findings	F
AgNP	AgNP	60-120	60-120	n/a	n/a	1 mg/L on day 31;, 10 mg/L on day 61, and 50 mg/L on day 91	1 mg/L on day 31;, 10 mg/L on day 61, and 50 mg/L on day 91	See "concentration"	See "concentration"	No adverse effects on nitrogen removal; Increased abundance of annamox bacteria; No effects on ROS production or LDH release.	No adverse effects on nitrogen removal; Increased abundance of annamox bacteria; No effects on ROS production or LDH release.	Z e 2

Particle	Particle	Size (nm)	Size (nm)	Surface charge	Surface charge	concentration	concentration	Duration	Duration	Major Findings	Major Findings	F
NZVI	NZVI	n/a	n/a	zero	zero	0.4-5000 ppb	0.4-5000 ppb	310 days	310 days	Significant enhancement if nitrogen removal rate; Enhanced EPS production; Intermediate dose more efficient in increasing annamox bacteria metabolism	Significant enhancement if nitrogen removal rate; Enhanced EPS production; Intermediate dose more efficient in increasing annamox bacteria metabolism	e 2

Figure

Figure 1: A schematic showing the types of ENPs commonly found in the WWTPs and their fate and effects on the microorganisms in the activated sludge. Several processes may happen to ENPs entering the WWTP: 1) aggregation including both intra- and inter-species aggregation; 2) dissolution which produces metal ions that could be toxic; 3) transformation such as the loss of surface coating; and 4) organics adsorption, NPs with large surface area may be covered by organics in the wastewater due to adsorption. Products of these processes, along with the original ENPs can cause damage to the microorganisms by several organisms: 1) weakening cell membrane by adsorption and aggregation onto the membrane; 2) extracellular ROS damage the membrane through lipid oxidation; 3) piercing through the membrane; 4) dissolved ions interact with important enzymes; 5) intracellular ROS damages damage the DNA, protein and other vital biomolecules; 6) dislodging the EPS from the bacteria by strong adsorption; 7) ENPs internalized into bacteria and damage biomolecules and metabolic functions; 8) wrapping around the bacteria to trap and isolate it from the microenvironment.

Figure 1: The fate and effects of ENPs in activated sludge