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Review

The Inhibition of Engineered Nano-ZnO in the Biological Nitrogen Removal Process: A Review

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Abstract: Engineered nano-ZnO is extensively utilized in both production and daily life, leading to its inevitable entry into the wastewater treatment system through various pathways. Nitrogen removal microorganisms in wastewater treatment systems are highly susceptible to environmental impacts. The antibacterial properties of nano-ZnO can impede the biological nitrogen removal (BNR) process and adversely affect the nitrogen removal performance. A comprehensive understanding of the inhibitory effect and mechanism of nano-ZnO on the BNR process is crucial in devising appropriate countermeasures to ensure optimal nitrogen removal performance. This review provides an overview of the sources of nano-ZnO in the environment, its impact on the BNR process, and the inhibition mechanism, and proposes potential methods that can mitigate the inhibitory effect of nano-ZnO. Additionally, future prospects are also discussed. This review serves as a foundation for a deeper understanding of the inhibition of engineered nano-ZnO on the BNR process and aids in guiding efforts to maintain the nitrogen removal performance in the presence of engineered nano-ZnO.

Keywords: nano-ZnO; inhibition; nitrification; denitrification; anammox; wastewater treatment

Citation: Ma, T.-F.; Ma, H.-X.; Wu, J.; Yu, Y.-C.; Chen, T.-T.; Yao, Y.; Liao, W.-L.; Feng, L. The Inhibition of Engineered Nano-ZnO in the Biological Nitrogen Removal Process: A Review. *Water* **2024**, *16*, 17. https://doi.org/10.3390/w16010017

Academic Editors: Antonio Albuquerque and Qiulai He

Received: 29 November 2023
Revised: 17 December 2023
Accepted: 18 December 2023
Published: 20 December 2023

1. Introduction

Nanomaterials are defined as materials possessing at least one dimension in the nano-scale range of 1–100 nm. These materials can be broadly classified into the following two categories: natural nanomaterials and artificial nanomaterials. The latter, also referred to as engineered nanomaterials, are characterized by their internal or surface structure possessing at least one dimension in the nano-scale range [1]. The 21st century has witnessed rapid growth in nanotechnology, leading to the widespread use of various nanomaterials in daily human life and production.

Engineered nano-ZnO, hereinafter referred to as nano-ZnO, is a representative metal oxide nanomaterial that exhibits a surface effect, quantum size effect, and small size effect. It is used in wide-ranging applications in various industries, including oxide semiconductors, additives, coatings, cosmetics, electronic equipment, and catalysts [2,3]. In cosmetics, nano-ZnO is primarily used as a sunscreen due to its ability to shield ultraviolet radiation through reflection, scattering, and absorption [4]. In the rubber industry, it is considered the most effective activator and is widely used in the rubber curing process [5]. Additionally, nano-ZnO has been extensively used in the photocatalytic degradation of dyes and other pollutants [6,7]. Its antibacterial properties have also made it a popular choice for food packaging [8,9]. The global production of ZnO nanoparticles is estimated to be significant.

It is estimated that the global production of ZnO nanoparticles (ZnO NPs) in the year 2020 reached a total of 58,000 metric tons [10].

Owing to the widespread and extensive utilization of nano-ZnO, it is inevitable that it is discharged into wastewater treatment systems (WWTSs) in the form of industrial or domestic wastewater during its production, use, transportation, and disposal processes. Research has indicated that nanomaterials are primarily captured in WWTSs [11–13], where biological treatment processes can capture 80–90% of NPs [14,15]. As one of the most commonly used nanomaterials, nano-ZnO has been detected in WWTSs for a long time. As early as 2008, the concentration of nano-ZnO in activated sludge from WWTSs in Europe and the United States was approximately 17.1 mg/kg and 23.2 mg/kg, respectively [16].

The removal of nitrogen from wastewater is a crucial objective of WWTSs. The primary approach for nitrogen removal in wastewater is through biological means. The conventional nitrogen removal process is primarily executed through nitrification and denitrification, with nitrifiers and denitrifiers performing these processes in separate reaction units. The discovery of aerobic denitrifying bacteria has made it possible for nitrification and denitrification processes to occur in the same reaction unit [17]. Anammox technology, which has garnered significant attention in recent years, employs anammox bacteria to transform ammonium nitrogen and nitrite nitrogen into nitrogen under anaerobic conditions [18]. However, these nitrogen-removal bacteria are all sensitive to environmental factors such as dissolved oxygen (DO) [19–21], the C/N ratio [22,23], temperature [24–26], pH [27,28], and so on. Numerous studies have confirmed the negative impact of nano-ZnO on the biological nitrogen removal (BNR) process in wastewater due to its antibacterial properties [29–31]. However, there is still a lack of comprehensive and profound understanding regarding the impact and inhibition mechanism of nano-ZnO on BNR processes.

This paper presents a comprehensive review of recent studies that study the impact of nano-ZnO on the BNR process. It covers the sources of nano-ZnO in the environment, its adverse effects on the BNR process, the corresponding inhibition mechanism, and potential approaches to mitigate the inhibition effect of nano-ZnO. This review serves as a foundation for a more profound comprehensive understanding of the inhibition of nano-ZnO on the BNR process and provides guidance for maintaining the nitrogen removal performance under the presence of nano-ZnO.

2. Sources of Nano-ZnO in the Environment

Nano-ZnO is primarily introduced into the environment through industrial manufacturing processes, consumer product usage, and agricultural applications. These sources contribute to the widespread presence of nano-ZnO in the environment, ultimately leading to its enrichment in WWTSs.

Nano-ZnO can be introduced into the environment during industrial manufacturing processes, which frequently employ it in electronics, photocatalysis, sunscreen, and other applications [32–37]. During these processes, nano-ZnO can be discharged into the air, soil, or water through dust and wastewater emissions or waste disposal. Furthermore, nano-ZnO can be derived from the utilization and disposal of consumer goods. A variety of everyday products, including cosmetics, sunscreens, coatings, plastics, and textiles, may contain nano-ZnO as an additive [38–41]. When these products are utilized, cleaned, or discarded, nano-ZnO can enter environmental systems. For instance, when clothing containing nano-ZnO is laundered, nano-ZnO may detach from the surface of the clothing and enter the wastewater. Additionally, the use of nano-ZnO in agricultural and rural areas can also result in the release of nano-ZnO into the environment. In agriculture, nano-ZnO is extensively employed in fertilizers, pesticides, and animal feed [42–44]. These applications can enhance crop growth, safeguard plants from disease, and improve the efficiency of animal feeding. However, during its use, some nano-ZnO may enter the environment through soil and water.

3. Effect of Nano-ZnO on BNR Process in WWTSs

Owing to the widespread utilization of nanomaterials in various industries and daily life, their discharge into WWTSs in the form of industrial or domestic wastewater is inevitable during their production, usage, transportation, and disposal processes. Research has indicated that nanomaterials are primarily trapped in WWTSs, with biological treatment processes capable of capturing 80–90% of NPs [45]. Nano-ZnO, as one of the most commonly employed nanomaterials, has been detected in WWTSs for a long time. In 2008, the concentration of nano-ZnO in wastewater treatment plant sludge was approximately 17.1 mg/kg and 23.2 mg/kg in Europe and the United States, respectively.

In recent years, the impact of nano-ZnO on the BNR process in wastewater treatment has emerged as a research hotspot. Nevertheless, current research mainly focuses on laboratory studies, specifically on the impact of the concentration and exposure duration of nano-ZnO on the BNR process (as presented in Table 1). The following sections summarize and discuss the effects of the concentration and exposure mode of nano-ZnO on the BNR process.

3.1. Effect of the Nano-ZnO Concentration on the BNR Process

Most studies have shown that the inhibitory effect of the nano-ZnO concentration increases in the lower concentration range [46]. For instance, in a study conducted on pure cultured ammonia-oxidizing bacteria *Nitrosomonas europaea*, it was discovered that the ammonia nitrogen removal efficiency of *N. europaea* decreased by approximately 22.9% after being treated with 10 mg/L of ZnO NPs for 6 h [47]. Furthermore, when the concentration of ZnO NPs was increased to 20 mg/L, the ammonia nitrogen removal efficiency of Nitrosomonas europaea at the fourth hour decreased by roughly 67% [48]. In nano-ZnO exposure experiments with pure cultured aerobic denitrification bacteria *Pseudomonas stutzeri* PCN-1, the total nitrogen removal of *P. stutzeri* PCN-1 decreased from 100% to 1.7% as the concentration of ZnO NPs increased from 1 mg/L to 128 mg/L [49]. Similar phenomena were also observed in a study of activated sludge systems and granular sludge systems. For instance, Zhang et al. found that the removal efficiencies of TN decreased from 75.6% to 70.8% when the concentrations of added ZnO NPs in the reactor increased from 10 mg/L to 50 mg/L [3]. Similarly, Song et al. observed that the specific anammox activity of anammox granular sludge exhibited a significantly decreasing trend with the increase in ZnO NPs' concentration within the range of 5 mg/(gVSS) to 20 mg/(gVSS). When the concentration of ZnO NPs surpassed 20 mg/(gVSS), the specific anammox activity of anammox granular sludge was almost entirely lost [50].

It is also reported that the introduction of high concentration (450–2000 mg/L) ZnO NPs can result in a significant reduction in the removal efficiency of ammonia nitrogen in the activated sludge system [51]. However, as the concentration of ZnO NPs increases, the removal efficiency of ammonia nitrogen does not experience a further decline, and the decrease in ammonia nitrogen removal efficiency remains within the range of 13–14% [51]. Due to the limited research on the BNR exposed to a high concentration of nano-ZnO, the underlying mechanisms behind this phenomenon require further investigation. It is speculated that this may be related to the state of high-concentration ZnO NPs in wastewater, such as their aggregation, distribution, and surface electrical properties.

3.2. Effects of the Exposure Mode of Nano-ZnO on BNR Process

Most studies on the impact of engineered nanomaterials on BNR indicate that the acute inhibitory effect of nanomaterials on BNR typically occurs at the beginning of exposure, but this system can usually recover after long-term operation [52]. As the system operates for a longer period of time, the added nanomaterials are adsorbed and enveloped by microbial extracellular substances in the system, which reduces their inhibitory effect. At the same time, the toxicity of metal and metal oxide nanomaterials released by metal ions also decreases or loses their toxicity due to coordination reactions and/or adsorption [53]. Meanwhile, the BNR performance of the system gradually recovers. However, in real

scenarios, there are also cases where wastewater contains nanomaterials for a long time, so the long-term exposure of the BNR process to nanomaterials cannot be ignored.

In studies investigating the impact of nano-ZnO on the BNR process, both short-term and long-term exposure may produce different inhibitory effects. For example, in short-term exposure experiments (6 h), ZnO NPs at concentrations of 1 mg/L, 25 mg/L, and 50 mg/L all showed dose-dependent inhibitory effects on the TN removal performance of activated sludge but had no inhibitory effect on ammonia nitrogen removal [54]. However, after long-term exposure to 10 mg/L of ZnO NPs for 45 days, the reactor showed an almost complete loss of TN and the ammonia nitrogen removal performance [55]. Similarly, in the study of aerobic granular sludge, He et al. found that short-term exposure to 10 mg/L of ZnO NPs resulted in a decrease of 16.4% and 5.7% in the removal efficiencies of TIN and ammonia nitrogen, respectively, compared to the control group [56], while long-term exposure further reduced the removal rates of TIN and ammonia nitrogen, with a decrease of about 25.2% and 7.4%, respectively, compared to the control group [57].

Furthermore, it is noteworthy that the intermittent exposure mode has the potential to augment the resistance and/or tolerance capacity of the BNR system to nano-ZnO [58], thereby enhancing this system's ability to counteract the inhibitory impact of nanomaterials. This is discussed later.

Table 1. The influences of nano-ZnO on the BNR process.

Concentration (mg/L)	Reactor Type	BNR Process	Experimental Duration	Influences	Reference
10	Flasks	Nitrification, *N. europaea*	6 h	Cell morphology, cell density, membrane integrity, and ammonia monooxygenase activity were impaired. The ammonia nitrogen removal efficiency was reduced by about 22.9%.	[47]
20, and 200	Flasks	Nitrification, *N. europaea*	4 h	The ammonia nitrogen removal efficiency of Nitrosomonas europaea at the 4th h decreased by about 67%. There was a reduction in the cell size by 40–45%. The specific oxygen uptake rate was seriously depressed.	[48]
1, 4, 16, 64, and 128	Serum bottles	Denitrification, *P. stutzeri* PCN-1	48 h	TN removal efficiency decreased from 100% to 1.70% with the increase in ZnO NPs from 1 to 128 mg/L. The gene expression of *napA*, *nirS*, *norB*, and *nosZ* were all inhibited.	[49]
1, 10, and 50	SBR	Nitrification and denitrification	4.5 h	After increasing the concentration of ZnO NPs from 10 mg/L to 50 mg/L, the reactor's removal of TN decreased from 75.6% to 70.8%.	[3]
5, 10, 20, 50, and 100	UASB	Anammox	24 h	When the concentration of ZnO NPs was within the range of 5 mg/(gVSS)~20 mg/(gVSS), the specific anammox activity of anammox granular sludge tended to decrease with an increase in ZnO NP concentration. When the concentration of ZnO NPs was higher than 20 mg/(gVSS), the specific anammox activity of anammox granular sludge was almost completely lost.	[50]
450, 900, 1500, and 2000	Aerobic bioreactors	Nitrification and denitrification, activated sludge	8 h	The removal efficiency of ammonia nitrogen by activated sludge was inhibited, but its removal efficiency did not decrease with the increase in the concentration of ZnO NPs and was maintained at about 59%.	[51]

Table 1. *Cont.*

Concentration (mg/L)	Reactor Type	BNR Process	Experimental Duration	Influences	Reference
1, 25, and 50	SBR	Nitrification and denitrification, activated sludge	6 h	Denitrification processes were depressed, while nitrification processes were not under different levels of ZnO NP exposure. TN removal efficiencies decreased from 69.7% (control) to 64.9% and 62.2% after being exposed to 25 and 50 mg/L ZnO NPs. The main denitrifying functional genes, including *narG*, *nirK*, *norB*, and *nosZ*, generally displayed decreasing trends in their relative expressions with the increase in ZnO NP concentrations.	[54]
1, and 10	SBR	Nitrification and denitrification, activated sludge	30 d (1 mg/L ZnO NPs) 60 d (10 mg/L ZnO NPs)	Exposure to 1 mg/L ZnO NPs did not affect the performance of ammonia and TN. After exposure to 10 mg/L ZnO NPs for 45 d, the reactor almost completely lost TN and ammonia nitrogen.	[55]
10, 50, and 100	SBR	Nitrification and denitrification, aerobic granules	6 h	Ammonia removal efficiencies decreased from 98.85% (control) to 93.17%, 91.69%, and 77.79%, respectively, after being exposed to 10, 50, and 100 mg/L ZnO NPs. TIN removal efficiencies also dropped from 96.65% (control) to 80.30%, 74.3%, and 57.43%, respectively.	[56]
5, 10 and 20	SBR	Nitrification and denitrification, aerobic granules	45 d for each ZnO NPs concentration	The removal efficiencies of ammonia nitrogen and TIN decreased by 24.75% and 36.14% after being exposed to 20 mg/L ZnO NPs for 45 d.	[57]

4. Inhibition Mechanism of Nano-ZnO to the BNR Process

The antibacterial mechanism of nano-ZnO, which is detailed in a prior review [59], typically encompasses the release of zinc ions, the generation of reactive oxygen species (ROS), and contact-mediated impairment. Nonetheless, these inhibitory mechanisms are primarily grounded in pure culture investigations of bacterial strains that are relevant to human health. Certain studies have been undertaken to explore the inhibitory effects of nano-ZnO on the BNR process. The following sections primarily delineate the inhibitory mechanism of the nitrification process, the denitrification process, and the anammox process.

4.1. Nitrification Process

The nitrification process refers to the gradual oxidation of ammonium (NH_4^+) to nitrate salts (NO_3^-) via microorganisms through the action of ammonia monooxygenase (AMO), hydroxylamine oxidoreductase (Hao), and nitrite oxidoreductase (NXR). This process is divided into the following two stages: ammonia oxidation (NH_4^+-NO_2^-) and nitrite oxidation (NO_2^--NO_3^-), which are completed using ammonia-oxidizing microorganisms and nitrite-oxidizing bacteria (NOB), respectively. Ammonia-oxidizing microorganisms include ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea. AOB and NOB are collectively referred to as nitrifying bacteria. From the perspective of the types of microorganisms involved, current research has mainly focused on nitrifying bacteria. These studies can be broadly divided into the following three directions: microbial community structure, cell physiology, and gene expression, depending on the research direction.

In terms of the microbial community structure, AOB, as a type of environmentally sensitive microbe, may experience a decrease in relative abundance with the addition of a certain concentration of nano-ZnO [46,60]. The main reason for this is that AOB growth and activity are inhibited by nano-ZnO, while other bacterial groups can be promoted. For example, Wang et al. [60] found that, when activated, sludge in an SBR was exposed to 20 mg/L of ZnO NPs while the growth of some typical AOB, such as *Nitrosococcus* sp.

and *Nitrosomonas* sp., were inhibited, while some types of denitrifying bacteria, such as *Nitratiruptor* sp. and *Pseudomonas* sp., were promoted.

In terms of cell physiology, nano-ZnO can cause damage to cell morphology, cell density, cell membrane integrity, and key enzyme activity, thereby inhibiting nitrification processes. When the model AOB strain *Nitrosomonas europaea* was exposed to 10 mg/L of nano-ZnO for 6 h, Yu et al. found that these cells underwent significant deformation, most of the multi-layer membranes of the cells could not be distinguished, cavities appeared inside the cells, and the cell density decreased by 34.7 ± 7.0% compared to the control group. The integrity of the cell membrane decreased by about 6% and was accompanied by physiological damage, while the strain's removal rate of ammonia nitrogen decreased by 22.9 ± 0.7% [47]. Similarly, Wang et al. observed damage to cell membrane integrity when *N. europaea* was exposed to 20 mg/L and 50 mg/L of nano-ZnO for 4 h [60]. Damage to bacterial cell walls and cell membrane integrity was also frequently observed in the study of other metal and metal oxide nanomaterials [61–64]. The possible reason for this is that NPs accumulate on the outer surface of the cell membrane, neutralize the surface potential of the cell membrane, increase surface tension, and depolarize the membrane, ultimately causing changes and rupture to the cell membrane structure and damage to cell wall/membrane integrity [61,62,65]. In addition, the uneven surface structure and rough edges of ZnO nanomaterials can also wear down the cell membrane [66,67]. The addition of nano-ZnO also leads to the inhibition of key enzyme activity in the nitrification process. Daraei et al. found that 1–50 mg/L of ZnO NPs inhibited the activity of AMO and NXR in activated sludge, and the inhibition increased with the increase in the ZnO NP concentration [68]. Similarly, Wang et al. found that 20 and 50 mg/L of ZnO NPs significantly inhibited the activities of AMO and NXR in traditional activated sludge, with the highest inhibition rates reaching 39.24% and 44.84%, respectively [60].

In order to further understand the inhibitory mechanism of nano-ZnO on nitrification processes, researchers studied the impact of nano-ZnO exposure on the genetic level of typical nitrification processes. Yu et al. [47] monitored the global transcriptional expression of the model strain N. europaea under short-term exposure to nano-ZnO using the whole-genome microarray technique. They found that the most abundant differentially downregulated genes were those related to cell wall/membrane biogenesis and post-translational modification, protein turnover, and chaperone encoding. The downregulation of cell wall/membrane biogenesis genes may partially explain why the impairment of the cell membrane was not repaired in time. At the same time, an up-regulation of genes related to ammonia oxidation processes (such as *amoA1*, *amoB1*, *amoC1*, and *amoC2*) was observed, which could be a response of the bacteria to the inhibition of AMO. Ye et al. [31] found that after the long-term exposure of activated sludge to 10 mg/L of ZnO NPs, the abundance of *amoA* and *nxrA* genes in the system decreased significantly compared to the control group. Phan et al. [69] enriched the nitrifying bacterial community in activated sludge and exposed it to 1, 5, and 10 mg/L nano-ZnO. They found that after 6 h of exposure, the transcription levels of *amoA* and *hao* genes in the community were significantly decreased compared to the control group, indicating that the presence of nano-ZnO can directly or indirectly affect the expression of these key genes in nitrification processes.

4.2. Denitrification Process

The denitrification process refers to the process in which nitrates are transformed into nitrogen gas through a series of reduction reactions under microbial action, which is an important part of the nitrogen cycle. Currently, there are numerous research reports on the effects of various nanomaterials on microbial denitrification processes. However, research on the impact of nano-ZnO on denitrification processes is relatively limited.

Like many other nanomaterials reported, nano-ZnO can also affect denitrification processes by altering the microbial community structure. ZnO NPs can reduce the diversity of denitrifying bacteria communities and change the dominance of some denitrifying bacteria. For example, Chen et al. found that under low and high concentrations of ZnO

NPs, the abundance of the *nirS* gene in denitrifying bacteria decreased by 83.8% and 95.8%, respectively, indicating a significant decrease in the abundance of denitrifying bacteria in the community. At the same time, the relative abundance of some bacterial genera in the denitrifying bacterial community also changed, such as an increase in the relative abundance of *Pseudomonas* and a decrease in the relative abundance of *Bacillus* [70]. Similarly, Chen et al. also reported the inhibitory effect of high doses of ZnO NPs (5 mg/L) on denitrifying bacterial genera in activated sludge, including *Diaphorobacter* species, *Thauera* species, and those in the *Sphaerotilus Leptothrix* group [71]. Cheng et al. also found that the relative abundance of denitrifying bacteria in the denitrifying granular sludge system significantly decreased after being exposed to 2.5 mg/L ZnO NPs, with the relative abundance of the dominant denitrifying bacterium *Castellaniella* decreasing from 51.0% to 8.0% [72]. These studies indicate that the presence of nano-ZnO can affect the microbial community's structure, reduce the relative abundance of denitrifying bacteria in the microbial community, and thus affect the denitrification process.

The inhibition of crucial denitrification enzyme activities in denitrifying bacteria is a significant mechanism through which nano ZnO impedes the bacterial denitrification process. The bacterial denitrification process primarily depends on four denitrifying enzymes, namely nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (NOS). These enzymes are encoded by the gene clusters of *narGHJI* (encoding respiratory nitrate reductase), *napABC* (encoding periplasmic nitrate reductase), *nirSECF*, *norCBQDEF*, and *nosRZD*, respectively. Nano-ZnO can affect bacterial denitrification by inhibiting the activity of key denitrification enzymes [30,31]. For example, Ma et al. found that exposure to 0.75 mM ZnO NPs significantly inhibited the activity of key enzymes in aerobic denitrifying bacteria, with NAR activity decreasing by 38.8% and NIR activity decreasing by 56.4% [73]. Similarly, Chen et al. observed that, under the action of 5 mg/L ZnO NPs, the highest decrease in NIR activity in the system could reach 32.8% [70]. The inhibition of these key denitrification enzyme activities may be related to the ROS generated by nanomaterials [74]. For example, Chen et al. found a good linear relationship between the activity of NAR and NIR in the denitrifying strain *P. stutzeri* PCN-1 and the amount of ROS generated under the action of ZnO NPs, indicating that the decrease in the enzyme's activity could be related to the generation of ROS [49]. In addition, as one of the important mechanisms of the antibacterial effect of nano-ZnO, the dissolution and release of zinc ions can also inhibit key denitrification enzymes, such as NAR [75] and NOS [76], thereby inhibiting bacterial denitrification [30].

In addition, genomic studies (such as genomics, transcriptomics, proteomics, etc.) on the effect of nano-ZnO on denitrifying bacteria have shown that it can indirectly inhibit denitrification by affecting other metabolic processes of denitrifying bacteria. Reports have indicated that nano-ZnO can inhibit key enzyme activities in the glycolysis process, such as glucokinase, phosphofructokinase, and pyruvate kinase [30,31], and also impact succinyl-CoA synthetase, succinate dehydrogenase, and malate dehydrogenase in the tricarboxylic acid cycle [31], leading to insufficient electron donors and a decreased electron transfer chain efficiency in denitrification, ultimately adversely affecting this process [31,77]. In addition, ZnO NPs can cause denitrifying bacteria to accumulate intracellular PHB, which could compete for the electrons used in microbial denitrification [30]. Luo et al. [74] found that 10 mg/L and 50 mg/L of ZnO NPs significantly inhibited purine metabolism, arginine biosynthesis, and alanine, aspartate, and glutamate metabolism in aerobic denitrifying bacteria at low temperatures, and 50 mg/L of ZnO NPs led to the downregulation of ribosomes and upregulation of iron death. The impact of ZnO NPs on bacterial metabolic processes adversely affects bacterial growth and division, protein synthesis, cell membrane elasticity, intracellular biosynthesis, and the ability to resist external pressure, indirectly leading to poor denitrification performance.

In addition, nanomaterials have excellent adsorption capabilities due to their high specific surface area. They may also adsorb trace metal elements such as Fe, Mo, or Cu, which are required by denitrifying bacteria. These metal elements are essential for

denitrifying enzymes [78–80], and their reduction has been shown to lead to a decrease in key enzyme activity in model strains [81–83]. Therefore, the adsorption of trace metal elements by nano-ZnO may also be one of the reasons for the inhibition of denitrifying bacterial activity [30].

4.3. Anammox Process

The anammox process uses ammonium nitrogen as an electron donor to reduce nitrite nitrogen to nitrogen gas, thereby achieving denitrification [18]. Due to its advantages, such as no need for a carbon source, low sludge production, and no need for aeration, anammox technology is considered a more economical and promising BNR technology than traditional nitrification–denitrification technology [84,85]. However, the practical application of anammox still faces challenges due to the long generation cycle of anammox bacteria and their sensitivity to environmental stimuli. In recent years, the impact and mechanism of zinc oxide nanoparticles on the anammox process have gradually received attention as a nanomaterial that has been shown to affect traditional BNR processes. Similar to the mechanism of biological toxicity on nitrification and denitrification processes, zinc oxide nanoparticles have adverse effects on the anammox process by disrupting cell membrane integrity, inhibiting key enzyme activity, and disrupting metabolic processes. The main mechanism includes zinc ion release and the production of ROS.

Nano-ZnO has been widely reported to inhibit anammox bacteria by releasing zinc ions [50,58,86]. Heme c, an important cofactor for key enzymes such as NIR, hydrazine synthase, and hydrazine dehydrogenase in anaerobic ammonia oxidation, plays a catalytic and electron transfer role in the substrate for intermediate conversion [87]. Its content is an indicator for evaluating anaerobic ammonia oxidation activity [88,89]. Studies have found that an increase in the concentration of zinc ions and ZnO NPs leads to a decrease in the content of heme c, indicating that the release of zinc ions from ZnO NPs is one of the biotoxic mechanisms that inhibit anammox activity [86,90]. A possible mechanism for this is the fact that zinc ions bind to thiol groups on proteins involved in the electron transfer chain, thereby affecting bacterial metabolism [91]. The zinc ions released by nano-ZnO can replace the divalent cations on the active site of key enzymes in anammox bacteria, thereby affecting bacterial metabolism and inhibiting bacterial activity. For example, zinc ions can replace copper ions on the active site of AMO, making it unable to oxidize NH_3 to NH_2OH [90].

The production and accumulation of ROS caused by nanomaterials is one of the mechanisms that inhibit anammox bacteria [92,93]. The accumulation of ROS produced by ZnO NPs and the death of anammox bacteria caused by the ROS have been reported [86]. However, some studies found that ZnO NPs do not cause oxidative damage to anammox [58,94]. This could be related to the fact that most studies operate anammox reactors under light-avoiding conditions [95]. Under anaerobic and dark conditions, ZnO NPs in the reactor may not produce ROS or produce very limited ROS, leading to a decrease in oxidative toxicity. Further research is needed to investigate the production and accumulation of ROS in anammox reactors to clarify the inhibitory mechanism of ROS on anammox bacteria.

5. Mitigation Strategies for Inhibition Effects

In order to mitigate the adverse effects of nano-ZnO on the wastewater BNR system, several research directions can be considered in the future.

5.1. Adding Agents or Substances to Reduce the Concentration and/or Toxicity of Nano-ZnO

By adding appropriate agents or substances to the wastewater, the concentration and/or toxicity of nano-ZnO in the wastewater can be reduced, thus reducing the adverse effects of nano-ZnO on the BNR treatment unit of the wastewater. For example, Khort and his colleagues found that adding natural organic matter mainly composed of fulvic and humic acid to the ZnO NPs solution could adsorb ZnO NPs to form colloids with higher stability, which helps aggregation and sedimentation [96]. It has also been reported

that the addition of HA can reduce the concentration of zinc ions released by ZnO NPs, thereby alleviating the toxicity of ZnO NPs to bacteria [97]. At the same time, the use of EDTA or S^{2-} has also been reported to reduce the adverse effects of ZnO NPs on the BNR system [95,98], mainly because EDTA and S^{2-} can chelate with zinc ions [99] and undergo a sulfidation reaction [100], thereby reducing the concentrations of Zn (II) released by nano-ZnO. Therefore, selecting or developing suitable additives for the BNR process of wastewater can help to reduce and/or alleviate the impact of nano-ZnO on BNR. Of course, the above measures can also be considered for the pretreatment of wastewater containing nano-ZnO before entering the wastewater BNR unit.

5.2. The Modification of Nano-ZnO

Prior research on the surface modification of nano-ZnO has primarily focused on the impact of such modifications on enhancing the bactericidal efficacy of nano-ZnO. However, it is worth noting that appropriate modification techniques can also mitigate the toxicity of nano-ZnO [101]. By implementing suitable surface modifications, the environmental stability of nano-ZnO can be improved, thereby reducing its zinc ion release in aquatic environments and its ability to penetrate bacterial cells. This, in turn, can alleviate the adverse effects of nano-ZnO on the BNR process. Recent studies have demonstrated that the surface modification of ZnO NPs using poly (methacrylic acid), silica, or serum can reduce the cytotoxicity of ZnO NPs [102]. Additionally, hydrophobic ZnO NPs obtained through surface modification using dodecyl trichlorosilane have exhibited lower biotoxicity [103]. Consequently, the synthesis of nitrogen removal from bacteria-friendly nanomaterials through surface modification and other means represents a promising avenue for future research.

5.3. Artificial Way to Enhance the Adaptability of Nitrogen Removal Bacteria to Nano-ZnO

The enhancement of nitrogen removal from bacteria through artificial means to improve its adaptability to nanomaterials is a promising avenue for research. Ma et al. obtained a strain (Re) that demonstrated resistance to ZnO NPs by subjecting the wild strain to a prolonged exposure of 1 mM of ZnO NPs [104]. Transcriptomic analysis revealed that the up-regulated KEGG pathways of the Re strain, such as nitrogen metabolism, purine metabolism, and cationic antimicrobial peptide resistance, were associated with the adaptation mechanism of the Re to the ZnO NPs [104]. Bacteria can also resist nano-ZnO by secreting more EPS and upregulating zinc ion efflux proteins [58,105]. Therefore, the cultivation of nitrogen removal strains with good adaptability to nanomaterials and methods such as domestication and gene editing can aid in maintaining the performance of BNR systems in the treatment of wastewater containing nano-ZnO.

Furthermore, appropriately regulating the operational conditions of the BNR system can enhance its adaptability to nano-ZnO. For instance, Gao et al. discovered that endogenous N-acyl-homoserine lactone (AHL)-based quorum sensing regulation is advantageous for the adaptability of the BNR system to ZnO NP exposure [55]. By externally adding AHLs (N-Butyryl-DL-homoserine lactone, N-hexanoyl-L-homoserine lactone, and N-Decanoyl-L-homoserine lactone) to the BNR system, the ammonia oxidation process damaged by ZnO NPs can be effectively recovered [106]. Research has found that appropriate dissolved oxygen conditions are beneficial for AOB to cope with long-term ZnO NP pressure [107]. By adjusting the system's DO and adding external AHLs, the adaptability of the BNR system to ZnO NP biotoxicity was strengthened [108].

6. Conclusions and Prospects

The widespread use of nano-ZnO in production and daily life inevitably leads to its entry into wastewater treatment systems. Numerous studies have confirmed the adverse effects of nano-ZnO on the BNR process of wastewater and, to varying degrees, have revealed its inhibition mechanism. However, most experiments are laboratory-based and do not simulate the diversity and complexity of wastewater from municipal sewage plants. Therefore, future research needs to explore the impact of BNR processes in actual

wastewater containing nano-ZnO. It is also necessary to pay attention to the characteristics of nano-ZnO in actual wastewater and how to effectively detect it. The development of relevant detection/identification technology is essential because understanding the true state of nano-ZnO in actual wastewater is the basis for subsequent research. In addition, research on the response and adaptation of different BNR processes under the inhibitory effect of nano-ZnO is still relatively limited. In the future, a lot of in-depth research on this mechanism is still needed, especially in combination with continuously developing omics technologies such as transcriptomics, proteomics, metabolomics, and red genomics. Finally, based on a deep understanding of the inhibition mechanism of nano-ZnO and the adaptation mechanism of BNR processes, research on relevant artificial enhancement measures should also be extensively carried out.

Author Contributions: Conceptualization, T.-F.M., H.-X.M. and L.F.; resources, T.-F.M., J.W. and L.F.; writing—original draft preparation, T.-F.M., H.-X.M. and Y.-C.Y.; writing—review and editing, T.-T.C., Y.Y. and W.-L.L.; supervision, T.-F.M. and L.F.; project administration, T.-F.M. and L.F.; funding acquisition, T.-F.M. and L.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Chongqing Science and Technology Plan Project (cstc2019 jxjl20003, and CSTB2023NSCQ-MSX0963), the Science and Technology Research Program of the Chongqing Municipal Education Commission (Grant No. KJQN202000846), the Chongqing Technology and Business University Research Program (Grant No. 2056021), and the China Postdoctoral Science Foundation (Grant No. 2020M683218).

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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