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Effects of nanoparticles on anaerobic, anammox, aerobic, and algal-bacterial granular sludge:
a comprehensive review

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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1 **Abstract**

2 Nanoparticles (NPs) are of significant interest due to their unique properties, such as large
3 surface area and high reactivity, which have facilitated advancements in various fields.
4 However, their increased use raises concerns about environmental impacts, including on
5 wastewater treatment processes. This review examines the effects of different nanoparticles on
6 anaerobic, anammox, aerobic, and algal-bacterial granular sludge used in wastewater treatment.
7 CeO₂ and Ag NPs demonstrated adverse effects on aerobic granular sludge (AGS), reducing
8 nutrient removal and cellular function, while anaerobic granular sludge (AnGS) and anammox
9 granular sludge (AxGS) showed greater resilience due to their higher extracellular polymeric
10 substance (EPS) content. TiO₂ NPs had fewer negative effects on algal-bacterial granular sludge
11 (ABGS) than on AGS, as algae played a crucial role in enhancing EPS production and
12 stabilizing the granules. The addition of Fe₃O₄ NPs significantly enhanced both aerobic and
13 anammox granulation by reducing granulation time, promoting microbial interactions,
14 improving granule stability, and increasing nitrogen removal efficiency, primarily through
15 increased EPS production and enzyme activity. However, Cu and CuO NPs exhibited strong
16 inhibitory effects on aerobic, anammox, and anaerobic systems, affecting EPS structure,
17 cellular integrity, and microbial viability. ZnO NPs demonstrated dose-dependent toxicity, with
18 higher concentrations inducing oxidative stress and reducing performance in AGS and AnGS,
19 whereas AxGS and ABGS were more tolerant due to enhanced EPS production and algae-
20 mediated protection. The existing knowledge gaps and directions for future research on NPs
21 are identified and discussed.

22 **Keywords:** aerobic granular sludge, algal-bacterial granular sludge, anaerobic granular sludge,
23 anammox granular sludge, nanomaterials, wastewater treatment.

1 **1. Introduction**

2 In recent years, nanoparticles (NPs) have garnered significant interest due to their larger surface
3 areas compared to their bulk forms (Baig et al., 2021), greater reactivity (Grommet et al., 2020),
4 and tunable properties (Wong et al., 2020). These unique characteristics have driven
5 advancements in nanoscience and the use of NPs in diverse fields such as cosmetics (Fytianos
6 et al., 2020), biomedicine (Liu et al., 2021), food analysis (Garkani Nejad et al., 2021),
7 electronics (Zavanelli and Yeo, 2021), paints (Ganguli and Chaudhuri, 2021), and
8 environmental remediation (Lu and Astruc, 2020; Saleem et al., 2022). With the increased
9 production and utilization of NPs, their accidental or intentional release into the environment
10 has become a concern, prompting considerable attention to their potential ecological impacts
11 (Liu et al., 2022; Sajid, 2022). Several studies have demonstrated that nanomaterials (NMs) are
12 toxic to bacteria (Pardhi et al., 2020), algae (Saxena et al., 2020), fungi (Ameen et al., 2021),
13 plants (Juárez-Maldonado et al., 2021), and animals (Umapathi et al., 2022) in the environment.
14 Due to the increasing production of NPs and their high stability, these materials enter
15 wastewater treatment plants and accumulate in the sludge, potentially causing negative
16 consequences (Kedves et al., 2020).

17 In the literature, few studies have measured the concentrations of NPs in different wastewater
18 treatment plants, with levels ranging from ng/L to mg/L. Lazareva and Keller (2014) found that
19 the annual per capita release of metal-based NPs into wastewater treatment plants can be as
20 high as 35 grams. Meanwhile, Westerhoff et al. (2011) reported that the concentration of
21 titanium-dioxide (TiO₂) NPs in wastewater can reach up to 1.2 mg/L, while another study found
22 TiO₂ present in sewage at levels of up to 4 mg/L (Shutao Wang et al., 2017). In Switzerland,
23 the yearly released amounts of Ag and TiO₂ NPs to wastewater treatment plants are 3.27 and
24 249.22 tons, respectively (Mueller and Nowack, 2008). The concentrations of TiO₂ NPs in
25 effluent wastewater in the EU, U.S., and Switzerland were 2.01, 1.01, and 2.48 µg/L,
26 respectively (Gottschalk et al., 2009), leading to a continuously increasing content of NMs in
27 surface waters. Finally, Cervantes-Avilés and Keller (2021) collected influent and effluent
28 wastewater samples from a southern California municipal wastewater treatment plant, and the
29 contents of 13 different metal-based NPs were measured using single-particle inductively
30 coupled plasma mass spectrometry (spICP-MS). The contents of NPs detected in the influent
31 varied from 2.6 ng/L for Cd-based NPs to 10,700 ng/L for Ti-based NPs, while removal
32 efficiencies ranged from 70% to 99%.

1 Given that the most common wastewater treatment technologies are aerobic and anaerobic
2 activated sludge (AS) processes, numerous studies have investigated the potential negative
3 effects of NPs on AS. For example, Nguyen and Rodrigues (2018) reported that graphene (G)
4 and graphene-oxide (GO) NMs at 5 mg/L reduced the chemical oxygen demand (COD),
5 ammonia, and phosphate removal efficiencies of aerobic AS by 60%-70%, 55%-70%, and 50%-
6 70%, respectively. In the case of TiO₂ NPs, the removal of nutrients gradually decreased with
7 increasing NP concentrations from 2 to 60 mg/L (Li et al., 2017). At 10 mg/L, cell viability in
8 flocs significantly decreased, the microbial community shifted (Zhou et al., 2019), and the
9 oxygen uptake rate was reduced (Cervantes-Avilés et al., 2017). Similar observations were
10 made with copper oxide (CuO) NPs (Hou et al., 2015; Sen Wang et al., 2017; X. Wang et al.,
11 2017) and zinc oxide (ZnO) NPs (Daraei et al., 2021; Wang et al., 2020; Ye et al., 2021). In the
12 case of anaerobic floccular digestion, ZnO NPs at 30 and 150 mg/g total suspended solid (TSS)
13 inhibited methane production by 8% and 75%, respectively (Mu and Chen, 2011), while another
14 study reported a 25% and 80% decrease in methane production at the same ZnO NP
15 concentrations (Mu et al., 2011).

16 Despite being the most widespread technology in wastewater treatment, the AS process has
17 several disadvantages, such as high energy demand (Espinosa-Ortiz et al., 2023), low sludge
18 stability (Kedves et al., 2020), large amounts of sludge generation (Elmansour et al., 2022),
19 extensive space requirements (Waqas et al., 2020), and sensitivity to environmental changes
20 (Adav et al., 2008). Therefore, the application of new wastewater treatment processes is
21 inevitable (Garcia et al., 2022). Anaerobic granular sludge (AnGS), anammox granular sludge
22 (AxGS), aerobic granular sludge (AGS), and algal-bacterial granular sludge (ABGS) are
23 relatively new technologies in wastewater treatment, offering numerous advantages over
24 activated floccular sludge. These advantages include high-strength wastewater treatment (Show
25 et al., 2020), reduced bioreactor land space requirements and energy consumption (Hamza et
26 al., 2022; Zhao et al., 2021), lower sludge production (Sun et al., 2023), high resistance to toxic
27 materials (Liang et al., 2020), dense sludge (Samaei et al., 2023), fast settling velocity (Purba
28 et al., 2020), and high biomass retention (Franchi et al., 2024). Due to these benefits, AGS
29 (Semaha et al., 2023; Tsertou et al., 2023; van Dijk et al., 2021), AnGS (Caizán-Juanarena et
30 al., 2020), and AxGS (Abma et al., 2010; Driessen and Hendrickx, 2021) technologies are
31 already being used on an industrial scale to treat industrial and domestic wastewaters. For
32 ABGS, there is information from lab-scale experiments showing that bioreactors can efficiently
33 treat municipal wastewater (Ji et al., 2021; Purba et al., 2023; Zhang et al., 2022).

1 The aim of this review paper is to comprehensively evaluate the impacts of various
2 nanoparticles on different types of granular sludge used in wastewater treatment, including
3 anaerobic granular sludge, anammox granular sludge, aerobic granular sludge, and algal-
4 bacterial granular sludge. It primarily discusses (a) the interaction mechanisms between
5 different NPs and granular sludge, focusing on the physicochemical properties of the NPs that
6 influence their behavior and impact on sludge stability and microbial activity; (b) the short-term
7 and long-term effects of NP exposure on the performance of different granular sludge systems,
8 including changes in nutrient removal efficiencies, sludge characteristics, and microbial
9 community dynamics; (c) the toxicity mechanisms of NPs on microbial communities within
10 granular sludge, identifying key pathways and genetic responses that mediate tolerance or
11 susceptibility to NP-induced stress; and (d) the potential recovery mechanisms of granular
12 sludge systems following NP-induced inhibition, investigating the role of various
13 environmental and operational factors in mitigating adverse effects and enhancing sludge
14 resilience.

15 **2. Types of nanomaterials and their properties**

16 Nanomaterials exhibit unique physical, chemical, and biological properties that are distinct
17 from their bulk counterparts (Hiebner et al., 2020). These properties originate due to their
18 nanoscale dimensions, high surface area to volume ratio, and quantum effects. The most
19 common NMs include carbon-based, metal, and metal oxide NPs.

20 *2.1. Carbon-based nanomaterials*

21 Graphene is a single layer of carbon atoms arranged in a two-dimensional honeycomb lattice.
22 It is known for its exceptional electrical conductivity, mechanical strength, and thermal
23 conductivity (Seifi and Kamali, 2021). High surface area (2630 m²/g) and electron mobility (up
24 to 200,000 cm²/V s) of graphene make it suitable for applications in electronics, energy storage,
25 and composite materials (Iqbal et al., 2020; Liu et al., 2020). Graphene oxide is derived from
26 the oxidation of graphite, introducing oxygen-containing functional groups such as hydroxyl,
27 epoxy, and carboxyl groups (Yan et al., 2021). These groups make GO hydrophilic and
28 chemically reactive (Chouhan et al., 2020). GO is an insulator, but its electrical properties can
29 be partially restored through reduction processes to form reduced graphene oxide (rGO), which
30 finds applications in sensors, energy storage, and as a reinforcement in composites (*Table 1*)
31 (Olabi et al., 2021; Xue et al., 2022). Single-walled carbon nanotubes (SWCNTs) are composed
32 of a single graphene sheet rolled into a cylindrical shape with diameters ranging from 0.4 to 2

1 nm (Shafiq et al., 2021). They exhibit remarkable electrical conductivity (up to 10^6 S/cm), high
2 thermal conductivity (~ 700 W/m K), and mechanical strength (Zhan et al., 2022); therefore,
3 these NMs are used in nanoelectronics, conductive composites, and sensors (Fazi et al., 2023;
4 Kharlamova and Kramberger, 2021). Multi-walled carbon nanotubes (MWCNTs) consist of
5 multiple concentric graphene cylinders with diameters ranging from 2 to 100 nm (Kazemi et
6 al., 2021). They have slightly lower electrical conductivity and thermal conductivity compared
7 to SWCNTs but offer higher mechanical strength due to their multi-layer structure (Gao et al.,
8 2020; Mansouri Sarvandani et al., 2021). Therefore, MWCNTs are utilized in applications such
9 as structural reinforcements, conductive films, and drug delivery systems (Samal et al., 2022;
10 Thakur et al., 2023).

11 2.2. Metal-based nanomaterials

12 One of the most common metal-based nanomaterials is nanoscale zero-valent iron (nZVI)
13 because it can degrade pollutants such as chlorinated organic compounds (Venkateshaiah et al.,
14 2022) and heavy metals through reduction processes (Tarekegn et al., 2021). nZVI NPs are
15 usually used for environmental remediation due to their high reactivity with contaminants (Ken
16 and Sinha, 2020). Copper (Cu) NPs exhibit excellent electrical and thermal conductivity
17 (Muhammed Ajmal et al., 2021), antimicrobial activity (Jayarambabu et al., 2020), and catalytic
18 properties (Dan et al., 2021), so they are widely used in conductive inks, antimicrobial coatings,
19 and as catalysts in chemical reactions (*Table 1*) (Siddiqi and Husen, 2024). Silver (Ag) NPs are
20 known for their strong antimicrobial properties, optical characteristics, and high electrical
21 conductivity (Pryshchepa et al., 2020; Tabassum et al., 2024). For this reason, Ag NPs are
22 applied in medical devices, wound dressings, conductive inks, and as catalysts (Alshameri and
23 Owais, 2022; Kalantari et al., 2020).

24 2.3. Metal oxide nanomaterials

25 CuO, ZnO, and TiO₂ NPs are widely used in gas sensors, catalysis, as antibacterial agents,
26 sunscreens, photocatalysis, and as catalysts in various chemical reactions (Ahmed et al., 2020;
27 Baran Aydın et al., 2022; Sabzehei et al., 2020). These nanomaterials exhibit high surface area,
28 catalytic and photocatalytic activity, high exciton binding energy, and UV absorption
29 characteristics, making them suitable for chemical sensors and environmental applications
30 (*Table 1*) (Kumar et al., 2022; Shinde et al., 2022). Nickel oxide (NiO) NPs exhibit good
31 electrical conductivity, chemical stability, and catalytic properties (Sivagami and Asharani,
32 2022), while iron oxide (Fe₃O₄) NPs exhibit magnetic properties, high surface area, and half-

1 metallicity (Nguyen et al., 2021). NiO NPs are widely used in battery electrodes, fuel cells, and
 2 as catalysts in organic synthesis (Rakhimbek et al., 2024), whereas the magnetic nature of Fe₃O₄
 3 makes them ideal for applications in magnetic resonance imaging (MRI) and targeted drug
 4 delivery systems (Fattahi Nafchi et al., 2022).

5 **Table 1.** Properties and applications of carbon-, metal-, and metal oxide-based nanomaterials.

	Type	Shape	Color	Stability	Applications	References
Carbon-based NMs	G	Sheet	Black	High	Electronics, composite materials, energy storage	(Liu et al., 2020)
	GO	Sheet	Brown	Moderate	Biomedicine, sensors, environmental applications	(Kornilov and Gubin, 2020)
	SWCNT	Tube	Black	High	Electronics, composite materials, drug delivery	(Rathinavel et al., 2021)
	MWCNT	Tube	Black	High	Electronics, composite materials, filtration	(Mansouri Sarvandani et al., 2021)
Metal-based NMs	Ag	Spherical, rod-like, cubic	Yellowish-brown	Moderate to high	Antibacterial, medical imaging, electronics, catalysis	(Alshameri and Owais, 2022)
	Cu	Spherical, rod-like	Reddish-brown	Low	Antibacterial, conductive inks, catalysis	(Siddiqi and Husen, 2024)
	Ni	Spherical	Black	Moderate to high	Catalysis, magnetic materials, hydrogenation	(Hassan et al., 2022)
	nZVI	Spherical	Black	Low	Environmental remediation, pollutant degradation	(Ken and Sinha, 2020)
Metal oxide-based NMs	ZnO	Spherical, rod-like	White	High	UV protection, antimicrobial, sensors	(Noman et al., 2022)
	CuO	Spherical, rod-like	Black	Moderate	Antimicrobial, catalysts, sensors	(Nassar et al., 2022)
	TiO ₂	Spherical, rod-like, tubular	White	High	Photocatalysis, UV protection, sensors	(Al-Etaibi and El-Asasery, 2020)
	CeO ₂	Spherical, rod-like	Yellow	High	Catalysts, UV protection, biomedical	(Wang et al., 2021)
	NiO	Spherical, rod-like	Green	Moderate	Catalysts, battery materials, sensors	(Rakhimbek et al., 2024)
	Fe ₃ O ₄	Spherical, cubic	Black	High	Magnetic materials, biomedical, catalysts	(Nguyen et al., 2021)
	SiO ₂	Spherical	White	High	Catalysts, drug delivery, sensors	(Razzaghi et al., 2023)
	MgO	Spherical	White	High	Antimicrobial, catalysts, sensors	(Manjunatha et al., 2021)
MnO ₂	Spherical, rod-like	Black	Moderate	Catalysts, rechargeable battery electrode, sensors	(Xu et al., 2019b)	

6

1 Cerium oxide (CeO₂) NPs are known for their high oxygen storage capacity and redox
2 properties; therefore, this type of NP is used as catalysts in automotive exhaust systems to
3 reduce emissions, in fuel cells to enhance efficiency, and for environmental remediation due to
4 their ability to scavenge reactive oxygen species (Wang et al., 2021; Xu et al., 2023).

5 **3. Types of granular sludge wastewater treatment technologies**

6 Anaerobic, anammox, aerobic, and algal-bacterial granular sludge technologies apply dense
7 microbial aggregates to enhance wastewater treatment. Anaerobic and anammox systems
8 operate under anaerobic and anoxic conditions, producing biogas and removing nitrogen
9 through ammonium oxidation. Aerobic and algal-bacterial systems facilitate the simultaneous
10 removal of organic matter, nitrogen, and phosphorus. These technologies are efficient in
11 biomass retention, fast settling, and resilience, making them suitable for treating high-strength
12 and complex wastewater.

13 *3.1. Anaerobic granular sludge*

14 Anaerobic digestion (AD) is a biological process where microorganisms degrade organic matter
15 without oxygen, producing biogas (Nie et al., 2021). AnGS consists of dense microbial
16 aggregates that settle rapidly, aiding in the separation of treated liquid from sludge (Show et
17 al., 2020). The granules form through microbial aggregation and the production of extracellular
18 polymeric substances (EPS), influenced by factors like seed sludge and environmental
19 conditions. Various models, such as the "spaghetti" and "multi-layer" models, describe the
20 structural development of these granules (Lim and Kim, 2014). AnGS has several advantages
21 over conventional sludge, including higher biomass retention, efficient organic matter
22 degradation under high loading, and rapid settling (Zhao et al., 2021). It offers greater resilience
23 to environmental fluctuations, making it more stable under varying conditions (Ma et al., 2013).
24 The granules have an organized structure, with different microbial communities in distinct
25 zones that facilitate the convert of organic compounds into biogas (Wang et al., 2020). AnGS
26 is effective in treating various high-strength wastewaters, such as those from the food, beverage,
27 pulp, paper, and chemical industries, and municipal sewage (Bakraoui et al., 2020; Fuentes et
28 al., 2021). It is particularly suited for industrial applications due to its resilience to high organic
29 loads and pollutant removal efficiency, while biogas production contributes to renewable
30 energy generation (Xiong et al., 2020).

31 *3.2. Anammox granular sludge*

1 Anammox (anaerobic ammonium oxidation) granular sludge is an advanced wastewater
2 treatment technology that utilizes anaerobic ammonium oxidation to efficiently remove
3 nitrogen (Adams et al., 2022). These granules form through a complex process involving
4 physicochemical and biological interactions, with EPS facilitating the aggregation of anammox
5 bacteria (Wang et al., 2020). Various models, including the selection pressure and positive ion-
6 bonding models, explain the granulation process, influenced by factors like hydraulic retention
7 time and nitrogen loading. The granules multilayered structure, incorporating hydroxyapatite
8 (HAP) cores, enhances their settling velocity and stability, contributing to efficient nitrogen
9 removal (Xue et al., 2023). AxGS systems provide higher biomass retention, allowing for
10 greater volumetric loading and specific surface areas, resulting in more efficient nitrogen
11 removal and reduced costs compared to traditional nitrification-denitrification processes (Lin
12 et al., 2023). This technology also reduce energy consumption and greenhouse gas emissions,
13 as they do not require organic carbon sources or extensive aeration (Zhuang et al., 2020). AxGS
14 is used to treat a variety of nitrogen-rich wastewaters, such as landfill leachate, industrial
15 effluents, and agricultural runoff (Madeira and de Araújo, 2021). Applications include the
16 treatment of slaughterhouse wastewater, swine wastewater, and sludge digestion liquids
17 (Ishimoto et al., 2021; Silveira et al., 2021). Additionally, AxGS systems can recover
18 phosphorus through the formation of anammox-HAP granules, offering simultaneous nitrogen
19 removal and phosphorus recovery, enhancing nutrient management in wastewater treatment
20 (Xue et al., 2022).

21 3.3. Aerobic granular sludge

22 Aerobic granules are dense microbial aggregates with excellent settling properties, allowing for
23 efficient biomass separation from treated wastewater (Liébana et al., 2023; Tsertou et al., 2023).
24 Formed under specific operational conditions, often in sequencing batch reactors (SBRs),
25 selective pressures such as short settling times and high shear forces promote granulation
26 (Kedves et al., 2020). The formation process involves microbial cell aggregation, enhanced by
27 EPS and quorum sensing (QS), which regulates microbial interactions and stabilizes the
28 granules (Peng et al., 2022). Quorum sensing (QS), mediated by acyl-homoserine lactones
29 (AHLs), plays a critical role in the aerobic granulation process by facilitating microbial
30 communication, enhancing EPS production (which stabilizing the structure of the granules),
31 and regulating community interactions. The transition from AHL-quenching to AHL-producing
32 bacteria during granulation accelerates the aggregation and stability of microbial communities

1 (Pan et al., 2024). Hydrodynamic shear forces and feast-famine conditions further shape these
2 aggregates into dense, stable granules (de Sousa Rollemberg et al., 2018).

3 Aerobic granules possess a layered structure with distinct aerobic, anoxic, and anaerobic zones,
4 enabling the simultaneous removal of organic material, nitrogen, and phosphorus (Liu et al.,
5 2009). Compared to conventional activated sludge (CAS), AGS offers higher biomass
6 retention, better settling, and resistance to high organic and inorganic loads (Purba et al., 2020).
7 Additionally, AGS systems reduce sludge production, lower energy requirements, and are more
8 cost-effective, with studies indicating space reductions of 50-75% and energy savings of 20-
9 40% over CAS systems (Hussain et al., 2024). AGS is versatile, treating a wide range of
10 wastewaters, including domestic sewage and industrial effluents containing toxic or refractory
11 substances like phenols and heavy metals. It has been successfully applied at lab scale for
12 wastewaters from industries such as dairy, fish canning, landfill leachate, and textiles (Dababat
13 et al., 2023; Hussain et al., 2024; Saad et al., 2023). Full-scale implementations worldwide have
14 proven the practical applicability and efficiency of AGS in real-world wastewater treatment (de
15 Sousa Rollemberg et al., 2018; Nancharaiah and Kiran Kumar Reddy, 2018).

16 *3.4. Algal-bacterial granular sludge*

17 ABGS or microalgal-bacterial granular sludge (MBGS) represents a novel wastewater
18 treatment technology that combines the advantages of algae and bacteria, forming robust
19 granules for efficient nutrient removal and system stability (Kant Bhatia et al., 2022). These
20 granules develop in sequencing batch reactors (SBRs), with microalgae performing
21 photosynthesis to produce oxygen, which is utilized by bacteria, enhancing overall treatment
22 efficiency (Fard and Wu, 2023). Small granules (<1 mm) exhibit a uniform structure, while
23 larger granules (>1 mm) develop a double-layered configuration, with a microalgae-dominated
24 outer layer and a bacteria-rich inner core. This arrangement facilitates oxygen utilization and
25 improves nutrient removal through a high-porosity structure (Ji and Liu, 2021; Zhang et al.,
26 2022). MBGS offers several advantages over CAS and AGS systems, including reduced energy
27 consumption due to the internal oxygen source provided by photosynthetic algae and enhanced
28 CO₂ sequestration, which reduces greenhouse gas emissions (Sanchez-Sanchez et al., 2023).
29 The system supports simultaneous nitrification, denitrification, and phosphorus removal,
30 improving treatment efficiency and stability under varying environmental conditions (Fard and
31 Wu, 2023). MBGS has shown high removal rates for organic matter, ammonia, and phosphorus
32 in various types of wastewaters, including municipal, industrial, and agricultural (Cai et al.,
33 2022; Xiong et al., 2023). Additionally, MBGS is effective in treating wastewater containing

1 hazardous chemicals and heavy metals. The microalgae within the granules aid in assimilating
2 and detoxifying these pollutants, contributing to resource recovery from wastewater (Wang et
3 al., 2023; Yang et al., 2021).

4 **4. Effects on anaerobic granular sludge**

5 Li et al. (2015) reported that the SWCNTs at a concentration of 1000 mg/L significantly
6 enhanced the efficiency of substrate utilization and methane production rates. The COD
7 concentration decreased more rapidly in the presence of SWCNTs, dropping to 189 mg/L in 24
8 hours compared to 260 mg/L in the control. Methane production was initially faster in the
9 SWCNT-treated reactors, with the methane production rate constant nearly doubling from
10 0.0097 to 0.0194 h⁻¹, though the maximum methane yield did not show a significant difference.
11 The microbial community analysis revealed a higher abundance of acetotrophic methanogens,
12 primarily *Methanosaeta concilii*, and a dominant presence of *Clostridium* among the bacteria,
13 suggesting a robust syntrophic interaction facilitated by the enhanced electrical conductance
14 and protective EPS matrix. Ambuchi et al. (2017) also demonstrated that the addition of Fe₃O₄
15 NPs at 750 mg/L and MWCNTs at 1500 mg/L to AnGS significantly enhanced biogas and
16 methane production during the treatment of beet sugar industrial wastewater. Fe₃O₄ NPs
17 increased biogas production to 25144.4 mL/g VSS and methane production to 8374.9 mL/g
18 VSS, while MWCNTs resulted in 21876.0 mL/g VSS of biogas and 7313.2 mL/g VSS of
19 methane. COD removal efficiency reached 95% with Fe₃O₄ NPs, compared to 89% with
20 MWCNTs and 92% in the control. Microbial community analysis revealed that Fe₃O₄ NPs
21 enriched the archaeal population, particularly the *Euryarchaeota* phylum (63%), while
22 MWCNTs induced the growth of *Bacteroidetes* (11%) and *Firmicutes* (8%) phyla. This
23 indicates that the nanoparticles not only improved the degradation process but also altered the
24 microbial community structure, favoring methanogenic archaea with IONPs and fermentative
25 bacteria with MWCNTs. Additionally, both NPs stimulated the production of EPS, providing
26 protection to microbial cells against cytotoxic effects. He et al. (2017) also reported a positive
27 effect of nZVI at 838 and 1675 mg/L on AnGS. At this concentration the methane production
28 increased by 15% and 30%, while the concentration of EPS in the sludge significantly
29 decreased, dropping from 180 to 160 and 140 mg/g VSS. The nZVI particles were primarily
30 adsorbed on the surface of the sludge, preventing significant internal cellular damage. The
31 microbial community analysis revealed a shift towards hydrogenotrophic methanogens,
32 particularly *Methanobacteria*, which increased due to the hydrogen produced from nZVI
33 reactions, while acetoclastic methanogens involved in glucose degradation were reduced.

1 When the AnGS were exposed to Ni NPs, the bioreactor performance continuously decreased
2 by increase NPs content to 1, 50, and 200 mg/g TSS over 6 days. At a concentration of 200
3 mg/g TSS, glucose degradation slowed markedly, taking up to 3-5 days compared to just 0.5
4 days in the control. Methane production also dropped substantially, from 5.61 to 2.00 mmol
5 over four cycles. The presence of 200 mg/g TSS Ni NPs resulted in volatile fatty acid (VFA)
6 accumulation and a decrease in pH to 6.37. The microbial community structure was altered,
7 with a reduction in the diversity of bacterial populations, notably those involved in fatty acid
8 transformation, while methanogenic archaea in the inner layers of the granules were less
9 affected, indicating a stratified response to the NP stress (He et al., 2019). Ma et al. (2013) when
10 compared the effect of CeO₂ NPs on AnGS and AS, they found that total short-chain fatty acid
11 (SCFA) production in AnGS increased by 30-40% at 5 and 50 mg/g VSS but decreased by 35%
12 at 150 mg/g VSS. Conversely, AS showed a consistent reduction in SCFA production by 15-
13 20% across all tested concentrations. Methane production remained unaffected for both sludge
14 types at all NP dosages. Examination of the microbial community indicated an increase in the
15 ratio of dead cells by 2-8% in granular sludge exposed to 150 mg/g VSS, while flocculent
16 sludge exhibited significant cell membrane damage and increased lactate dehydrogenase (LDH)
17 release at both 5 and 150 mg/g VSS. Additionally, granular sludge showed an increase in EPS
18 production by 30% and 40% at 5 and 150 mg/g VSS, respectively, whereas flocculent sludge
19 showed a decrease in EPS production by 35% and 30% at the same dosages. ZnO NPs at 10
20 and 50 mg/g TSS had negligible effects on the EPS and methane production over eight days.
21 However, at higher concentrations (100 and 200 mg/g TSS), protein levels dropped from 540
22 mg/L in the control to 230 and 160 mg/L, with among this, methane production was reduced
23 by 55% and 80%. Additionally, the microbial community within the sludge showed increased
24 cell death, particularly among methanogenic *Archaea*, as evidenced by a rise in dead cells in
25 the granules center, correlating with the observed decline in overall physiological activity (Mu
26 et al., 2012).

27 Li et al. (2022) compared the effects of TiO₂, ZnO, and CuO NPs at 10, 20, 50, 100, 150, and
28 200 mg/L over 2 days and revealed that CuO NPs were the most toxic to AnGS, significantly
29 reducing methanogenic activity by 80% for acetoclastic methanogens and 50% for
30 hydrogenotrophic methanogens at a concentration of 200 mg/L. ZnO NPs also demonstrated
31 inhibitory effects, with methanogenic activity decreasing by 45% for acetoclastic methanogens
32 and 25% for hydrogenotrophic methanogens at the same concentration. In contrast, TiO₂ NPs
33 did not inhibit methanogenesis and instead stimulated an increase in EPS production. The

1 microbial community analysis indicated that the toxic effects of CuO and ZnO NPs led to a
2 shift in methanogenic and acidogenic microbial populations, highlighting the protective role of
3 EPS against nanoparticle toxicity. CuO NPs caused a significant increase in reactive oxygen
4 species (ROS) production and lactate dehydrogenase (LDH) release to 140% and 280%,
5 indicating severe cytotoxicity and cell membrane damage. Li et al. (2017) also investigated the
6 impacts of TiO₂, ZnO, and CuO NPs, but only at 5 mg/L and over 90 days. They reported that
7 TiO₂ NPs reduced biogas production only by 30% and methane yield by 15%, primarily
8 affecting the outer structure of the granules. CuO NPs exhibited the highest toxicity, completely
9 suppressing methane yields after 39 days and consistently inhibiting glucose conversion. ZnO
10 NPs temporarily stimulated methanogenesis for up to 5 days but ultimately reduced glucose
11 degradation to 30% and completely suppressed methane production by day 52. The study also
12 observed significant structural collapse and cell lysis in the AnGS exposed to CuO and ZnO,
13 caused by the generation of ROS and the release of metal ions. Changes in the microbial
14 community included the suppression of acidogens and acetogens, leading to altered SCFA
15 production and accumulation. Gonzalez-Estrella et al. (2013) examined the potential effects of
16 Ag, Al₂O₃, CeO₂, Fe, Fe₂O₃, Mn₂O₃, SiO₂, TiO₂, Cu, ZnO, and CuO NPs at 1,500 mg/L on the
17 methanogenic activity in AnGS. Cu NPs completely inhibited methanogenic activity at
18 concentrations of 1,500 mg/L, with IC₅₀ values of 62 mg/L for acetoclastic and 68 mg/L for
19 hydrogenotrophic methanogens. ZnO NPs also demonstrated considerable toxicity, with IC₅₀
20 values of 87 mg/L for acetoclastic and 250 mg/L for hydrogenotrophic methanogens. CuO NPs
21 affected acetoclastic methanogens with an IC₅₀ of 223 mg/L but had no significant impact on
22 hydrogenotrophic methanogens. The primary mechanism of toxicity was attributed to the
23 release of metal ions (Cu²⁺ and Zn²⁺) from the NP. Ag, Al₂O₃, CeO₂, Fe, Fe₂O₃, Mn₂O₃, SiO₂,
24 TiO₂ NPs had no significant inhibition of methanogenic activity (*Table S1*).

25 The nZVI reacted with water, forming FeO(OH) and iron oxide (which adsorbed onto the
26 surface of the granules), producing hydrogen gas in the process, thus increasing the relative
27 abundance of hydrogenotrophic methanogens and leading to higher biogas yields (He et al.,
28 2017). The improved performance is likely due to the enhanced electrical conductivity provided
29 by Fe₃O₄, SWCNTs, and MWCNTs, which facilitates more efficient direct interspecies electron
30 transfer between syntrophic bacteria and methanogens (Ambuchi et al., 2017; Li et al., 2015).
31 In contrast, metal- and metal oxide-based nanoparticles such as Ni NPs, ZnO NPs, and CuO
32 NPs negatively affect AnGS performance. There are several possible reasons for this. First,
33 these nanoparticles can release Ni²⁺, Zn²⁺, and Cu²⁺ ions, which penetrate into the granules and

1 inhibit the activity of methanogenic microorganisms residing there (Altaş, 2009; Wang et al.,
2 2016). The greater the concentration of nanoparticles, the more ions are present in the medium,
3 thus hindering the biogas production process. Additionally, another explanation could be the
4 Trojan-horse mechanism, where the nanoparticles penetrate the cell membrane and release
5 metal ions inside the cell, inhibiting its function (He et al., 2019; Limbach et al., 2007). Lastly,
6 direct physical interactions between AGS microorganisms and NPs can severely compromise
7 the integrity of the cytoplasmic membrane, allowing the NPs to penetrate into the cells (Wang
8 et al., 2016).

9 **5. Effects on anammox granular sludge**

10 Weng et al. (2023) demonstrated that nZVI added to the anammox system, at concentrations
11 ranging from 0 to 3.0 mM, dissolved and released Fe^{2+} ions, peaking at 2.52 mg/L in EPS
12 solutions within 60 minutes. These nanoparticles adsorbed onto the EPS and coated the surface
13 of the AxGS, leading to the formation of iron oxides like Fe_3O_4 . The presence of nZVI
14 significantly altered the morphology of the sludge, with a noticeable aggregation and deposition
15 of NPs. Additionally, nZVI penetrated the bacterial cells within the sludge, forming highly
16 absorbable substances and resulting in structural changes. Elreedy et al. (2021) presented
17 different impact of G and Fe_2O_3 NPs on the anammox granular sludge. The optimal
18 concentration of G (10 mg/L) significantly enhanced nitrogen removal, achieving NH_4^+ -N and
19 NO_2^- -N removal efficiencies of 85% and 95%, respectively. This improvement was
20 accompanied by a 15% increase in hydrazine dehydrogenase (HDH) enzyme activity and better
21 EPS formation, leading to improved bacterial granulation. In contrast, Fe_2O_3 NPs at 100 mg/L
22 increased nitrogen removal mainly through abiotic adsorption (90% efficiency) without
23 enhancing HDH activity and causing oxidative stress. The microbial community analysis
24 revealed that the abundance of the anammox-related genus *C. Jettenia* increased from 12% to
25 12% with G, while it decreased to 8% with Fe_2O_3 NPs, indicating that G supported anammox
26 bacterial growth better than Fe_2O_3 NPs.

27 Xu et al. (2020) investigated the impact of Fe_3O_4 NPs at 2-200 mg/L over a six-month period.
28 It was found that the nitrogen removal efficiency remained stable around 90% even with Fe_3O_4
29 concentrations up to 200 mg/L. SAA initially decreased slightly but then increased significantly
30 from 290 to 380 mg TN/g VSS at the highest nanoparticle concentration. The presence of Fe_3O_4
31 enhanced sludge characteristics, including an increase in VSS to 35 g/L and heme c content to
32 $2.7 \mu\text{mol/g}$ VSS. The microbial community analysis revealed that the relative abundance of
33 *Candidatus Kuenenia* increased by 35% at 200 mg/L Fe_3O_4 , indicating a positive effect on the

1 dominant bacteria responsible for the anammox process. Yun et al. (2023) observed similar
2 when the amount of Fe₃O₄ NPs was 2.4 g/L, the maximum total nitrogen loading rate (TNLR)
3 reached 0.82 mg N/m³ d, significantly higher than the 0.54 mg N/m³ d) observed in the control.
4 The Fe₃O₄ NPs also promoted better sludge granulation by decreasing the start-up time from
5 37 to 33 days and increasing the production of EPS, resulting in more compact and stable
6 granular sludge. The relative abundance of *Candidatus Kuenenia* was also higher after
7 introduction NPs at 2.4 g/L (33%) compared to the control reactor (26%). In contrast, Zhang et
8 al. (2024) found that Fe₃O₄ NPs a concentration of 1000 mg/L caused a significant decline in
9 nitrogen removal efficiency (NRE) to 60% and nitrogen removal rate (NRR) to 0.8 g/L d,
10 primarily due to increased ROS production, which damaged the membrane integrity and
11 metabolic processes of the anammox bacteria. The microbial community analysis revealed that
12 the relative abundance of *Planctomycetota*, particularly the genus *Candidatus Brocadia*,
13 decreased from 31% to 25%, indicating inhibited growth. However, other phyla like *Chloroflexi*
14 and *Bacteroidota* increased, suggesting enhanced resistance and stability of the sludge under
15 nanoparticle stress. Recovery mechanisms were observed, with increased expression of genes
16 related to oxidative stress defense, allowing the community to eventually restore its nitrogen
17 removal performance.

18 Ma et al. (2022) found that magnesium oxide (MgO) NPs at 2 and 5 mg/L did not influence the
19 AxGS performance, but at concentrations of 20 and 50 mg/L significantly reduced the specific
20 anammox activity (SAA) by 24% and 37% of the original value, respectively. The NRE also
21 dropped dramatically, reaching 26% at the highest concentration of 50 mg/L. Additionally, the
22 microbial community structure was altered, with an increase in microbial diversity and richness
23 indices, while the dominant genus, *Candidatus Kuenenia*, maintained a high abundance despite
24 the reduced nitrogen removal performance. The addition of 40 mg/L bull serum albumin (BSA)
25 effectively mitigated the toxicity of nanoparticles, restoring anammox activity to 97% of its
26 initial level. Manganese dioxide (MnO₂) NPs, at concentrations ranging from 1 to 200 mg/L,
27 significantly enhanced the nitrogen removal efficiency, with a high efficiency of 90% observed
28 at 200 mg/L. SAA also increased, reaching 660 mg TN/g VSS day at the highest concentration.
29 The EPS and sludge settleability improved, with EPS production increasing from 360 to 480
30 mg/g VSS. Additionally, the relative abundance of the *Candidatus Kuenenia* increased from
31 17% to 24% with the addition of 200 mg/L NPs, indicating a positive correlation between NP
32 concentration and microbial enhancement (Xu et al., 2019b). Zhang et al. (2018) demonstrated
33 that anammox granules maintained good nitrogen removal efficiency (87%), while the SAA

1 increased significantly by 36% despite exposure to Ag NPs at concentrations of up to 50 mg/L.
2 The EPS content also rose, with a significant increase in protein secretion and a decrease in
3 polysaccharides, suggesting an adaptive defense mechanism. Contrary to MnO₂ and Ag NPs,
4 NiO NPs at concentrations up to 10 mg/L, the nitrogen removal performance of the anammox
5 system was enhanced, showing a TNRE of 90%. However, higher concentrations of NiO NPs
6 (10-60 mg/L) led to significant decrease in reactor performance, with TNRE dropping to 35%
7 at 60 mg/L. The SAA and heme c content increased initially but decreased drastically at higher
8 NP concentrations, with SAA falling from 240 to 120 mg TN/g VSS day at 60 mg/L. The
9 abundance of *Candidatus kuenenia* initially increased but then dropped to 20% at 60 mg/L,
10 before recovering to 23% after stopping addition of NiO NPs (Xu et al., 2019a).

11 Zhang et al. (2018b) investigated the shock-effects of ZnO NPs and it was found that
12 concentrations of 1-5 mg/L NPs had no significant impact on reactor performance. However, a
13 10 mg/L shock led to a 90% reduction in nitrogen removal capacity within three days. Despite
14 this initial inhibition, the resistance and resilience of reactors improved with repeated shock,
15 ultimately enhancing its stability. Microbial community analysis revealed that transient
16 disturbances increased the relative abundance of anammox bacteria, particularly the genus
17 *Kuenenia*, indicating a shift towards a more resilient community despite a reduction in overall
18 diversity. Song et al. (2018) revealed that ZnO NPs significantly inhibited the activity of
19 anammox granules at concentrations ranging from 5 to 100 mg/g VSS. The IC₅₀ was found to
20 be 12 mg/g VSS, indicating substantial inhibition at relatively low levels. At 5 mg/g VSS, the
21 SAA decreased by 12%, while at 10 mg/g VSS, the SAA dropped by 48%. Severe inhibition
22 was observed ≥ 20 mg/g VSS, with almost complete loss of activity within 2 hours. The
23 inhibition was primarily due to the release of zinc ions from the ZnO NPs, which affected both
24 nitrite and ammonium conversion kinetics. Zhao et al. (2019) also observed the inhibitory effect
25 of ZnO NPs, wherein the SAA reduction were 18%, 41%, and 64% at concentrations of 5, 50,
26 and 150 mg/L, respectively. The nanoparticles reduced the content of EPS up by 49%,
27 worsening the protective matrix around the microorganisms. This reduction in EPS resulted
28 more susceptible granules to the NPs and released zinc ions, which led to increased production
29 of ROS and decreased cell viability. Similarly to the above, Sari et al. (2020) found that acute
30 exposure to concentrations of up to 200 mg/L caused severe inhibition, with an 80% reduction
31 in nitrogen removal rates. Long-term exposure revealed that the anammox granules could
32 maintain stable nitrogen removal efficiency up to 70 mg/L, but at 100 mg/L the ZnO NPs

1 resulted a significant reduction in bioreactor performance ($\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ removal
2 decreased to 1.5% and 5.5%).

3 Zhang et al. (2017) revealed that exposure to Cu NPs at 5 mg/g SS significantly inhibited
4 anammox activity, reducing it to 47% compared to the control. This exposure also caused
5 damage to cell membranes, with LDH levels rising to 110%, and increased extracellular N_2H_4
6 concentration by 16-fold. The presence of CuO NPs or ZnO NPs did not notably change the
7 toxicity of Cu NPs. However, the introduction of EDTA or S^{2-} mitigated the adverse effects,
8 increasing anammox activity to around 80%. Zhang et al. (2018c) also observed that the CuO
9 NPs had no negative effect on granules even at 160 mg/L. However, at a concentration of 5
10 mg/L, Cu NPs reduced SAA by 91%, dehydrogenase activity by 95%, and EPS amount by 44%.
11 The microbial community analysis revealed that exposure to Cu NPs caused a decline in the
12 abundance of key functional genes and a shift in community structure, whereas CuO NPs had
13 a lesser impact, maintaining stable microbial populations and activity. Cheng et al. (2020)
14 investigated the joint effects of Cu NPs and oxytetracycline (OTC), which materials at
15 concentrations of 0.5 mg/L caused a slight inhibition on bioreactor performance. Inhibition
16 became more pronounced at 1 mg/L, and during the first shock phase with 5 mg/L Cu NPs and
17 2 mg/L OTC, performance rapidly deteriorated, with ammonia levels increasing to 260 mg/L
18 and nitrite to 220 mg/L. However, the resistance of anammox bacteria improved after the
19 second shock (2.5 mg/L Cu NPs and 2 mg/L OTC), enhancing recovery. Fu et al. (2021)
20 examined the impact of 5 mg/L Cu NPs on different types of anammox granules: antibiotic-
21 exposed granules (R1) and normal granules (C1). The nitrogen removal efficiency of R1
22 decreased by 20%, compared to a 9% decrease in C1, over a two-week period. SAA in both
23 granules dropped significantly, with a 56% reduction in C1 and a 52% reduction in R1 by day
24 52. The abundance of *Candidatus Kuenenia* fell by 28% in C1 and 36% in R1. Zhang et al.
25 (2017b) compared the potential negative effects of Cu, CuO, ZnO, and Ag NPs, during which
26 they established that while CuO, ZnO, and Ag NPs did not significantly impact anammox
27 sludge at concentrations up to 50 mg/g SS, Cu NPs exhibited notable toxicity. Cu NPs at a
28 concentration of 1.25 mg/g SS significantly inhibited anammox activity, with a IC_{50} at 4.6 mg/g
29 SS for granules and 3.3 mg/g SS for flocs. Exposure to 5 mg/g SS Cu NPs led to a marginally
30 accumulation of N_2H_4 approximately 16 times higher than the control (Table S2).

31 Overall, Fe_3O_4 and MnO_2 NPs were found to enhance sludge properties and nitrogen removal
32 efficiency, with Fe_3O_4 supporting microbial growth at concentrations up to 200 mg/L, and
33 MnO_2 showing significant improvement at concentrations up to 200 mg/L. In contrast, ZnO

1 and Cu NPs were the most toxic, with ZnO causing substantial inhibition at relatively low
2 concentrations (IC₅₀ at 12 mg/g VSS) and Cu NPs exhibiting significant toxicity even at 5 mg/g
3 SS.

4 **6. Effects on aerobic granular sludge**

5 Liu et al. (2017) investigated firstly the effect of GO (60 mg/L) on the phosphorus removal of
6 AGS. The addition of GO significantly reduced the net phosphorus uptake from 4.4 mg/L to
7 2.6 mg/L, indicating a 41% decrease in removal efficiency. The intracellular and extracellular
8 phosphorus contents decreased by 65% and 20% of their original values, respectively, showing
9 a markable reduction in the ability to retain phosphorus. EPS decreased by 20%, primarily due
10 to a reduction in protein content, while polysaccharides and humic-like substances remained
11 relatively stable. In contrast, Guo et al. (2018) observed that GO significantly enhanced the
12 bioactivities of ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), as
13 resulted an increased ammonium uptake rate, which was 3.5 times higher compared to the
14 control, and an increase in EPS production, reaching 140 from 120 mg/g VSS. Kedves et al.
15 (2020) investigated the chronic effects of GO in concentrations ranging from 5 to 95 mg/L on
16 removal of COD, PO₄-P, and nitrogen. At lower concentrations (15, 25, and 35 mg/L), the
17 removal efficiency for COD and NH₄-N remained stable. However, higher concentrations (55,
18 75, and 95 mg/L) significantly inhibited the removal efficiency, with COD removal dropping
19 to 75% and NH₄-N removal efficiency to 82% at 95 mg/L. The presence of GO also negatively
20 impacted phosphorus removal, reducing the efficiency to 68% at the highest concentration. The
21 study observed an increase in mixed liquor suspended solids (MLSS) and EPS contents at lower
22 concentrations, which decreased notably at 95 mg/L. The microbial community structure was
23 significantly affected, with reduced diversity at higher NP concentrations. Strains like
24 *Paracoccus* sp., *Klebsiella* sp., and *Acidovorax* sp. demonstrated resilience to GO exposure,
25 whereas others were adversely impacted, indicating a shift in microbial community
26 composition due to nanoparticle stress. In another experiment, where the concentration of GO
27 (15-115 mg/L) was increased in a single bioreactor, it was observed that as the concentration
28 of GO were increased continuously, significant declines in the efficiency of COD, NH₄-N, and
29 TP removal were observed. The COD removal efficiency, which initially ranged around 95%,
30 dropped to 60% at a GO concentration of 115 mg/L, while NH₄-N removal efficiency fell from
31 99% to 90%. TP removal efficiency was also notably affected, decreasing to below 57% at the
32 highest NP concentration. The study also noted a decrease in microbial activity, with the
33 specific oxygen uptake rate (SOUR) decreasing from 42 to 33 mg O₂/g MLVSS h and the

1 specific ammonia oxidation rate (SAOR) declining from 5 to 4 mg N/g MLVSS h. EPS
2 production initially increased, reaching 12 mg/g MLVSS at 55 mg/L GO, but declined to 5.5
3 mg/g MLVSS at 115 mg/L NP. Despite these negative impacts, the AGS system demonstrated
4 a strong recovery capability once the addition of GO was stopped, with $\text{NH}_4\text{-N}$ and COD
5 removal efficiencies returning to near-initial levels (Kedves et al., 2021).

6 Zheng et al. (2019) during their investigation found that the TN removal efficiency dropped
7 from 80% to 73% at 1 mg/L and 67% at 5 mg/L CeO_2 NPs, while TP removal efficiency
8 decreased from 83% to 73% and 64%, respectively. The presence of CeO_2 NPs increased the
9 production of PS and PN in both loosely bound EPS (LB-EPS) and tightly bound EPS (TB-
10 EPS). Specifically, PS levels rose to 86 mg/g VSS at 1 mg/L and 94 mg/g VSS at 5 mg/L, while
11 PN concentrations increased to 100 mg/g VSS and 140 mg/g VSS, respectively. When the
12 addition of CeO_2 NPs stopped and the influent COD was raised, the TN and TP removal
13 efficiencies gradually recovered, although they remained slightly lower than the control. In case
14 of Ag NPs at 5 and 50 mg/L, the results indicated that the microbial activity was significantly
15 inhibited, with the ammonia oxidizing rate decreasing by 33% and the oxygen respiration rate
16 dropping by up to 45% at the highest NP concentration. Denitrification rates were also affected,
17 showing a 6.8% inhibition. Biomass production was reduced, with the biomass in the reactors
18 containing 5 mg/L and 50 mg/L Ag NPs decreasing to 5.7 g/L and 3.5 g/L, respectively,
19 compared to the control 7 g/L. Despite these reductions, the sludge maintained its granular size
20 (approximately 900 μm) and good settling ability. The microbial community structure showed
21 slight changes, while the dominant microbial populations remained stable. Additionally, there
22 was an increase in ROS and LDH release, indicating oxidative stress and cell membrane
23 damage, particularly at the higher nanomaterial concentration (Quan et al., 2015). Jiang et al.
24 (2022) compared the impact of SiO_2 and TiO_2 NPs at 50 mg/L. They found that SiO_2 NPs
25 negatively affected sludge settleability, increasing the sludge volume index (SVI_{30}) by 65% and
26 reducing protein secretion by 30%. In contrast, TiO_2 NPs improved settleability and increased
27 protein secretion. Despite these differences, both types of NPs did not significantly reduce the
28 overall removal efficiency of COD and aniline. However, TiO_2 NPs significantly inhibited
29 nitrification and denitrification processes, tripling the effluent $\text{NH}_4^+\text{-N}$ concentration. The
30 microbial community analysis showed that both nanoparticles reduced microbial diversity. SiO_2
31 had a lesser inhibitory effect on nitrifying bacteria compared to TiO_2 , which strongly inhibited
32 functional strains involved in nitrogen removal.

1 When the effects of nanoscale nZVI were compared on AGS and AS, it was found that at low
2 concentrations (5 mg/L), nZVI had minimal impact on the performance of AGS, with no
3 significant changes in COD, TN, and TP removal efficiencies after 60 days. However, higher
4 concentrations (50 and 100 mg/L) led to noticeable declines in these parameters, particularly
5 for AS. For instance, the TN removal efficiency in AS decreased from 63% to 51%, while in
6 AGS declined from 70% to 61% at 100 mg/L nZVI. The microbial community structure also
7 showed resilience in AGS, with minor changes, whereas AS showed significant alterations,
8 including the disappearance of certain microorganisms like *Euryarchaeota* and *Crenarchaeota*.
9 Additionally, ROS production increased significantly in AS at higher nZVI concentrations,
10 indicating oxidative stress, whereas AGS showed only a slight increase. LDH release, an
11 indicator of cell membrane damage, was significant in AS but negligible in AGS, suggesting
12 that the dense structure and higher EPS content of AGS provided better protection against nZVI
13 toxicity (Daraei et al., 2019). Liang et al. (2017) demonstrated that introducing Fe₃O₄ NPs at a
14 concentration of 50 mg/L into an AS system significantly improved the aerobic granulation
15 process. The presence of NPs reduced the granulation time from over 45 days to just 20 days
16 and enhanced biomass retention, with concentrations only slightly decreasing from 3.7 to 3.4
17 g/L MLSS. The granules formed in the NPs reactor were more compact and stable compared to
18 the control, exhibiting increased concentrations of EPS, particularly PN (95 mg/g VSS) and PS
19 (44 mg/g VSS). Nanoparticles also improved the surface hydrophobicity of the granular sludge,
20 as indicated by increased contact angles, and maintained a higher COD removal rate, reaching
21 95% by day 90. Furthermore, the microbial community in the presence of NPs showed reduced
22 growth of filamentous bacteria, leading to a more robust granule structure and improved overall
23 sludge performance. Pan et al. (2024) reported similar observations when the effect of Fe₃O₄
24 NPs was investigated at 10, 50, and 100 mg/L on granulation. At a concentration of 50 mg/L,
25 the NPs reduced the time required to achieve over 82% granulation by 90 days, compared to
26 the control. This concentration also improved COD removal efficiency to 91% and PO₄³⁻-P
27 removal efficiency to 94%. Furthermore, the addition of Fe₃O₄ NPs led to increased production
28 of EPS by 48%, which facilitated better sludge aggregation and stability. Finally, based on their
29 thorough investigations, they concluded that when granulation formation was the fastest, the
30 relative abundance of AHL-producing bacteria (such as *Psychrobacter*, *Thermomonas*, and
31 *Nitrosomonas*) was higher than that of AHL-quenching bacteria, suggesting that QS may
32 influence the granulation process. However, this observation should be interpreted cautiously,
33 as other factors could also contribute to granulation, and further studies, including the use of
34 QS mutants, are required to confirm a causal role of QS.

1 The ZnO NPs under shock load at concentrations of 10, 50, and 100 mg/L showed that higher
2 concentrations of NPs (50 and 100 mg/L) led to increased COD removal efficiency, reaching
3 up to 97%, compared to the control (88%). However, the TN removal rate decreased
4 significantly at 100 mg/L ZnO NP concentrations, dropping from 93% to 57%, respectively.
5 The phosphorus removal process remained unaffected across all concentrations. The microbial
6 activity within the sludge showed inhibition in ammonia oxidizing activity and phosphorus
7 release and uptake rates, while oxygen respiration rates increased notably, especially at 10 mg/L
8 ZnO NP concentrations (He et al., 2017b). Another study investigated the chronic response of
9 ZnO NPs at 5, 10, and 20 mg/L, wherein He et al. (2017a) also observed a slightly increase in
10 COD removal efficiency (from 90% to 99%) and a drop in ammonia and TN removal rate (from
11 100% to 75% and 65%) when the amount of ZnO NPs was 20 mg/L. Phosphorus removal was
12 relatively stable, with a slight reduction from 98% to 89%. The SOUR decreased by 34%,
13 indicating inhibited respiration and catabolic microbial activity. Despite the increased
14 production of EPS, the microbial diversity and richness were significantly reduced at higher
15 ZnO NP concentrations, with notable shifts in the relative abundances of key functional species
16 involved in nitrogen and phosphorus removal. Cheng et al. (2019) revealed that short-term
17 exposure to 1 mg/L ZnO NPs stimulated the specific denitrification activity (SDA) of granular
18 sludge by 10%, whereas higher concentrations (5 mg/L and 10 mg/L) inhibited SDA by 23%
19 and 36%, respectively. At even higher concentrations (50-200 mg/L), SDA decreased
20 significantly. Continuous exposure to 2.5 mg/L ZnO NPs resulted in a significant decline in the
21 reactor performance, with TN and COD removal rate dropping sharply. The addition of
22 phosphate (310 mg/L) mitigated these adverse effects, enhancing TN and COD, although the
23 removal efficiencies decreased once phosphate was withdrawn.

24 Long-term effect of Cu NPs at 1 and 2 mg/L did not cause significant negative effect on AGS
25 nutrient removal. When exposed to 5 mg/L Cu NPs, the TN removal drastically decreased from
26 99% to 48%. Additionally, the SDA and DHA were significantly reduced by 45% and 99%,
27 indicating inhibited sludge functionality. The microbial community analysis showed a decrease
28 in the relative abundance of key denitrifying bacteria such as *Castellaniella*, and a shift in
29 community composition, with a notable increase in the abundance of *Bacteroidetes* and
30 *Chloroflexi* at higher Cu NP concentrations. When the addition of NPs were stopped, after 2-3
31 days the nitrogen removal efficiency gradually increased and completely recovered after 25
32 days (Cheng et al., 2019). In contrast, CuO NPs at concentrations of 5, 20, and 50 mg/L over
33 90 days, led to significant increases in reactive oxygen species (up to 190%) and lactate

1 dehydrogenase release (up to 340%), indicating cellular stress and membrane damage. TN
2 removal efficiency improved with higher CuO NP concentrations, reaching 82% at 50 mg/L,
3 while TP removal efficiency decreased significantly, dropping to 53% at 20 mg/L. The
4 microbial community analysis revealed that higher CuO NP concentrations increased the
5 abundance of nitrogen-removal bacteria like *Nitrosomonas* and *Nitrospira* but decreased the
6 presence of phosphorus-removal bacteria such as *Acinetobacter* and *Pseudomonas*. This shift
7 in microbial populations showed the selective pressure exerted by CuO NPs, favoring
8 organisms that enhance nitrogen removal while inhibiting those involved in phosphorus
9 processing (Zheng et al., 2017). Li et al. (2023) investigated the effects of CuO NPs and
10 ciprofloxacin (CIP) on nutrient removal in AGS systems. When the influent wastewater
11 contained only CuO NPs at 5 mg/L, the bioreactor performance and the sludge properties kept
12 stable. In contrast, 5 mg/L each of CuO NPs and CIP significantly inhibited the removal of
13 phosphorus, with long-term stress reducing phosphorus removal efficiency by 62% compared
14 to the control. Nitrogen removal efficiency also decreased, with a notable reduction from 73%
15 in the control to 63% in the combined nanoparticle and antibiotic treatment, COD removal
16 efficiency also dropped from 85% to 67%. The study also observed significant changes in the
17 microbial community, including a decrease in the relative abundance of key phosphorus-
18 accumulating organisms and a reduction in nitrogen-oxidizing bacteria, indicating a toxic effect
19 of the NPs and CIP on the microbial composition and functional metabolic pathways within the
20 sludge (Table S3).

21 GO did not have a negative effect on the anoxic and anaerobic processes of the AGS; however,
22 the aerobic ammonia and COD removal efficiencies decreased after the addition of ≥ 25 mg/L
23 GO. This decreasing trend is closely related to the reduction in the amount of EPS, suggesting
24 that GO may have easily embedded itself into the EPS on the surface of the granules due to the
25 high airflow rate, causing physical damage to the surface microorganisms responsible for the
26 removal of aerobic organic matter, ammonia, and EPS production (Kedves et al., 2020). ZnO
27 NPs do not significantly affect COD and phosphate removal. Their negative impact arises from
28 inhibiting the activity of nitrifying and denitrifying microorganisms in the nitrogen removal
29 cycle. Similarly, Cu and CuO nanoparticles do not affect COD removal efficiency but
30 significantly hinder phosphorus and nitrogen removal. All three nanoparticles exert their
31 harmful effects by releasing Cu^{2+} and Zn^{2+} ions, which alter and weaken the structure of EPS,
32 simultaneously reducing its protective role (Cheng et al., 2019; Zheng et al., 2017).

33 **7. Effects on algal-bacterial granular sludge**

1 Li et al. (2015) examined the impact of TiO₂ NPs on aerobic granulation in an ABGS system
2 over a period of 100 days, with TiO₂ NP concentrations of 10, 30, and 50 mg/L. They found
3 that the addition of NPs enhanced the granulation process, leading to larger and more stable
4 granules compared to the control. The biomass concentration increased steadily, with MLVSS
5 values reaching approximately 5.4-5.5 g/L in the contaminated ABGS bioreactor and 5.6-5.9
6 g/L in the control. The COD removal efficiency remained high at around 96% and the nitrate
7 removal efficiency was consistently high at 98-100% in both reactors at 10 mg/L NPs, but
8 nitrate removal efficiency significantly decreased at higher TiO₂ NP concentrations (\geq 30
9 mg/L), resulting nitrate accumulation from 4 mg/L to 50 mg/L. During the experiment, the
10 amount of PS remained stable, but with increasing concentrations of TiO₂ to 10, 30, and 50
11 mg/L, the amount of PN also increased from 18 to 49, 53, and 64 mg/g VSS. Additionally, the
12 microbial community structure showed an increase in *Gammaproteobacteria*, which are
13 associated with enhanced nitrification, and a decrease in the TM7 phylum, linked to improved
14 granule stability.

15 Xiao et al. (2024) demonstrated that over 95% of ZnO nanoparticles (ZnO NPs) at 10 mg/L
16 were adsorbed by MBGS within 40 days, primarily through interactions with –OH functional
17 groups and protein structures. The introduction of ZnO NPs impaired nutrient removal, leading
18 to reductions in COD, NH₄⁺-N, and PO₄³⁻-P efficiencies by 7%, 25%, and 6.5%, respectively,
19 with nitrification processes being particularly affected. ZnO NPs significantly damaged cell
20 membranes, as evidenced by an increase in LDH release from 2.9 to 4 U/gprot. Furthermore,
21 genes associated with biological processes were upregulated, while genes involved in
22 intracellular biosynthesis, such as those linked to glutathione synthesis, were inhibited. Key
23 metabolic genes like *acs* and *glnA* were notably downregulated, adversely impacting cellular
24 metabolism. The suppression of glycosyl transferase and glycoside hydrolase genes further
25 disrupted intracellular glycogen hydrolysis, compromising energy production and overall
26 degradation efficiency of MBGS. Another study evaluated the impact of ZnO NP and
27 established that at concentrations of 0.1 and 1.0 mg/L, the ZnO NPs did not significantly
28 influence the removal of COD, NH₄⁺-N, and PO₄³⁻-P. However, a higher concentration of 10
29 mg/L significantly reduced the removal efficiencies of NH₄⁺-N by 8.8% and PO₄³⁻-P by 14%,
30 demonstrating a notable adverse effect. The presence of ZnO NPs at this higher concentration
31 also led to a significant increase in superoxide dismutase (SOD) enzyme activity, indicating
32 oxidative stress within the granular sludge. While low concentrations slightly promoted EPS
33 and increased from 84 to 110 and 94 mg/g VSS, a concentration of 10 mg/L inhibited their

1 production and declined to 67 mg/g VSS. Furthermore, ZnO NPs significantly altered the
2 microbial community, decreasing the abundance of key prokaryotic groups like *Proteobacteria*,
3 which are critical for nitrogen and phosphorus removal, while increasing the relative abundance
4 of *Cyanobacteria*, known for their metal sequestration properties. These changes likely
5 contributed to the observed reduction in nutrient removal efficiency and underline the potential
6 of *Cyanobacteria* to mitigate ZnO NP toxicity through biosorption and bioaccumulation
7 mechanisms (Table S4) (Xiao et al., 2022).

8 The impact of TiO₂ and ZnO nanoparticles on algal-bacterial granular sludge systems varies
9 significantly, with TiO₂ NPs showing beneficial effects on granule stability, promoting
10 enhanced granulation and nutrient removal through controlled algal growth and increased EPS
11 production. Conversely, ZnO NPs, particularly at higher concentrations, exhibited toxicity by
12 inhibiting nutrient removal, damaging cellular integrity, and altering microbial metabolism
13 through the release of Zn²⁺ ions and the generation of oxidative stress (Xiao et al., 2022). These
14 findings suggest that while TiO₂ NPs may enhance the long-term performance of such systems,
15 careful management of ZnO NP exposure is necessary to avoid adverse effects on wastewater
16 treatment efficiency.

17 **8. Comparison of the effects of the same nanomaterials on different types of granular** 18 **sludges**

19 It is important to understand how individual nanoparticles affect different types of granular
20 sludge systems, how they influence the structure of granules, and which granular sludge
21 technology is the most resilient to this type of contaminant.

22 *8.1. Effects of CeO₂ NPs*

23 The effects of CeO₂ NPs were investigated in AGS and AnGS systems. The authors found that,
24 at certain concentrations, no negative effects were observed due to the increase in EPS.
25 However, the aerobic granules showed a decrease in the removal of COD, ammonia, and TP at
26 5 mg/L CeO₂ NPs, as the removal of these nutrients is carried out by aerobic microorganisms
27 on the surface of the granules (Zheng et al., 2019). In AnGS, while the methane and ROS
28 production remained stable (even at 150 mg/g VSS), the production of short-chain fatty acids,
29 synthesized by microorganisms on the surface of the granules, decreased (Ma et al., 2013).
30 These results suggest that CeO₂ NPs were not able to penetrate inside the granules (as confirmed
31 by fluorescence microscopy). Thus, their negative effects may manifest under both aerobic and
32 anaerobic conditions by attaching to microorganisms located on the surface of the granules and

1 penetrating through the membrane into the cells, potentially causing cell death (Qiu et al., 2015;
2 Zhuo et al., 2021). However, further studies are necessary to gain a more detailed understanding
3 of this process.

4 *8.2. Effects of Ag NPs*

5 No adverse effects of Ag NPs were observed on AnGS and AxGS during batch experiments
6 (Gonzalez-Estrella et al., 2013; Zhang et al., 2017b). During long-term exposure, Ag NPs at 50
7 mg/L had no negative effects on anammox granules; ROS and LDH levels remained stable, and
8 EPS production as well as specific anammox activity were enhanced (Zhang et al., 2018a). In
9 contrast, the long-term presence of Ag NPs at 50 mg/L resulted in a decrease in the relative
10 respiration rate, relative ammonia-oxidizing rate, and EPS content in AGS (Quan et al., 2015).
11 These differences can be attributed to several factors: (i) under anammox conditions, no ROS
12 is generated (Zhang et al., 2018a), so Ag NPs could not have caused oxidative cell damage
13 (ROS increased by 28% in AGS); (ii) the amount of EPS in the control sludge differs, with
14 AxGS containing 310 mg/g VSS (Zhang et al., 2018a), while AGS has only 70 mg/g VSS (Quan
15 et al., 2015), therefore we assume that AxGS can bind/capture more nanoparticles, resulting in
16 fewer nanoparticles reaching the cell surface. Due to these factors, as the concentration of Ag
17 NPs increased, the structure of the sludge began to disintegrate, and more dead cells appeared
18 in the interior of the aerobic sludge, as observed in CLSM studies (Quan et al., 2015), while no
19 similar effects were observed in anammox sludge granules.

20 *8.3. Effects of TiO₂ NPs*

21 Li et al. (2022) showed unaffected ROS production, LDH release, and a 69% increase in EPS
22 for AnGS during batch tests after the addition of TiO₂ NPs at 200 mg/L. In contrast, Li et al.
23 (2017) observed negative effects of TiO₂ NPs at 5 mg/L after long-term exposure (90 days). By
24 the end of the experiments, the glucose removal rate had declined by 16%, and methane yield
25 by 30%, suggesting that it is necessary to explore the long-term effects of NPs. TiO₂ NPs at 50
26 mg/L in AGS had a positive effect on EPS content (which increased by 20%) and its structure
27 also changed (the intensity of tyrosine-like, aromatic-like, tryptophan-like proteins, and humic-
28 like substance peaks increased). However, in the effluent water, the ammonia concentration
29 increased from 1 mg/L to 5 mg/L after 20 days (Jiang et al., 2022). In contrast, in ABGS,
30 ammonia removal remained stable even at 50 mg/L NPs after 20 days, while the EPS content
31 increased twofold (Li et al., 2015). These differences between AGS and ABGS suggest that
32 algae may play a crucial role in cell protection and stability, which can be explained by the

1 following: (i) microalgal-bacterial consortia are able to secrete more EPS (Liu et al., 2018;
2 Zhang et al., 2020), which may mitigate the harmful effects of the TiO₂ NPs and the relatively
3 small amount of titanium ions released from the TiO₂ NPs, (ii) algae play an important role in
4 the removal of ammonia (Zhou et al., 2022), and (iii) algae are more tolerant to metal
5 contaminants and are thus able to remove nutrients from wastewater (Xiao et al., 2023).

6 8.4. Effects of Fe₃O₄ NPs

7 The effect of Fe₃O₄ NPs at 50 mg/L on aerobic granulation was investigated in two studies.
8 Liang et al. (2017) and Pan et al. (2024) found that the granulation time was shortened by 55%
9 and 80%, respectively, while the EPS content of the sludge, compared to the control, was higher
10 by 20% and 30%. Liang et al. (2017) also showed that peak intensity increased, especially for
11 O—H and C=O bonds, which are the main bonds in the structure of PS and PN. While Pan et
12 al. (2024) found that Fe₃O₄ NPs enhanced the growth of EPS producing microorganisms like
13 *Terrimonas* and *Devosia*, the amount of produced EPS was higher, which enhances cell-cell
14 adhesion and accelerates the granulation process. The addition of Fe₃O₄ NPs at 2,400 mg/L
15 enhanced anammox granulation by promoting the secretion of PN, strengthening the granule
16 structure and leading to improved system stability. The magnetic field provided by these
17 nanoparticles increased cell permeability and enzyme activity, contributing to faster adaptation
18 and reducing the startup time of the anammox process by 4 days. Regarding nitrogen removal,
19 the addition of Fe₃O₄ NPs achieved a higher total nitrogen loading rate (TNLR) of 0.8 kg
20 N/m³/day compared to the control (UASBC), which had a TNLR of 0.5 kg N/m³/day, aligning
21 with an increased abundance of anammox bacteria (AnAOB) by 32% (Yun et al., 2023). During
22 long-term exposure to Fe₃O₄ NPs at 200 mg/L, negligible impacts on nitrogen removal
23 performance were observed, while the SAA and EPS of anammox sludge increased by 54%
24 and 75%, respectively (Xu et al., 2020). Heme c also increased by 67%, as Fe₃O₄ NPs ionized
25 into Fe²⁺ and Fe³⁺ ions and generated a magnetic field. The addition of Fe₃O₄ NPs significantly
26 enhanced both aerobic and anammox granulation processes by reducing granulation time,
27 promoting microbial interactions, improving granule stability, and increasing nitrogen removal
28 efficiency, primarily due to increased EPS production and enhanced enzyme activity.

29 8.5. Effects of Cu NPs

30 The authors observed a long-term negative effect of Cu NPs in both aerobic and anammox
31 granules, even at low concentrations (5 mg/L). The NRE and EPS content in AGS decreased
32 by 48% and 15%, respectively (Cheng et al., 2019), while in AxGS bioreactors, the NRE and

1 EPS declined by 2-60% and 34%-44% (Fu et al., 2021; Zhang et al., 2018c). However, after
2 the withdrawal of NPs, nitrogen removal in both AGS and AxGS completely recovered after
3 30 days. The relatively strong inhibitory effect of Cu NPs is attributed to the release of Cu²⁺
4 ions from the nanoparticles (Cheng et al., 2019). As a result, both the Cu NPs and the released
5 Cu²⁺ ions adsorbed onto the negatively charged EPS, disrupting its structure and reducing its
6 protective function, which caused further damage to the cell membranes and ultimately led to
7 cell death (Fu et al., 2021; Zhao et al., 2019).

8 *8.6. Effects of CuO NPs*

9 In the case of CuO NPs at 5 mg/L, glucose degradation declined by 65% after 90 days of
10 exposure, while biogas production stopped after 75 days in AnGS (Li et al., 2017). In contrast,
11 AGS bioreactor performance remained stable at 5 mg/L of CuO NPs, but at higher
12 concentrations (20 and 50 mg/L), there was a significant decrease in biomass, EPS, and TP
13 removal, due to decreased activities of polyphosphate kinase (PPK) and exophosphatase (PPX).
14 In parallel, LDH release increased by 300% and 340%, and ROS increased by 180% and 190%
15 after long-term exposure (Li et al., 2023; Zheng et al., 2017). The bioreactor performance and
16 sludge properties of AxGS did not change even at 160 mg/L of CuO NPs (Zhang et al., 2018c).
17 The main difference between the three types of sludge is the EPS amount. The polymer content
18 is around 100 mg/g VSS in AnGS, while in AGS and AxGS it is approximately 190 and 300
19 mg/g VSS, respectively. Thus, we hypothesize that the aggregation-prone CuO NPs (which do
20 not release large amounts of copper ions) had a reduced specific surface area (Zhang et al.,
21 2018c), causing them to primarily adhere to the surface of AxGS even at high concentrations,
22 due to the large amount of EPS. While AGS, with its relatively high EPS content, was able to
23 tolerate lower concentrations of CuO NPs, at higher concentrations the EPS could no longer
24 bind them. The NPs adhered to the surface of the microorganisms, inhibiting their activity and
25 reducing further production of protective EPS. However, this still did not completely inhibit the
26 anaerobic processes, suggesting that CuO NPs were not able to penetrate into the core of the
27 aerobic granules (Li et al., 2023). Finally, due to the low EPS content in AnGS, CuO NPs
28 reached the methanogenic microorganisms within the granules even at lower concentrations,
29 completely inhibiting their activity.

30 *8.7. Effects of ZnO NPs*

31 In AGS studies, ZnO NPs at 1 and 2.5 mg/L did not affect nutrient removal, while at higher
32 concentrations (≥ 5 mg/L), they had a negative impact on bioreactor performance, likely due to

1 increased ROS production and LDH release (Cheng et al., 2019; He et al., 2017a). The harmful
2 effects of ZnO NPs were observed at 10 mg/L in MBGS, where LDH release increased by 35%.
3 Based on the increased abundance of microalgae, which can secrete antioxidant enzymes, the
4 authors speculated that the algae may display a symbiotic behavior to protect cells from the
5 ROS produced by ZnO NPs (Xiao et al., 2024, 2022). In the case of AxGS, ZnO NPs began to
6 reduce nitrogen removal at different concentrations (usually above 30-50 mg/L). However, a
7 common observation was that LDH release did not increase, even at concentrations where
8 bioreactor performance declined; only ROS production increased when EPS production was
9 inhibited (Song et al., 2018; Zhang et al., 2018b; Zhao et al., 2019). Glucose degradation
10 declined by 50% after 90 days of exposure to 5 mg/L CuO NPs, while biogas production
11 stopped after 85 days in AnGS (Li et al., 2017). The toxic effects of ZnO NPs may result from
12 several factors: (i) ZnO NPs attach to the cell, damaging the membrane and leading to the
13 release of LDH (Xiao et al., 2024); (ii) since the ZnO NPs release Zn^{2+} ions in large amounts,
14 Zn^{2+} ions also cause cell damage as the EPS amount decreases by affecting the selective
15 permeability of the cell membrane (Vargas-Estrada et al., 2020); (iii) in the case of MBGS, the
16 shading effect of the NPs reduces the light reaching the algae, decreasing their activity (Xiao et
17 al., 2022). It can also be concluded that, in the case of ZnO NPs, AnGS is the most sensitive,
18 followed by AGS, then ABGS (due to the algae-bacteria symbiosis), with AxGS being the most
19 tolerant, presumably due to the larger amount of EPS in the sludge.

20 **9. Conclusions and prospect**

21 The review on the impact of various NPs on AnGS, AxGS, AGS, and ABGS has revealed both
22 opportunities and challenges in wastewater treatment. Both short-term and long-term exposures
23 to nanoparticles result in significant changes in nutrient removal efficiencies, sludge
24 characteristics, and microbial community dynamics. While some nanoparticles, like Fe_3O_4 and
25 MnO_2 , have been shown to enhance sludge properties and treatment efficiencies, others,
26 particularly ZnO and CuO, exhibit significant toxicity and inhibit microbial activity. The
27 toxicity of nanoparticles to microbial communities within granular sludge is mediated through
28 pathways such as ROS production, cell membrane damage, and disruption of metabolic
29 processes. The presence of nanoparticles induces shifts in microbial community structures,
30 favoring certain bacteria over others. Fe_3O_4 NPs enhance the abundance of anammox-related
31 bacteria, while CuO nanoparticles inhibit key functional bacteria involved in phosphorus
32 removal. Granular sludge systems exhibit varying degrees of resilience to nanoparticle-induced
33 inhibition. Recovery mechanisms are influenced by environmental and operational factors, such

1 as EPS production, microbial community adaptability, and external interventions such as the
2 addition of BSA or phosphate. Future research should focus on the following areas to optimize
3 the use of nanoparticles in wastewater treatment:

4 (a) Fe_3O_4 have demonstrated its potential in improving microbial activity and stability in case
5 granular sludge, further experiments should be focus on maximizing treatment efficiency while
6 minimizing potential negative effects. Explore the exact mechanism by which Fe_3O_4 NPs exert
7 their positive effect when added to the sludge, and develop synthetize methods that minimize
8 environmental impact while achieving a prolonged positive effect in the sludge (if the released
9 iron ions also play a role, the nanoparticles could be encapsulated).

10 (b) Since the effects of some common NPs on certain granular sludge systems are still unknown,
11 further investigation is required. For instance, the impact of CeO_2 NPs and TiO_2 NPs on AxGS
12 has not yet been studied, and the effects of GO have only been examined in AGS. In the case
13 of MBGS, only ZnO NPs and TiO_2 NPs have been investigated.

14 (c) It will be necessary to conduct studies that examine not only the effects of metal and metal
15 oxide-based NPs but also the impact of the corresponding metal ions in the same systems. Since
16 metal ion release from nanoparticles does not occur immediately, shock-load experiments
17 focused solely on nanoparticle-sludge interactions are also recommended.

18 (d) Future research should focus on the adaptive mechanisms that microbial communities utilize
19 in response to NP exposure. Investigating the production of EPS and other protective
20 mechanisms will provide insights into how microbial communities maintain resilience.
21 Advanced genetic and bioinformatic approaches could help identify microbial species that
22 exhibit higher tolerance to NP stress.

23 (e) Studies on the effects of NMs on granulated sludge are mostly conducted in laboratory
24 environments; thus, there is limited information on their effects in complex wastewater
25 environments containing a mix of municipal or industrial pollutants. The interactions between
26 NPs and heavy metals, organic pollutants, or pharmaceuticals could increase or decrease their
27 toxic effects. Future studies should focus on these interactions to develop comprehensive
28 treatment strategies.

29 (f) As the release of NMs into wastewater treatment plants grows, developing regulatory
30 frameworks will be necessary. Establishing regulatory guidelines for acceptable levels of
31 various nanomaterials, particularly those with known toxicity issues such as ZnO and CuO.

1 (g) Recovering and reusing nanoparticles from wastewater systems could provide significant
2 economic and environmental benefits. Research into efficient recovery techniques, such as
3 magnetic separation or biosorption, will be essential to minimize the environmental impact of
4 NP discharge.

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9

10 **CRediT authorship contribution statement**

11 **Alfonz Kedves:** Writing – original draft, Formal analysis, Conceptualization, Visualization.

12 **Zoltán Kónya:** Supervision, Writing – review & editing, Funding acquisition.

13

14 **Declaration of competing interest**

15 The authors declare that they have no known competing financial interests or personal
16 relationships that could have appeared to influence the work reported in this paper.

17

18 **Data availability**

19 No data was used for the research described in the article.

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Highlights

- Insights into granular sludge-NPs interactions and their mechanisms are provided.
- Nanoparticles influence sludge stability, performance, and microbial activity.
- Toxicity of nanomaterials is dose and time dependent.
- Granular sludges exhibit excellent recovery capability.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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