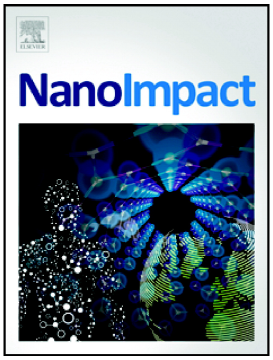

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Response to shock load of titanium dioxide nanoparticles on aerobic granular sludge and algal-bacterial granular sludge processes

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Abstract

Titanium dioxide nanoparticles (TiO_2 NPs) are extensively used in various fields and can consequently be detected in wastewater, making it necessary to study their potential impacts on biological wastewater treatment processes. In this study, the shock-load impacts of TiO_2 NPs were investigated at concentrations ranging between 1 and 200 mg L^{-1} on nutrient removal, extracellular polymeric substances (EPSs), microbial activity in aerobic granular sludge (AGS), and algal-bacterial granular sludge (AB-AGS) bioreactors. The results indicated that low concentration ($\leq$10 mg L^{-1}) TiO_2 NPs had no effect on microbial activity or the removal of chemical oxygen demand (COD), nitrogen, and phosphorus, due to the increased production of extracellular polymeric substances (EPSs) in the sludge. In contrast, the performance of both AGS and AB-AGS bioreactors gradually deteriorated as the concentration of TiO_2 NPs in the influent increased to 50, 100, and 200 mg L^{-1}. Specifically, the ammonia-nitrogen removal rate in AGS decreased from 99.9% to 88.6%, while in AB-AGS it dropped to 91.3% at 200 mg L^{-1} TiO_2 NPs. Furthermore, the nitrate-nitrogen levels remained stable in AB-AGS, while NO_3-N was detected in the effluent of AGS at 100 and 200 mg L^{-1}. Microbial activities change similarly as smaller decrease in the specific ammonia uptake rate (SAUR) and specific nitrate uptake rate (SNUR) was found in AB-AGS compared to those in AGS. Overall, the algal-bacterial sludge exhibited higher resilience against TiO_2 NPs, which was attributed to a) higher EPS volume, b) smaller decrease in LB-EPS, and c) the favorable protein to polysaccharide (PN/PS) ratio. This in turn, along with the symbiotic relationship between the algae and bacteria, mitigates the toxic effects of nanoparticles.

Keywords: aerobic granular sludge, algal-bacterial granular sludge, titanium dioxide nanoparticles, shock load, inhibition effect.

1. Introduction

Urbanization and the progress of civilization inevitably result in the production of large volumes of wastewater, which has led to a growing focus on and continuous research into effective nutrient removal in wastewater treatment [1,2]. In comparison to activated sludge flocs, aerobic granules present a more attractive option due to their dense structure containing diverse microorganisms [3,4]. The aerobic granular sludge (AGS) process offers several advantages, including adaptability to varying temperatures [5], resilience to toxic substances [6,7], and simultaneous nutrient removal capacities [8,9]. Consequently, aerobic granular sludge processes have garnered considerable attention in municipal wastewater treatment [10]. Recently, there has been growing interest in algal-bacterial granular sludge (AB-AGS) processes for wastewater treatment [11,12]. This innovative approach takes advantage of the synergistic relationship between algae and bacteria to facilitate nutrient cycling and pollutant removal through biosynthesis and metabolic processes [13]. Additionally, a symbiotic association can be established between algae and bacteria, wherein algae supply oxygen for bacterial organic oxidation, while bacteria provide CO_2 for microalgal photosynthesis [14,15]. During the granulation processes, microorganisms produce extracellular polymeric substances (EPS), that form a unique gel-like matrix, helping microbial cells to create more complex polymers, which has two main components: polysaccharides (PS) and proteins (PN). This matrix shields microorganisms from fluctuating and harsh external conditions [4]. EPS can be classified into two types based on their location: loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS), which together form dual protective layers [16]. Indicators such as specific oxygen uptake rate (SOUR), specific ammonia uptake rate (SAUR), specific nitrite uptake rate (SNIUR), specific nitrate uptake rate (SNUR), and specific phosphorus uptake rate (SPUR) are critical for understanding the microbial activity and overall health of the sludge [3,17]. These indicators reflect different aspects of the microbial metabolic processes, which are essential for evaluating the impact of hazardous materials on the performance of AGS and AB-AGS.

Nanomaterials are increasingly being used in the production of a wide range of industrial and consumer goods, thanks to their unique physical and chemical properties [18]. Titanium dioxide nanoparticles (TiO_2 NPs) find extensive use in various applications, including catalysts, paints, sunscreens, plastics, cosmetics, and industrial processes, owing to their chemical stability, high photocatalytic efficiency, and cost-effectiveness [19–22]. Previous studies have shown that the half-maximal inhibitory concentration (IC_{50}) of TiO_2 NPs is 37 mg L^{-1} for denitrifying bacteria

[23], 35.9 mg L^{-1} for algae [24], and only 11.9 mg L^{-1} for human cells [25]. TiO_2 NPs can exert their toxic effects by attaching to the cell surface, causing membrane damage, which leads to cell shrinkage and eventually cell death [26]. If they attach to the cell and penetrate the cell wall, they can cause irreversible damage to its function [27]. Furthermore, the negative impact of nanoparticles is influenced by the composition of the wastewater. Cervantes-Avilés et al. [28] investigated the effects of TiO_2 NPs in synthetic, raw, and filtered wastewater, and based on the results, it was concluded that the nanoparticles negatively affected nutrient removal differently in each case, owing to the varying compositions of the wastewaters. Finally, their toxic effects can also occur through the release of metal ions from the nanoparticles, which alters the selective permeability of the cell membrane, affecting intracellular osmotic pressure [29]. The widespread use of these nanoparticles has led to the release of TiO_2 NPs into the environment, where they are detectable in soil, aquatic environments, and wastewater [30]. Wastewater treatment plants serve as one of the final barriers for removing contaminants from municipal and industrial wastewaters through microorganisms. One study reported that the amount of TiO_2 NPs reaches 10.7 μg L^{-1} in influent wastewater at Southern California [31], while another study found TiO_2 NPs present in sewage at concentrations of up to 5 mg L^{-1} [32], and another investigation reported levels of these particles in sludge reaching up to 23 mg kg^{-1} [30]. Because of the potential adverse effects of TiO_2 NPs on biological systems, it is crucial to evaluate their influence on the effectiveness of biological wastewater treatment systems.

The study by Zheng et al. [18] found that the presence of TiO_2 NPs at a concentration of 50 mg L^{-1} decreased the efficiency of ammonia removal in activated sludge by 55%, attributed to a reduction in aerobic ammonia-oxidizing bacteria. In contrast, Li et al. [33] observed a decline in the removal rates of organic matter and phosphorus, when the nanoparticle concentration reached 10 mg L^{-1}, with no significant impact on ammonia removal. Cervantes-Avilés et al. [34] observed that increasing the concentration of TiO_2 NPs in the influent resulted in a decrease in the oxygen uptake rate and organic matter removal rate. Subsequent investigations by Zhou et al. and Li et al. [35,36] noted a decrease in floc size, dewaterability, and microbial diversity in activated sludge by TiO_2 NPs addition. In aerobic granules, sludge settleability was improved at 50 mg L^{-1} nanoparticle concentration due to the increased protein content along with rising nitrogen levels in effluent water [37]. However, TiO_2 NPs at concentrations of 30 and 50 mg L^{-1} negatively affected denitrification processes in AB-AGS, while organic matter, ammonia, and phosphorus removal remained stable [38]. To date, there has been no comparative study on the impact of TiO_2 NPs on AGS and AB-AGS wastewater treatment technologies under identical operating conditions.

The present study investigated and compared the short-term effects of TiO_2 NPs at concentrations ranging from 1 to 200 mg L^{-1} in two promising wastewater treatment systems, i.e. in AGS and AB-AGS. The removal rates of nitrogen compounds (ammonia, nitrite, and nitrate) and phosphorus were determined every 4 hours during the experiment. Additionally, at the end of each experimental phase, the quantities of extracellular polymeric substances (EPSs) and microbial activity in the sludges were also assessed.

2. Materials and methods

2.1. Preparation and characterization of TiO_2 NPs

All chemicals were used as received without any further purifications. Based on the previously published technique, TiO_2 NPs were synthesized through a modified nonaqueous solvothermal process [39]. Titanium isopropoxide (TIP, 5 mL) was dissolved in 130 mL of anhydrous acetone, and after 20 minutes of stirring at room temperature, the solution was taken to a 250 mL Teflon-lined stainless-steel autoclave and kept at 180 °C for 10 hours. The yellowish sediment was gathered by centrifugation, repeatedly washed with ethanol and dried at 60 °C. In the last step, the powder was calcined at 450 °C for 2 hours to obtain pure white TiO_2 NPs. Structural and morphological characterization was done by using a Rigaku Miniflex-II X-ray diffractometer (Cu Kα radiation, $\lambda = 1.5406$ Å, 40 kV, 30 mA), a Bruker Vertex 70 FT-IR instrument (16 scans at 4 cm^{-1} resolution), and a Hitachi S-4700 Type II scanning electron microscope (SEM) with 10 kV accelerating voltage equipped with a Röntec QX2 energy dispersive X-ray spectrometer (EDX) [4].

2.2. Experimental procedure

The AGS sequencing batch reactors (SBRs) and the AB-AGS photo-sequencing batch reactors (PSBRs) were configured with an effective volume of 1.4 L and a hydraulic retention time of 8 h (detailed information can be found in *Supporting Information*). The average diameter of both types of granular sludge was 600 µm with mixed liquor suspended solids (MLSS) of 5.75 ± 0.15 g L^{-1}, and an initial sludge volume index (SVI_5) of 26.26 ± 0.13 mL g^{-1}, respectively. In each experiment, fresh granular sludge obtained from control bioreactors was utilized. TiO_2 NPs concentrations of 1, 5, 10, 50, 100, and 200 mg L^{-1} were applied to investigate the effect of shock loads for 24 hours. TiO_2 NPs were studied at environmentally relevant concentrations of 1 and 5 mg L^{-1} [40,41]; additionally, higher concentrations were selected for investigation due to their extensive large-scale production [42]. Before use, the nanoparticles were placed in deionized water and sonicated for 10 minutes to prevent the use of aggregated particles during the experiments. Then, the desired amount of particles were introduced into the bioreactors along with synthetic wastewater (SWW). The SWW consisted of the following components per liter of deionized water: 1200 mg COD as glucose, 110 mg NH_4-N as NH_4Cl, 20 mg PO_4-P added as KH_2PO_4, 200 mg $NaHCO_3$, 25 mg $CaCl_2$, 45 mg $MgSO_4$, and 1 mL of trace element solution [4].

2.3. Analytical methods

COD, nitrogen (including ammonia-nitrogen NH_3-N, nitrite-nitrogen (NO_2-N), and nitrate-nitrogen (NO_3-N)) and PO_4^{3-} contents of the effluent water were measured using test kits (Hanna Instruments) and a HANNA Instruments HI83306 Environmental Analysis Photometer. The concentration of titanium in the effluent was measured via Inductively-coupled plasma mass spectrometry (ICP-MS) using an Agilent 7900 instrument. The MLSS and SVI_5 of the sludge were measured as reported previously by Clesceri et al. [43]. The effect of TiO_2 NPs on microbial activity was determined by measuring the specific oxygen uptake rate (SOUR), specific ammonia uptake rate (SAUR), specific nitrite uptake rate (SNIUR), specific nitrate uptake rate (SNUR), and specific phosphorus uptake rate (SPUR) at the end of each phase (see *Supporting Information*). Furthermore, the amounts of loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) in the sludge was determined using a modified heat extraction method [44] (the sum of LB-EPS and TB-EPS constituted the total EPS amount). Briefly, a 10 mL sample was centrifuged at 5000*g* for 30 minutes. The resulting supernatant was passed through a 0.22 µm membrane filter, and the filtrate collected represented the LB-EPS fraction. Once the supernatant was discarded after LB-EPS collection, the remaining sludge was resuspended in a 0.9% (w/v) NaCl solution to its original volume. It was then heated to 80 °C for 30 minutes. The solution was centrifuged again at 10,000*g* for 20 minutes, and the supernatant was filtered using a 0.22 µm membrane. The resulting filtrate was used to analyze the TB-EPS fraction. Both LB- and TB-EPS samples were stored at −30 °C until further analysis. The polysaccharide (PS) and protein (PN) contents in the EPS were quantified using the Anthrone method (with glucose as the standard) and the modified Lowry method (with bovine serum albumin as the standard) as described by Frølund et al. [45]. EPS was calculated as the sum of PN and PS. All analyses were carried out in triplicate. The morphology of the sludge was investigated with a scanning electron microscope (SEM), the diameter of both types of granules were analyzed by using ImageJ 1.53a software [3].

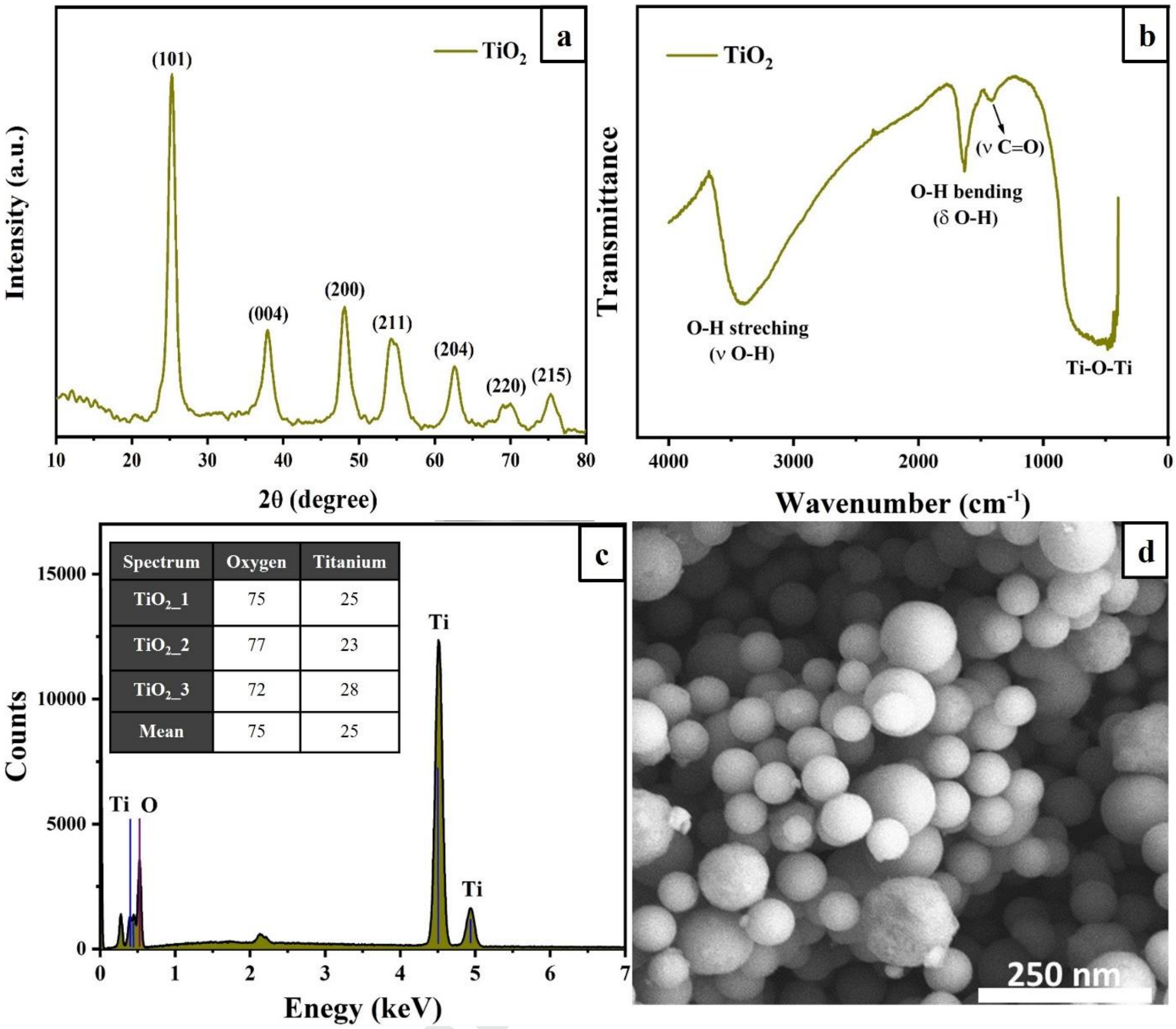
2.4. Statistical analysis

All measurements were conducted in triplicate, and the results were reported as the mean ± standard deviation (SD). To assess the significance of the findings, a one-way analysis of variance (ANOVA) was used. The significance level was set at 5% for each analysis.

3. Results and discussion

3.1. Characterization of TiO_2 NPs

The X-ray diffraction patterns of the TiO_2 nanocrystals are displayed in *Fig. 1a*. Reflections can be indexed as anatase TiO_2 NPs (JCPDS card file no. 21-1272), where the peaks appeared at 2θ of 25.2°, 37.8°, 48.1°, 54.6°, 62.6°, 69.7°, and 75.3° correspond to the (101), (004), (200), (211), (204), (220), and (215) crystal planes [46]. To obtain more compositional details on TiO_2 NPs, FT-IR measurement was performed, and the resulting spectrum is shown in *Fig. 1b*. The vibrational modes that are commonly attributed to the stretching of Ti–O–Ti of the TiO_2 NPs are observed at 410 and 780 cm^{-1}. Furthermore, the vibrational modes at around 1632 and 3420 cm^{-1} are associated with the bending mode of the physisorbed water molecules on TiO_2 NPs, respectively [47]. Energy dispersive X-ray (EDX) analysis was carried out to confirm the chemical composition of the as-prepared nanoparticles, and a typical EDX spectrum is shown in *Fig. 1c*. A Ti:O atomic ratio of 1:3 was found from the average measured at three different points of the sample (inset *Fig. 1c*). Scanning electron microscopy image of the TiO_2 NPs sample is displayed in *Fig. 1d*. The TiO_2 crystals have spherical morphology of a diameter ranging between 30 and 130 nm.



Spectrum	Oxygen	Titanium
TiO$_2$_1	75	25
TiO$_2$_2	77	23
TiO$_2$_3	72	28
Mean	75	25

Fig. 1. Characterization of the TiO$_2$ NPs. **a)** X-ray diffraction pattern; The **b)** FT-IR spectrum, **c)** EDX spectrum, and **d)** SEM image of the nanoparticles.

3.2. Impact of TiO$_2$ NPs on extracellular polymeric substances production in AGS and AB-AGS

The granular sludge owes its excellent tolerance to pollutants to the high amount of extracellular polymeric substances (EPSs). The EPS are produced by microorganisms and consist of two main components: proteins (PN) and polysaccharides (PS). These substances are able to adsorb different compounds, improve the settling ability of sludge. Moreover, they protect microorganisms against harmful materials, such as heavy metals or nanoparticles [16,37]. The PS and PN content of the initial AGS were 47.5 ± 4.1 and 56.5 ± 5.2 mg g^{-1} MLVSS (*Fig. 2a*), and 46.4 ± 3.9 and 56.5 ± 4.7 mg g^{-1} MLVSS in AB-AGS, respectively (*Fig. 2b*). Although nanoparticles at 1 mg L^{-1} did not cause any change in EPS, there was an increased polymer content in both granular sludges at 5 and 10 mg L^{-1}. This suggests, that TiO_2 NPs may have a toxic effect on microorganisms at these concentrations, as it was reported earlier in activated sludge [33]. Granular sludge, on the other hand, were able to secrete more EPS (mainly protein), thereby enabling the microorganisms to protect themselves against the nanomaterials [48].

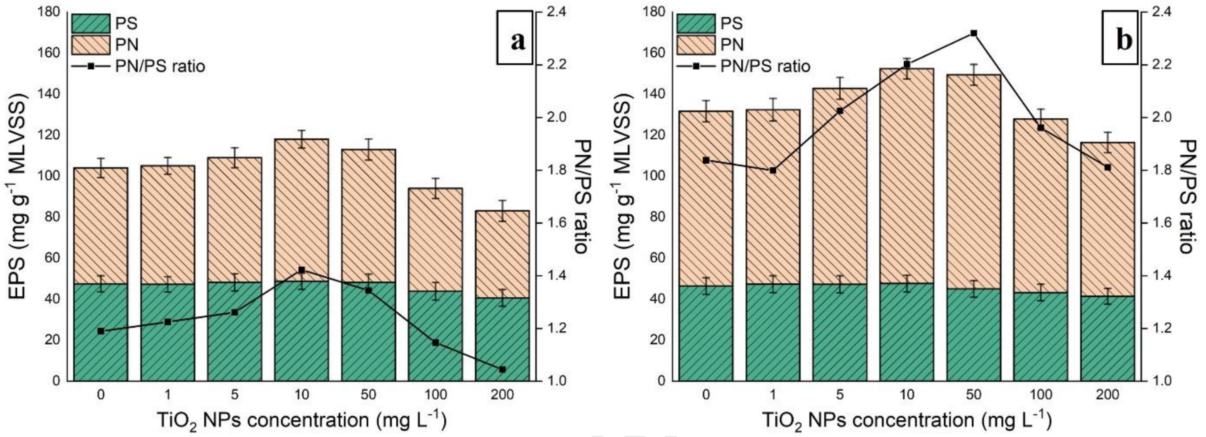


Fig. 2. Change in the extracellular polymeric substance (EPS) after introduction of titanium dioxide nanoparticles (TiO_2 NPs). EPS amount in **a)** aerobic granular sludge (AGS), and **b)** algal-bacterial aerobic granular sludge (AB-AGS) after addition of TiO_2 NPs.

With further increases in the TiO_2 NPs content, the extent of EPS showed a declining trend. At 50 mg L^{-1}, the PN content in AGS and AB-AGS was 64.8 and 104.4 mg g^{-1} MLVSS, while the PS remained relatively stable. This, in turn, increases the PN/PS ratio to 1.34 and 2.32, respectively. The results are in line with previous research, wherein 50 mg L^{-1} TiO_2 NPs had a positive effect on the EPS of AGS and AB-AGS [37,38]. The higher PN/PS ratio in the algal-bacterial sludge suggests more protected microorganisms in the center of the sludge against nanoparticles, as proteins are predominantly secreted inside the granules [49]. In AGS, both PS and PN marginally decreased, when the contamination level reached 100 and 200 mg L^{-1}. This resulted in 10% and 20% declines in EPS volume, respectively. The PN/PS ratio was also lower than that in the initial sludge. This implies that microorganisms inside the granules were exposed to TiO_2 NPs, which then lead to a reduction in their metabolic activity. Conversely, in AB-AGS, the percentage of polymers decreased only by 3% and 12%, respectively, while the PN/PS ratio was comparable to that in the control sludge. This suggests that microorganisms in the core of the granular sludge were more protected against titanium-dioxide than in aerobic granular sludge. Consequently, the negative effect of nanoparticles on wastewater treatment efficiency may be lower. The observed higher EPS content in the AB-AGS system can be attributed not only to bacterial secretion but also to the contribution of algae, which are an integral part of the algal-bacterial consortia. Algae in AB-AGS are known to secrete EPS, including both PS and PN, at different rates compared to bacteria [50,51]. Specifically, the algal contribution to EPS is significant in providing additional PS, which enhances the protective matrix of the granules [52]. This additional source of PS and PN from algae leads to a more robust EPS matrix, which supports the structural integrity of the granular sludge and provides greater resilience against environmental stressors, such as TiO_2 NPs. Therefore, the higher protein content observed in the AB-AGS is not solely a result of bacterial activity but is also driven by the algal production of EPS. This combined contribution from both algae and bacteria helps explain the improved resistance of AB-AGS to TiO_2 NPs, as the algal-generated EPS complements the bacterial EPS, creating a more protective and resilient granular structure.

In granular sludge tightly bound proteins (TB-PN) and tightly bound polysaccharides (TB-PS) are integral to the structural integrity and stability of the granules, mainly form the internal structure of the granules, contributing to their dense and compact nature. Loosely bound proteins (LB-PN) and loosely bound polysaccharides (LB-PS), on the other hand, play a crucial role in the initial aggregation and surface interactions of the sludge particles, it is present in the outer part of the granules, influencing the overall microbial activity and biofilm formation within the granules [16]. As shown in the *Figure 3*, the amount of TB-EPS in the initial AGS sludge is 2.1 times that of LB-EPS, while in AB-AGS it is only 1.7 times. This can be attributed to the fact that algae on the surface of AB-AGS are also capable of synthesizing EPS [53]. Increasing the amount of TiO_2 NPs to 10 mg L^{-1} resulted in an increase in LB-PS and LB-PN in both sludges, with a more pronounced increase in LB-PN. In AGS, the LB-PN increased from an initial 16.9 to 28.7 mg g^{-1} MLVSS, while in AB-AGS it increased from 29.6 to 40.2 mg g^{-1} MLVSS.

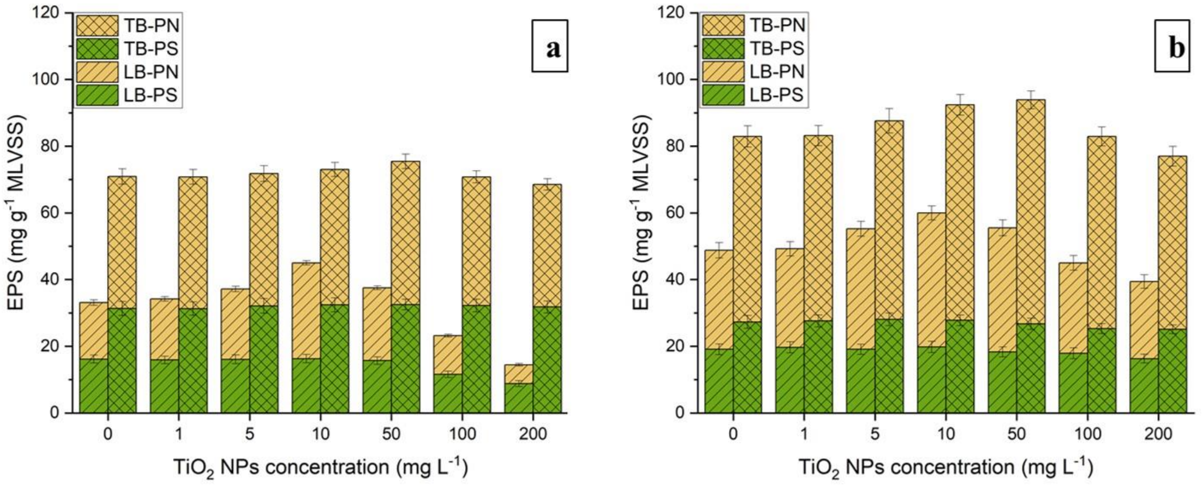


Fig. 3. Changes in loosely- and tightly bound polysaccharides and proteins (LB-PS, LB-PN, TB-PS, and TB-PN) after introduction of titanium dioxide nanoparticles (TiO_2 NPs). EPS amount in **a)** aerobic granular sludge (AGS), and **b)** algal-bacterial aerobic granular sludge (AB-AGS) after addition of TiO_2 NPs.

Nanoparticles at a concentration of 200 mg L^{-1} did not cause significant changes in the amount of TB-EPS, which may suggest that due to the structure of the granules, the toxic substance was unable to penetrate the interior of the granules (as they were able to produce sufficient TB-EPS), thus not affecting the predominantly anoxic and anaerobic microorganisms present inside. In contrast, there was a significant decrease in the volume of LB-EPS. For aerobic granules, the amount of LB-PS decreased by 45.6% compared to the initial amount, while the amount of LB-PN decreased by 66.6%. This large decrease suggests that aerobic microorganisms on the surface of the granules were unable to protect against the high concentration of nanoparticles, potentially reducing their nutrient removal capacity. Although the LB-EPS amount in AB-AGS also decreased, the amounts of LB-PS and LB-PN only decreased by 14.6% and 22.1%, respectively. These observations indicate that algae on the surface of the granules play an important role in EPS production and defense against toxic substances (*Fig. 4b and 4d*).

3.3. Impact the shock load of TiO_2 NPs on bioreactor performance

The surface of AGS (400 – 800 µm in diameter) exhibited various embedded microorganisms within the EPS matrix (*Fig. 4a*), while algae were also visible on the exterior of AG-AGS (500 – 1000 µm in diameter) (*Fig. 4b*).

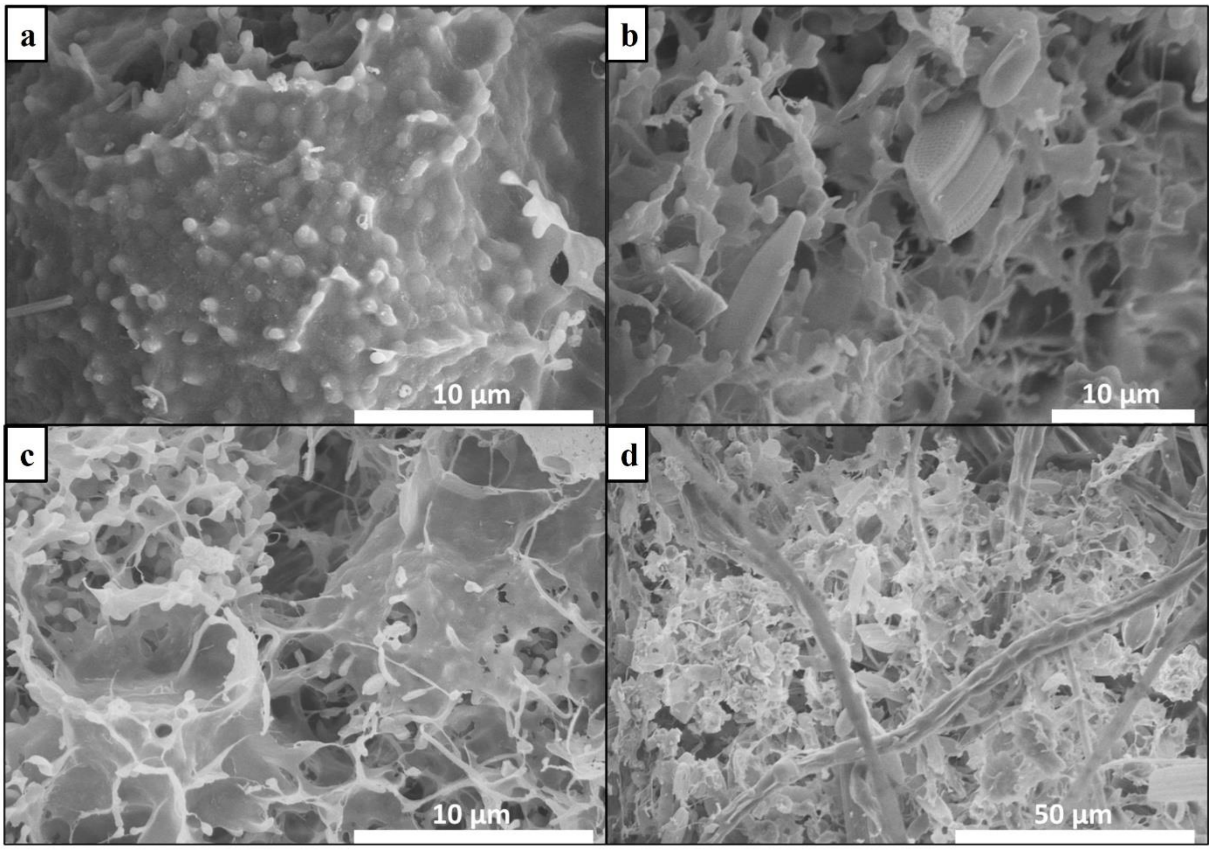


Fig. 4. Scanning electron microscope (SEM) images of granular sludge (GS). The exterior of the **a)** aerobic and **b)** algal-bacterial GS. The surface of the **c)** aerobic and **d)** algal-bacterial GS after the addition of 200 mg L^{-1} TiO_2 NPs.

Without the presence of nanoparticles, the effluent COD, ammonia-, nitrite-, nitrate-nitrogen, and PO_4^{3-} contents in AGS were 82 ± 5, 0.08 ± 0.02, 0.05 ± 0.01, 0.49 ± 0.03, and 0.54 ± 0.06 mg L^{-1}, respectively. In contrast, AB-AGS demonstrated more efficient nutrient removal with the corresponding concentrations of 74 ± 6.3, 0.05 ± 0.01, 0.03 ± 0.01, 0.45 ± 0.04, and 0.36 ± 0.05 mg L^{-1}, respectively. Upon addition of 50, 100, and 200 mg L^{-1} TiO_2 NPs into the bioreactors, the nutrient removal declined (*Fig. 5*). The performance of AGS and AB-AGS, however, remained stable at lower TiO_2 NP concentrations.

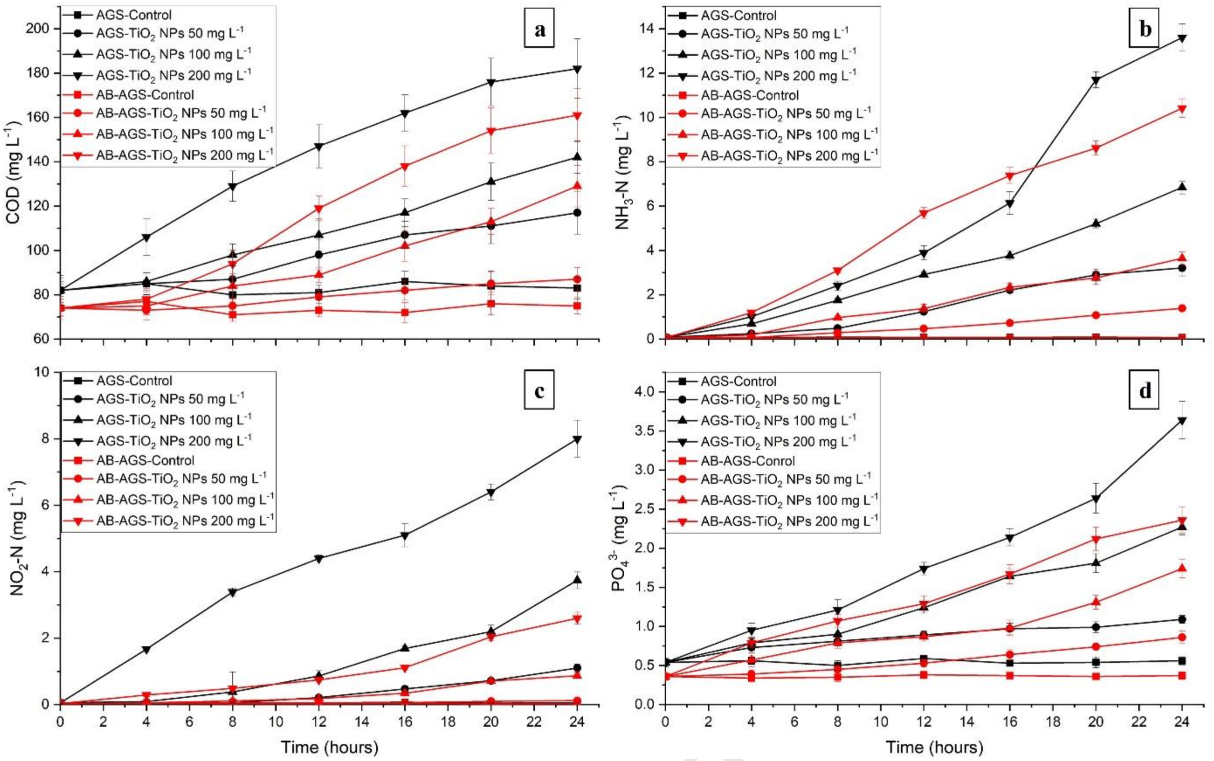


Fig. 5. Impacts of titanium dioxide nanoparticles (TiO$_2$ NPs) on the aerobic granular sludge (AGS) and algal-bacterial aerobic granular sludge (AB-AGS) performance. **a)** Chemical oxygen demand (COD), **b)** ammonia-nitrogen (NH$_3$-N), **c)** nitrite-nitrogen (NO$_2$-N), and **d)** phosphorus (PO$_4^{3-}$) contents.

The addition of 50 mg L^{-1} TiO$_2$ NPs to AB-AGS did not change the COD removal significantly, while in the AGS bioreactor, COD concentration in the effluent increased from 82 ± 2.3 to 117 ± 4.4 mg L^{-1} after 24 hours (*Fig. 5a*). Further increase in TiO$_2$ NPs concentration to 100 and 200 mg L^{-1}, on the other hand, resulted in reduced COD removal in both SBRs. The COD values in the effluent were 142 ± 3.8 and 183 ± 5.2 mg L^{-1} in AGS, and 129 ± 3.7 and 161 ± 4.1 mg L^{-1} in AB-AGS, respectively. Furthermore, at 200 mg L^{-1} nanoparticle concentration, no drop in the removal rate in the AB-AGS was observed after 4 hours of operation. This suggests higher tolerance for the latter compared to that of the aerobic granular sludge within a very narrow time interval. Li et al. [33] investigated the effect of TiO$_2$ NPs on activated sludge and found, that the nanoparticles at concentrations as low as 10 mg L^{-1} negatively influenced COD removal with a 10% drop after 1 day of operation. In another study, exposure of activated sludge to 50 mg L^{-1} TiO$_2$ NPs reduced the organic matter removal by 64% [36]. While Zhou et al. [35] and Cervantes-Avilés et al. [34] also reported a declined chemical oxygen demand reduction efficiency when the concentration of nanoparticles were lower than 50 mg/L. The significant differences between activated and granular sludges may originate from the differences in their structure. In activated sludge, microorganisms are more exposed to pollutants, as flocs have very loose structure. This allows the nanomaterials to attach to the surface of heterotrophic microorganisms and pass through the cell membrane [32]. In contrast to previous literature results, our findings suggest that heterotrophic microorganisms within the granules displayed increased resilience against the adverse effects of NPs. Compared to activated sludge, the elevated secretion of EPS within the granules could mitigate the toxicity of NPs on the sludge, which results in a lower decline in organic removal efficiency.

The removal rate of ammonia also decreased by the continuous increase in nanoparticle concentration. At 50, 100, and 200 mg L^{-1} TiO_2 NPs the NH_3-N removal efficiency dropped from 99.9% to 97%, 94.3%, and 88.6% in SBR, and from 99.9% only to 98.8%, 96.9%, and 91.3% in PSBR, respectively (*Fig. 5b*). The latter implies that the algal-bacterial consortia are more tolerant against contaminations, which is even more pronounced in the case of nitrite. Whilst addition of 200 mg L^{-1} nanoparticle to AB-AGS increased the nitrite concentration from 0.03 to 2.6 mg L^{-1}, the NO_2-N concentration grew from 0.05 to only 3.74 and 8.04 mg L^{-1} at 100 and 200 mg L^{-1} TiO_2 NPs, respectively (*Fig. 5c*).

During the experiments, the effluent nitrate concentration remained stable in the algal-bacterial sludge, whereas it slightly increased to 5.38 and 8.26 mg L^{-1} in AGS upon introducing 100 and 200 mg L^{-1} TiO_2 NPs, respectively. The differences observed in nitrite and nitrate removal efficiency between AGS and AB-AGS may be attributed to the amount of LB-EPS present, as nitrite- and nitrate-removing microorganisms are primarily located in the inner layers of the granules [54]. Therefore, microorganisms may be better protected against the negative effects of nanoparticles in an algal-bacterial sludge with higher LB-PS and LB-PN contents, due to the adhesion of toxic nanoparticles on the surface of the granules, they did not penetrate the anoxic and anaerobic zones where nitrite and nitrate removal also occurs. Additionally, significant changes were observed in the polymer matrix on the surface of AGS (*Fig. 5c*). This suggests that TiO_2 NPs were able to penetrate into the interior of the granules, inhibiting denitrifying microorganisms. Zheng et al. [18] investigated the impact of TiO_2 NPs on activated sludge, and found that the removal of ammonia declined to 24.4% at 50 mg L^{-1} nanoparticle contamination with stable nitrite and nitrate concentrations stable in the effluent wastewater. Studies on the effect of ≥50 mg L^{-1} ZnO NPs on AGS reported decreased nitrite and nitrate removal, similar to our results [55,56]. This discrepancy may arise from the low ammonia-to-nitrite conversion in the activated sludge, which in turn can be degraded first to nitrate and then to nitrogen by the microorganisms.

Cervantes-Avilés et al. [34] established that TiO_2 NPs at 1.5 and 2 mg/L caused a shift in bacterial diversity, resulting in a decline in organic matter and nitrogen removal efficiency in activated sludge. Studies by Li et al. [33] and Zheng et al. [18] have also shown that the addition of TiO_2 NPs can lead to significant shifts in the microbial community structure, particularly affecting the diversity of nitrifying and denitrifying bacteria. These changes can impact the overall efficiency of nitrogen removal, as well as the removal of organic matter. In our study, although we did not perform a detailed microbial diversity analysis, the observed decline in nitrification rate at higher concentrations of TiO_2 NPs (≥100 mg/L) suggests that the microbial community in both AGS and AB-AGS may have shifted. Specifically, the reduction in nitrifying bacteria could explain the decrease in ammonia and nitrite removal. These findings are consistent with previous reports, which indicate that TiO_2 NPs can exert selective pressure on microbial populations, reducing the abundance of key functional groups involved in nitrogen and organic matter cycling.

A significant difference was found in phosphate removal between the two bioreactors (*Fig. 5d*). At 100 and 200 mg L^{-1} titanium dioxide concentrations, the phosphate removal efficiency decreased from 97.3% to 88.6% and 81.8%, and from 98.2% to 91.3% and 88.2% in AGS and PSBR, respectively. The results show higher nanoparticle tolerance in AB-AGS, as the PO_4^{3-} removal at 200 mg L^{-1} TiO_2 NPs was nearly identical to that in AGS at 100 mg L^{-1}. This could be originated from the higher EPS amount in algal sludge. The P-accumulating organisms (PAOs) are embedded in the polymer [57,58], which prevent nanoparticles from attaching to cell surface and permeating into the cells. A further reason could be the high sensitivity of PAOs to various environmental factors, such as increased nitrite and nitrate concentrations [4,38]. Thus, higher nitrogen levels in AGS SBRs may further inhibit phosphorus removal. Moreover, algae uptake and store phosphorus from water [59,60], which also highlights the importance of algae in PO_4^{3-} removal in PSBRs. The greater resistance of AB-AGS to TiO_2 NPs compared to AGS can be attributed to several factors. Firstly, AB-AGS has a higher amount of EPS, which provides better protection to the microorganisms by adsorbing and sequestering nanoparticles, thereby reducing their toxic effects. Additionally, the symbiotic relationship between algae and bacteria in AB-AGS contributes to its resilience. The algae produce oxygen through photosynthesis, which supports bacterial respiration and metabolic activity [50], enhancing overall stability and resistance to pollutants. Moreover, the higher protein-to-polysaccharide ratio in AB-AGS suggests a stronger and more protective EPS matrix, further mitigating the impact of TiO_2 NPs. Finally, it is possible that due to filamentous growth, the specific surface area of the granules increased, which caused the nanoparticles to be more evenly distributed across the granules, rather than concentrated in one place. Thus, TiO_2 NPs did not inhibit the metabolism of the algae, as the negative effects of nanoparticles on algae (*see Fig. 4b and 4d*) are primarily exerted by adhering to their surface, preventing them from receiving sufficient light [24].

3.4. Impact of TiO_2 NPs on the microbial activity of AGS and AB-AGS

To assess the impact of TiO_2 NPs on the microbial activity, the SAUR, SNIUR, SNUR, and SPUR of AGS and AB-AGS were examined at various nanoparticle concentrations. As shown in *Fig. 6a*, the SAUR of aerobic granular sludge remained stable after the introduction of 1 and 5 mg L^{-1} nanoparticles, while it did not change in the algal-bacterial sludge even at 10 mg L^{-1} TiO_2 NPs. In AGS, the increase in TiO_2 NP concentration from 0 to 100 and 200 mg L^{-1} decreased the ammonia uptake from 4.71 to 3.51 and 2.97, and from 5.61 to 4.87 and 4.29 mg N (g MLVSS·h)$^{-1}$ in AGS and AB-AGS, respectively. The initial SNIUR in AGS and AB-AGS was 3.74 and 4.41 mg N (g MLVSS·h)$^{-1}$. This decreased first to 3.26 and 3.03, and then further to 4.29 and 3.94 mg N (g MLVSS·h)$^{-1}$ at 100 and 200 mg L^{-1} TiO_2 NP concentrations, respectively (*Fig. 6b*). The changes in SAUR and SNIUR indicate that elevated TiO_2 NPs concentration could influence the microbial activities of both ammonia- and nitrite-oxidizing microorganisms. Furthermore, the variations in SAUR and SNIUR were consistent with the NH_3-N and NO_2-N removal at all nanoparticle concentrations. Similar phenomena were observed in previous studies, where increasing amounts of ZnO and TiO_2 NPs led to a decrease in SAUR and SNIUR, consequently affecting the removal of NH_3-N and NO_2-N in activated sludge [33,61]. The SNUR in the control aerobic and algal-bacterial sludge was 15.4 and 16.7 mg N (g MLVSS·h)$^{-1}$, respectively. With TiO_2 NP concentrations of 50, 100, and 200 mg L^{-1}, the SNUR decreased by 4%, 12%, and 20% in AGS, respectively. In contrast, in AB-AGS, the nitrate uptake remained unchanged up to 50 mg L^{-1} TiO_2 NPs and only dropped by 3% and 6% at higher concentrations (*Fig. 6c*). This observation suggests that the nitrate removal in algal-bacterial sludge remained unaffected, which indicates that the increased titanium content did not inhibit the microbial activity of denitrifying bacteria. As shown in *Fig. 6d*, at 50, 100, and 200 mg L^{-1} NPs, the SPUR dropped by 7%, 11%, and 23%, and only by 3%, 7%, and 15% in AGS and AB-AGS, respectively. These differences explain the higher PO_4^{3-} concentrations in the effluent from AGS. The increase in titanium concentration in the influent resulted in greater inhibition of aerobic PAOs in the SBR compared to that in PSBR. The mild change in SPUR can also be attributed to several factors: i) heavy metal tolerance [62], ii) the algae's capability to accumulate and store a large amount of cellular polyphosphate [63], and iii) the enhanced removal of nitrogen by algae [64]. Therefore ammonia, nitrite, and nitrate did not hinder the activity of PAOs. For AB-AGS, the presence of algae not only aids in oxygen production but also contributes to the overall structural integrity and resilience of the granules. The algae secrete additional EPS, which enhances the overall protective ability of the sludge. Furthermore, the metabolic activities in AB-AGS are less affected due to the synergistic interactions between algae and bacteria, which maintain higher levels of microbial activity even under nanoparticle stress.

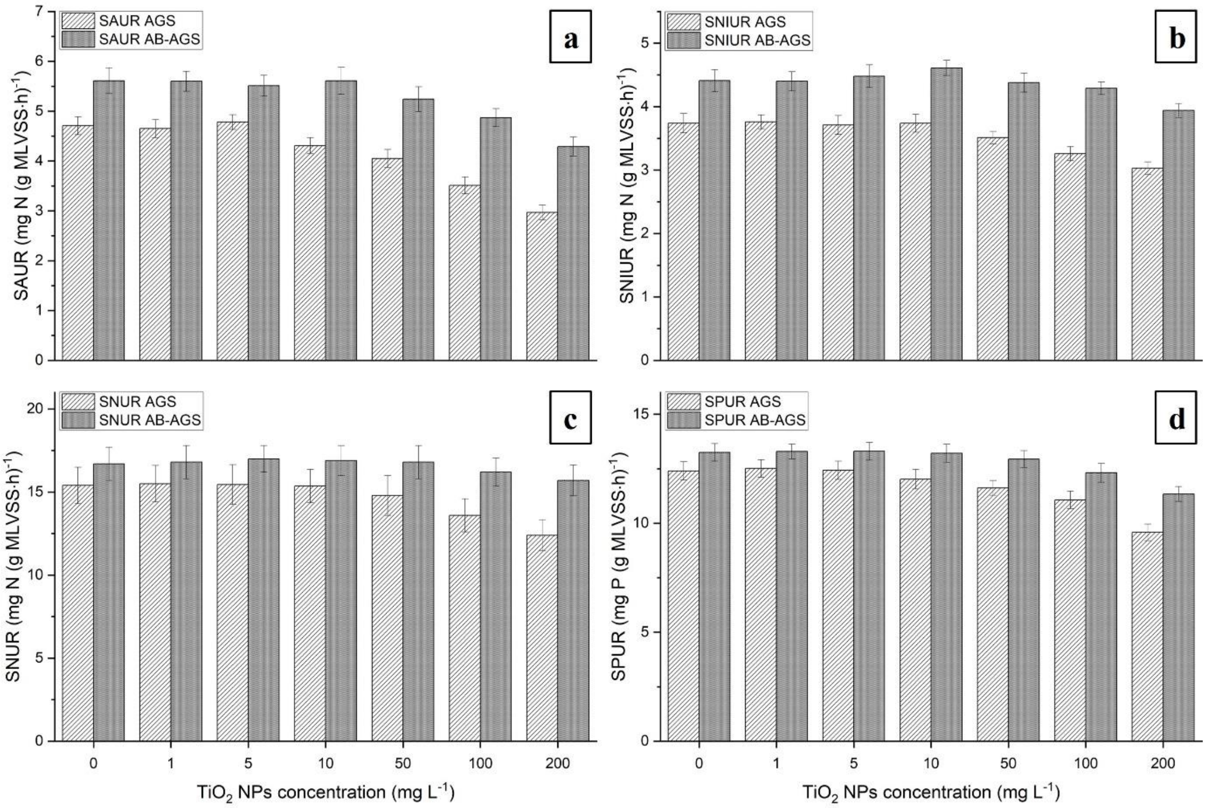


Fig. 6. Impact of titanium dioxide nanoparticles (TiO_2 NPs) on the microbial activities of aerobic granular sludge (AGS) and algal-bacterial aerobic granular sludge (AB-AGS). **a)** specific ammonia uptake rate (SAUR); **b)** specific nitrite uptake rate (SNIUR); **c)** specific nitrate uptake rate (SNUR); **d)** specific phosphorus uptake rate (SPUR).

4. Conclusions

The shock-load impact of titanium dioxide nanoparticles (TiO_2 NPs) on biological wastewater treatment processes was studied at concentrations ranging between 1 and 200 mg L^{-1}. TiO_2 NPs exerted various effects on nutrient removal, the level of extracellular polymeric substances

(EPSs), and the microbial activity in aerobic granular sludge (AGS) and algal-bacterial granular sludge (AB-AGS) bioreactors. The fluctuations in nutrient removal mirrored those in microbial activities across varying TiO_2 NP concentrations. Low nanoparticle concentrations ($\leq$10 mg L^{-1}) had no discernible impact on the bioreactors, whereas COD, nitrogen, and PO_4^{3-} removal decreased at concentrations $\geq$50 mg L^{-1}. At the highest titanium content, PO_4^{3-} removal efficiency declined from 97.3% to 81.8%, and from 98.2% to 88.2% in AGS and AB-AGS, respectively. The reduction in specific phosphorus uptake rate (SPUR) was less pronounced in algal-bacterial sludge, resulting in lower phosphorus levels in the effluent. The enhanced tolerance of AB-AGS to TiO_2 NPs may be attributed to a higher supply of LB-EPS and TB-EPS (especially lower drop in LB-EPS) and a higher protein-to-polysaccharide (PN/PS) ratio. These findings provide valuable insights into understanding the shock-load impact of TiO_2 NPs on the performance of both aerobic granular and algal-bacterial granular sludges.

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CRediT authorship contribution statement

Alfonz Kedves: conceptualization, data curation, formal analysis, validation, investigation, methodology, visualization, software, writing - original draft. **Çagdas Yavuz**: data curation, investigation, methodology, software, visualization, formal analysis, writing - original draft.

Orsolya Kedves: formal analysis, investigation, methodology, software, visualization. **Henrik Haspel**: formal analysis, funding acquisition, project administration, resources, writing - review & editing, supervision. **Zoltán Kónya**: formal analysis, resources, writing - review & editing, supervision, project administration, and funding acquisition. All authors have given approval to the final version of the manuscript.

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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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.

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Highlights

- Impact of TiO_2 NPs on the performance of AGS and AB-AGS bioreactors was studied.
- Microbial activity was stable at $\leq$10 mg L^{-1} NPs and declined at amounts $\geq$50 mg L^{-1}.
- TiO_2 NPs over 50 mg L^{-1} reduced the COD, nitrite, and phosphorus removal.
- TiO_2 NPs had more significant negative effects on AGS than AB-AGS.
- Higher PN and PN/PS ratio mitigated the toxicity of TiO_2 NPs in AB-AGS.