

Long-term effect of graphene oxide on the aerobic granular sludge wastewater treatment process

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Abstract

This study investigated the bioreactor performance, production of extracellular polymeric substance (EPS), and microbial activity of a granulated sequencing batch reactor (SBR) by increasing the graphene oxide nanoparticle (GO NP) concentration stepwise. The GO NPs reduced the removal of chemical oxygen demand (COD), ammonia, and phosphorus, whereas the nitrite and nitrate contents of the effluent were kept stable during the experiment. The ammonia-nitrogen (NH₄-N) and COD removal rates were decreased considerably from 99% and 95% to 96.2% and 78.6% at 55 mg/L GO NPs; furthermore, at 115 mg/L GO NPs, the NH₄-N and COD removal rates further decreased to 88.5% and 59.3%. The removal of phosphorus decreased even at small concentrations of graphene oxide (GO), and the inhibitory effect enhanced with an increase in the GO NP content. The increased amounts of nanoparticles significantly influenced the microbial activity of aerobic granular sludge (AGS). The specific oxygen uptake rate (SOUR) decreased from 42.04 to 33.14 mg O₂/g MLVSS*h, specific ammonia oxidation rate (SAOR) declined from 4.84 to 4.12 mg N/g MLVSS*h, specific phosphorus uptake rate (SPUR), and specific phosphorus release rate (SPRR) significantly decreased from 13.1 and 10.05 to 8.2 and 8.7 mg P/g MLVSS*h after 98 days. However, the specific nitrite and nitrate reduction rate (SNIRR and SNRR), and the specific nitrite oxidation rate (SNOR) remained relatively stable. The EPS content of sludge was initially 5.95 mg/g MLVSS, but the presence of GO up a concentration of 55 mg/L promoted the secretion of EPS and increased to 11.86 mg/g MLVSS. At higher GO concentrations, the secretion of EPS was inhibited. After 14 days, when the influent synthetic wastewater (SWW) did not contain GO NP, the AGS SBR performance showed a remarkable recovery capability.

Keywords: aerobic granular sludge, GO nanoparticles, microbial activity, extracellular polymeric substances, recovery, long-term effect

1. Introduction

Graphene oxide nanoparticles (GO NPs) are used extensively in the industry as fuel cells and batteries, and for catalysis. Furthermore, using GO NPs in biomedical applications is becoming more common [1–3]. With the increasing production and extensive applications, GO NPs are unavoidably released into the environment, such as into the soil and surface water [4–7]. Hence, previous studies described the biotoxic impact of nanoparticles (NPs) on phytopathogenic bacteria [8], bacteria biofilm [9], and marine ecosystem [10]. However, the negative impact of GO NPs on the soil microbial communities is reversible [6]. Nonetheless, few studies have examined the effect of graphene oxide (GO) on wastewater treatment processes. Owing to their long-term stability in aquatic systems, they finally reach the municipal biological wastewater treatment systems [11,12]. A survey found that in the case of wastewater treatment plants, nanomaterials are detectable at relatively high concentrations in the biosolid [13]. Thus, it is critical to discover the influence of GO NPs on biological wastewater treatment processes. Ahmed and Rodrigues (2013) investigated the acute effect of GO NPs on activated sludge (AS) and established that the particles significantly reduced the nutrient removals, and negatively affected the properties of sludge. Nguyen and Rodrigues (2018) reported that the chronic exposures of GO NPs negatively influenced the organic matter, nitrogen, and phosphorus removal rates in AS, resulting in a shift in the microbial community structure and diversity.

The aerobic granular sludge (AGS) technology is a novel and up-and-coming biological process for wastewater treatment [16,17]. AGS systems showed many unique attributes against conventional activated sludge (CAS) [18]; for example, a shorter settling time, the ability to withstand the toxic compounds, and strong recovery against environmental changes because of the aerobic and anaerobic belts within the granules. This AGS technology can remove the phosphorus, nitrogen, and organic matter using as energy sources in the same system and time [19–21]. The extracellular polymeric substances (EPS) play a vital role in the AGS technology because, during the granulation process, microorganism are embedded in the matrix; therefore, they are protected against toxic matter [19,22]. Hence, this technology can remove textile dyes [23], metal contaminants [24], and landfill leachate [25].

Previous studies investigated the long-term effects of zinc oxide nanoparticles (ZnO NPs), cupric oxide nanoparticles (CuO NPs), and silver nanoparticles (Ag NPs) on AGS wastewater treatment

technology. He et al. (2017a) reported that the ZnO NPs inhibited nitrification and denitrification, whereas the removal of organic matter and phosphorus remained stable. In contrast, Zheng et al. (2017) showed an increased total nitrogen (TN) removal rate and a decreased total phosphorus (TP) removal efficiency because of the increase in CuO NPs. Quan et al. (2015) observed a decline in the denitrification and nitrification processes at 50 mg/L Ag NP content.

Zheng et al. (2019) investigated the effect of cerium oxide nanoparticles (CeO₂ NPs) on AGS performance and examined the recovery ability of the bioreactor. They found that CeO₂ NPs inhibited the removal rate of nutrients, the production of EPS increased; however, when the addition of particles was stopped, the removal rate of TN and TP started to increase and finally recovered. In the case of GO NPs, there is limited data about their effect on AGS. Guo et al. (2018) and Liu et al. (2017) investigated the presence of GO particles in granular sludge and measured the removal of phosphorus and nitrogen during batch tests. As far as we know, no previous studies have been investigated the long-term effect of GO on AGS bioreactor performance. Furthermore, we do not have information about studies, where the bioreactor was continuously fed with GO NPs and the concentration of particles was also continuously increased. In addition, there is no data on its recovery after long-term exposure to GO NPs, which is an important characteristic of a biological system.

Therefore, this study's major purpose is to demonstrate the long-term effect of GO on the aerobic granulated sequencing batch reactor (SBR) regarding reactor performance, EPS production, microbial activity, and AGS recovery. Hence, the concentrations of GO NPs in the influent synthetic wastewater (SWW) were increased stepwise, from 0 to 115 mg/L. Finally, to examine the recovery of the system, the bioreactor was operated without NPs. This work might supply thorough knowledge on the effects of GO NP administration on AGS SBR.

2. Methods

2.1. Synthesis of GO NPs and configuration of the bioreactor

The particles were prepared following the modified Hummer's method and characterized as reported previously [31], (for details, see the supporting information, (Fig. S1)). An AGS SBR operation temperature was $20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ with an effective volume at 1.4 L (inner diameter of 65 mm and height of 455 mm) and configured as in our previous work investigating the chronic effects of AGS to the presence of GO NPs [31]. The AGS bioreactor was fed with SWW (pH = 7.3 ± 0.5), consisting of (mg/L) COD 1300 (glucose), $\text{NH}_4\text{-N}$ 120 (NH_4Cl), TP 20 (KH_2PO_4), NaHCO_3 200, CaCl_2 25, MgSO_4 45, and 1 mL trace element solution [26], that was contaminated with GO NPs (at 0, 15, 35, 55, 75, 95, 115, and 0 mg/L), except in the initial and final phases. The GO concentration was increased continuously, and the exposure was conducted in eight phases, each phase for 2 weeks (14 days), and up to 112 days. In the last phase, GO NPs were not added to the bioreactor, to investigate the self-recovery ability of the AGS system.

2.2. Analytical methods

Physicochemical analyses on the control and treated bioreactors were performed; the chemical oxygen demand (COD), ammonia-nitrogen ($\text{NH}_4\text{-N}$), nitrite- and nitrate-nitrogen ($\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$), and TP of the effluent were measured daily by using HACH kits (LCI400, LCK304, LCK341, LCK339, and LCK348) and a Hach-Lange DR5000 ultraviolet-visible spectrophotometer (UV-VIS). To determine the nutrient removal pathways of the AGS, the specific oxygen utilization rate (SOUR), specific ammonium- and specific nitrite oxidation rate (SAOR and SNOR), specific nitrite- and nitrate reduction rate (SNIRR and SNRR), specific phosphorus uptake and release rate (SPUR and SPRR) were measured at the end of each phase (on days 14, 28, 42, 56, 70, 84, 98, and 112). The conditions of measurements are described in the supporting information.

At the same time, several parameters were investigated, including mixed liquor suspended solids (MLSS), the concentration of mixed liquor volatile suspended solids (MLVSS), and the settleability (sludge volume index after five minutes of sedimentation-SVI₅). These parameters were analyzed as per standard methods [26]. The extracellular polymeric substances (EPS) of the granular sludge were extracted with a modified heating method [32]. The protein and

polysaccharide (PN and PS) amounts of the extracted polymeric substances were assessed using the Anthrone and modified Lowry patterns [33]. The granular sludge samples, which were previously fixed and spray-coated with gold [31], were noticed by scanning electron microscopy (SEM; Quorum Technologies, SC7620) to investigate the morphological and structural changes.

2.3. Statistical analysis

All measurements were performed in triplicate, and the measured data were expressed as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) was conducted to test the significance of the results. In each plot the significant level was set at 5%. The regression coefficient (R^2) of the model was 0.978, in the case if the last 14 days were ignored (days 99-112).

3. Results and discussion

3.1. Bioreactor performance and sludge properties during GO NP exposure

The AGS SBR was run for 112 days, in which the GO NP concentration was increased gradually every two weeks from 0 to 15, 35, 55, 75, 95, 115, and 0 mg/L. During the GO NP exposures, the COD, nitrogen compounds, and TP were measured daily in the effluent. The MLSS, MLVSS, and SVI₅ were determined at the end of the exposure phase.

The granules had a size of 300–500 μm , and a compact and dense structure (Fig. 1a and b). As shown in Fig. 1c there are many cocci on the surface and in the matrix of the EPS, while Fig. 1d shows the adherent particles on the interface of the granules.

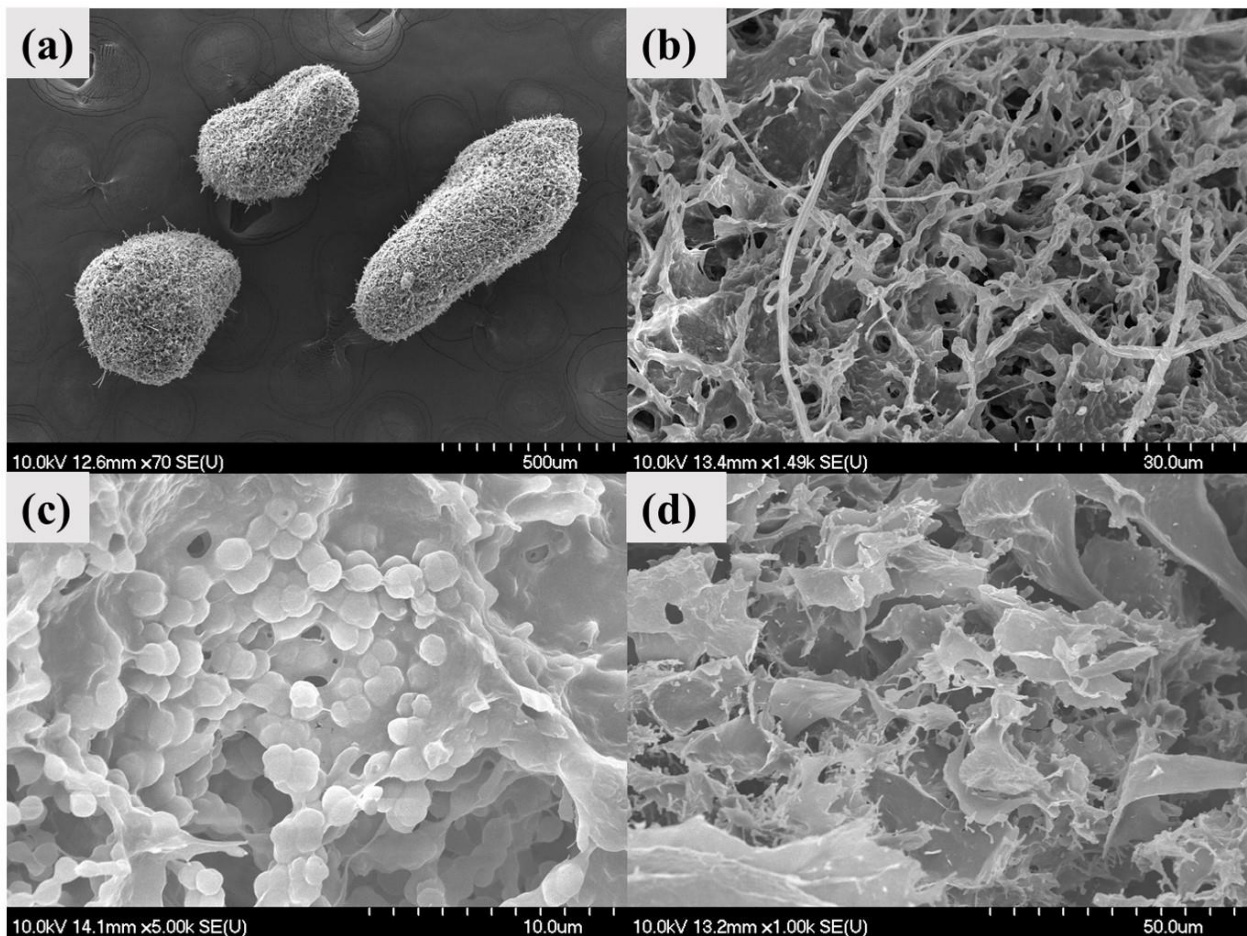


Fig. 1. SEM micrographs of AGS. (a) size of granular sludge, (b) surface of a granular sludge, (c) microorganisms on EPS, and (d) sheets of GO NPs on the granules.

Throughout the whole experiment, nitrite- and nitrate-nitrogen concentrations in the effluent remained stable (0.04 ± 0.01 and 0.5 ± 0.15 mg/L). Previous AGS studies reported similar observations, that is, the denitrification processes remained unaffected by adding CuO and Ag nanoparticles even at high amount [26,27]. Fig. 2. illustrates the effluent COD, NH₄-N, and TP contents, and the removal efficiency of these nutrients. Ammonia-nitrogen removal remained comparatively stable during the experiment. In the first phase, the removal efficiency of ammonia-nitrogen was 99%, and it declined to 88.5% at 115 mg/L GO, suggesting that the GO NPs did not cause considerable inhibition on nitrifying bacteria. Owing to the low concentrations of nitrite- and nitrate-nitrogen, considering that the removal efficiency of nitrogen was higher than 80%, the effluent nitrogen content complies with the limit permitted by Directive 91/271/EEC [34].

A stepwise increase in the GO NPs concentration caused an increased amount of COD in the effluent. In the first phase, the removal efficiency of COD was around 95%; however, this value fell below 90% at 35 mg/L graphene oxide, and the content of COD exceeded the permitted limit in the effluent (125 mg/L). The addition of 95 and 115 mg/L GO NPs caused a significant decrease in the removal of COD, and the effluent COD content was 410 and 530 mg/L, respectively. The increased pH values could cause high concentrations of COD in the effluent. In the first two periods, the pH was 7.3 ± 0.1 . The higher GO NP concentration caused a decline in pH; therefore, it required continuous adjustments. The accumulated GO NPs probably negatively influenced the aerobic organic matter-degrading microorganisms, while the anaerobic microorganisms, which are located inside the granules, remained unaffected. Therefore the amount of produced organic acids were accumulated in the system, as was observed in a previous study [35].

In the case of COD and NH₄-N, after 98 days, when the addition of GO NP was terminated, their removal efficiency started to increase. After 14 days, the NH₄-N and COD concentrations in the effluent were 0.5 and 103 mg/L, which are almost the same as those in the initial phase; thus, the system showed an excellent recovery ability. Our results suggest that the microbial metabolic activity (in the case of heterotrophic and nitrifying microorganisms) started to improve by termination of adding GO NPs.

Even at low concentrations, the nanoparticles negatively influenced the removal of TP. When the GO NP content in the effluent was 15 mg/L, after 14 days, the removal rate decreased to 89%, while at 115 mg/L of graphene oxide the removal efficiency fell below 57%. During the biological

phosphorus removal, the aerobic phosphorus uptake is more significant than the anaerobic phosphorus release [36]; probably, in our system the GO NPs could have a greater inhibitory effect on the biological phosphorus uptake. During the last phase, the TP remained relatively high in the effluent. After 112 days, it was 5 mg/L and showed worse recovery ability than COD and NH₄-N. Zheng et al. (2019) also observed that in the case of CeO₂ NP exposure, the COD removal efficiency rapidly recovered after a few days, while the TP removal efficiency did not completely recover, when no nanoparticles were added to the granular sludge. These results indicate that the GO NPs in the case of phosphorus removal caused a chronic effect and not only an acute effect.

The decreased biomass content in the bioreactor could explain the steadily decreasing removal rate of nutrients. In the first stage, the MLSS content was 6.2 g/L, while the highest concentration of particle (115 mg/L) caused a decrease of MLSS to 3.7 g/L. However, it subsequently increased to 4.4 g/L in the last phase. The SVI₅ showed a similar tendency. While it was initially 34 mL/g, the continuous addition of GO NPs decreased the settling velocity, and at day 98, the SVI₅ was 174 mL/g.

Two previous studies also reported in the case of activated sludge, the increasing concentration of GO NPs resulted in a negative effect on the removal of COD, ammonia, and phosphorus [14,15]. A preceding study showed that in granular sludge, the ZnO NPs negatively influenced the inorganic nutrient removal efficiency, due to the increased concentrations of nanoparticles under long-term exposure; however, the COD removal efficiency remained unaffected [17]. Zheng et al. (2017) reported similar observations, during a long-term exposure, the CuO nanoparticles at 50 mg/L reduced the biomass production of granular sludge and inhibited the biological removal of phosphorus. The impacts of the mentioned nanoparticles on the sludge are distinct, due to their structural, morphological, and chemical differences. Furthermore, the microbial community which degrades the nutrients in bioreactors was also different [31].

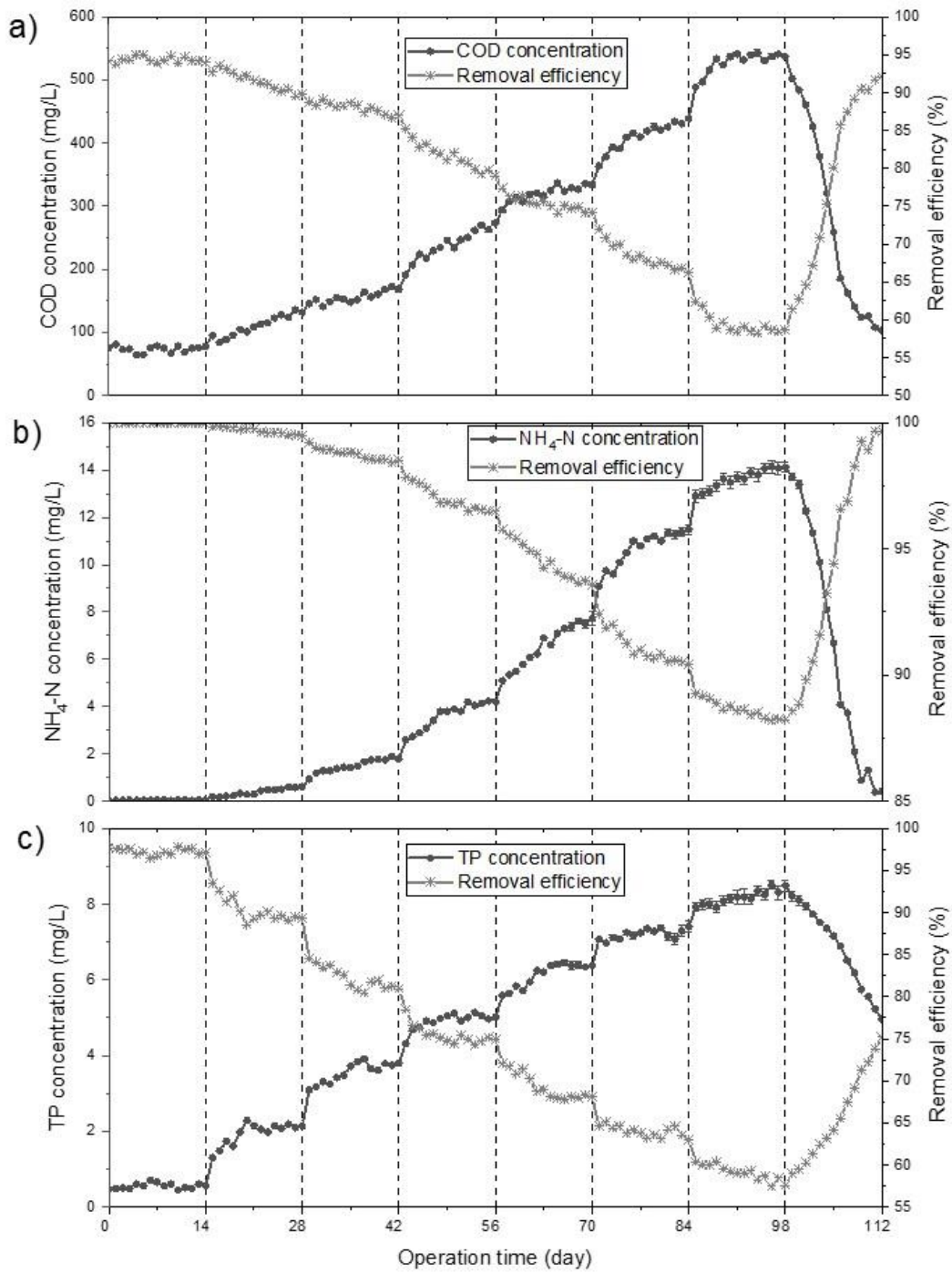


Fig. 2. Impacts of GO on the variations of the effluent (a) COD, (b) $\text{NH}_4\text{-N}$, and (c) TP contents.

3.2. Impact of GO particles on the microbial activity of aerobic granulated sludge

Fig. 3 demonstrated the impact of GO nanoparticles on the microbial activity of sludge. The SOUR increased significantly from 42.04 to 45.11, and 45.89 mg O₂/g MLVSS*h when the amount of GO NPs was increased to 15 and 35 mg/L (Fig. 3a). This indicates that the GO NPs at low amount had positive impact on the catabolic activity of heterotrophic microorganisms in the granular sludge. Previous examination reported a similar observation, where the SOUR of AGS increased due to the increased salinity of SWW [37]. However, at 75, 95, and 115 mg/L nanoparticles, a significant reduction in SOUR was observed ($p < 0.01$ and $p < 0.001$). The SOUR of granular sludge gradually decreased from 39.94 to 38.04, 34.93 and 33.14 mg O₂/g MLVSS*h by increasing GO NPs from 55 to 75, 95, and 115 mg/L, respectively. These results could interpret the reduced COD removal rate in the AGS bioreactor. Xu et al. (2017) also noticed that the SOUR decreased by the addition of Ag NPs in the case of activated sludge. In the last phase, when the addition of particles was completed, the SOUR increased to 39 mg O₂/g MLVSS*h, which result is in line with the effluent COD concentration.

Both the SAOR and SNOR decreased slightly due to the increased GO content (Fig. 3b). The SAOR and SNOR of AGS decreased from 4.84 and 3.85 at 0 mg/L GO to 4.12 and 3.6 mg N/g MLVSS*h at 115 mg/L GO. The SNOR did not change significantly during the experiment ($p > 0.05$); furthermore, significant differences in SAOR were observed at 75, 95, and 115 mg/L GO NPs ($p < 0.05$ and $p < 0.01$). The decreased SAOR of granular sludge could have caused the increased concentration of NH₄-N in the effluent. This indicates that graphene oxide could negatively inhibit the ammonia oxidization process. This phenomenon was also observed in the case of CAS after addition ZnO and CeO₂ NPs [39,40].

As shown in Fig. 3c, the GO NPs did not cause statistically significant differences in the SNRR of AGS ($p > 0.05$). However, a slightly significant decrease of SNIRR was observed after exposure of 75, 95, and 115 mg/L GO particles ($p < 0.05$), illustrating that the GO NPs did not strongly affect the denitrifying bacteria in granular sludge. These observations are in correspondence with the low NO₂-N and NO₃-N contents in effluent water. The SNRR or SNIRR of the sludge was constantly higher than the sum total of SNOR and SAOR during the experiment, which could explain that the effluent nitrite and nitrate contents remained stable. These results corroborate with previous

studies, wherein no obvious increased $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$ contents were found in the effluent, due to the higher amount of SNRR or SNIRR over SAOR and SNOR in the AGS [37,39].

The SPRR of granular sludge showed a more slowly decreasing trend compared with the SPUR with the increase of GO content (Fig. 3d). A significant decrease in the SPRR was found after addition of GO at 55 mg/L ($p < 0.05$), however, further increasing the nanoparticle concentrations, the SPRR was extremely significantly lower compared with the initial phase ($p < 0.001$), when the bioreactor was fed only with SWW. In contrast, the SPUR significantly decreased already at 15 mg/L GO ($p < 0.05$). Further increasing the amount of additional particles, a strong significant decrease in SPUR was observed ($p < 0.001$), illustrating that graphene oxide had earlier been inhibited phosphorus uptake under aerobic conditions (Fig. 3d). Previous studies reported similar observations, in which the reduced SPUR and SPRR of the sludge resulted in an increased phosphorus content due to the increased concentration of nanoparticles (ZnO, CeO₂, Ag, and Fe₃O₄ NPs) [38–41].

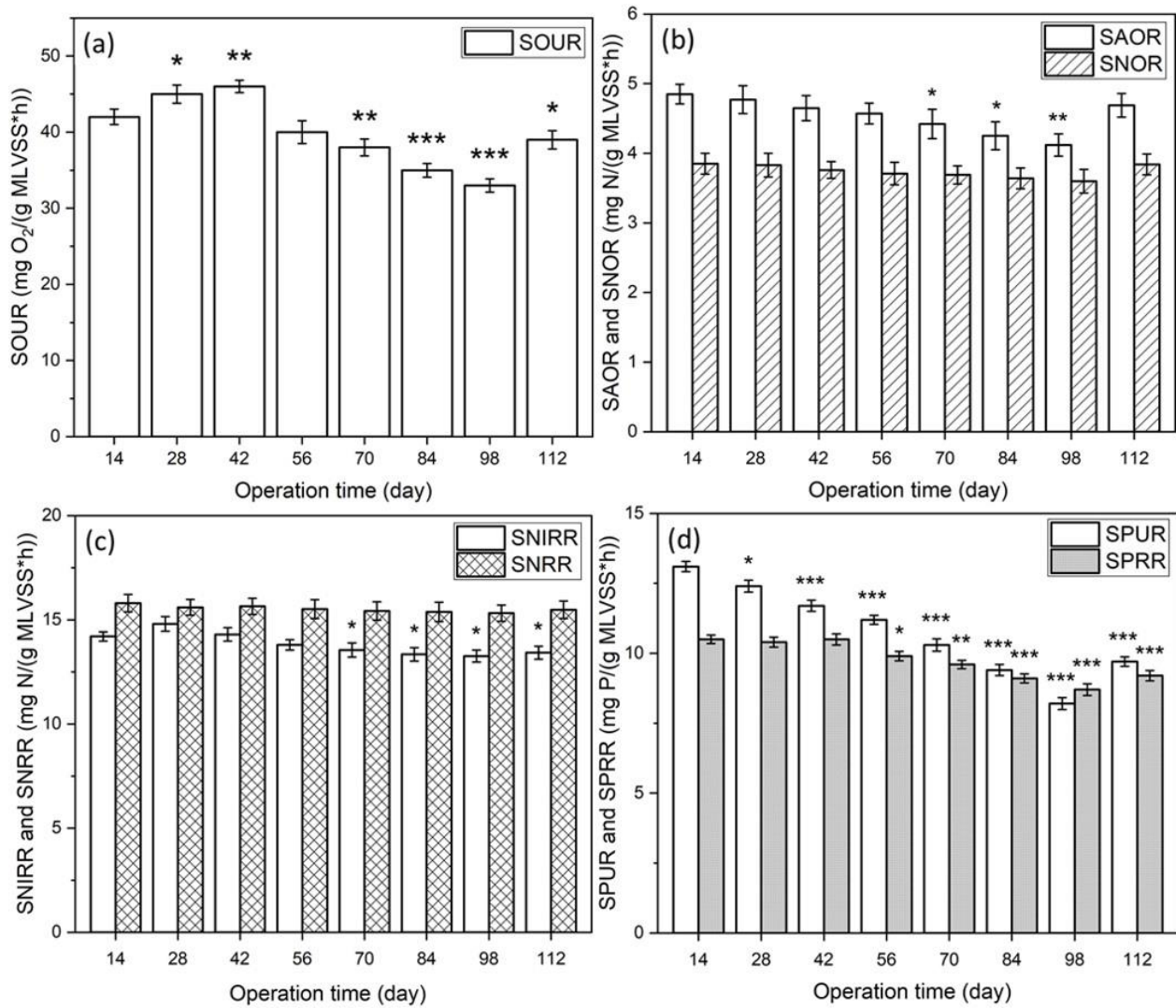


Fig. 3. Impacts of GO NPs on the microbial activities of AGS. (a) SOUR, (b) SAOR and SNOR, (c) SNIRR and SNRR, (d) SPUR and SPRR. The stars show statistical differences (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$) compared to the control (on day 14 and without GO NPs) (one-way ANOVA).

3.3. Effects of GO particles on EPS contents of AGS

Fig. 4. illustrated the amount of PS, PN, and EPS in the AGS that were recorded at the end of each phase. At the beginning of the experiment, the EPS content was 5.89 mg/g MLVSS, while the PS and PN contents were 3.31 and 2.70 mg/g MLVSS, respectively. Introducing the particles at the amount of 15, 35, 55, and 75 mg/L significantly stimulated the excretion of EPS, especially at 35

and 55 mg/L GO exposure, wherein the EPS were extremely significantly higher compared with the initial phase ($p < 0.001$). Similarly, the content of PN significantly increased to 4.69, 7.31, 8.69, and 9.93 mg/g MLVSS, while the content of PS remained relatively stable; therefore, the PN/PS ratios in the EPS increased from 0.82 to 1.53, 1.75, 2.74, and 2.51, respectively. Zheng et al. (2019) also showed similar observations in the case of CeO₂ NPs, where the PN production of AGS was higher than that of PS. In addition, based on Figs. 2 and 4, we observed that when the secretion of PN started to decrease (at 75 mg/L GO), the ammonia content in effluent increased gradually. At 95 and 115 mg/L particle exposure, the EPS amount in AGS was lower than that in the initial phase, decreased to 5.46 and 5.52 mg/g MLVSS, respectively.

In the last phase, when the bioreactor was fed only with SWW, the amount of EPS was substantially higher than that at the initial phase and increased to 8.61 mg/g MLVSS. Ending the administration of GO NPs into the bioreactor, the amount of EPS and PN was extremely higher in the sludge than in the initial sludge ($p < 0.01$ and $p < 0.001$), while the content of PS was no significantly different ($p > 0.05$). The remaining amount of GO NPs could not exert an additional negative effect on the granular sludge, suggesting that the long-term exposure of the nanoparticles did not cause permanent damage to the EPS-producing microbial community.

Similar observations were also reported in the case of CuO NPs, i.e., the ratio of PN/PS and the PN content of granular sludge increased considerably at 5 and 20 mg/L CuO NPs exposure; however, the concentration of EPS decreased extensively at 50 mg/L CuO NPs concentration [26]. In the case of ZnO NPs, the concentration of EPS was already decreased when the nanoparticle concentration was 20 mg/L [17]. In addition, Joshi et al. (2012) showed that the overproduction of EPS improved the aggregation of Ag NPs, thereby protecting the microorganisms against the stress caused by nanoparticles. The abovementioned observations and our results indicate that the EPS assuredly play a vital role against the negative effect of nanoparticles because a decline in the content of EPS redounded in an increased nutrient content in the discharge.

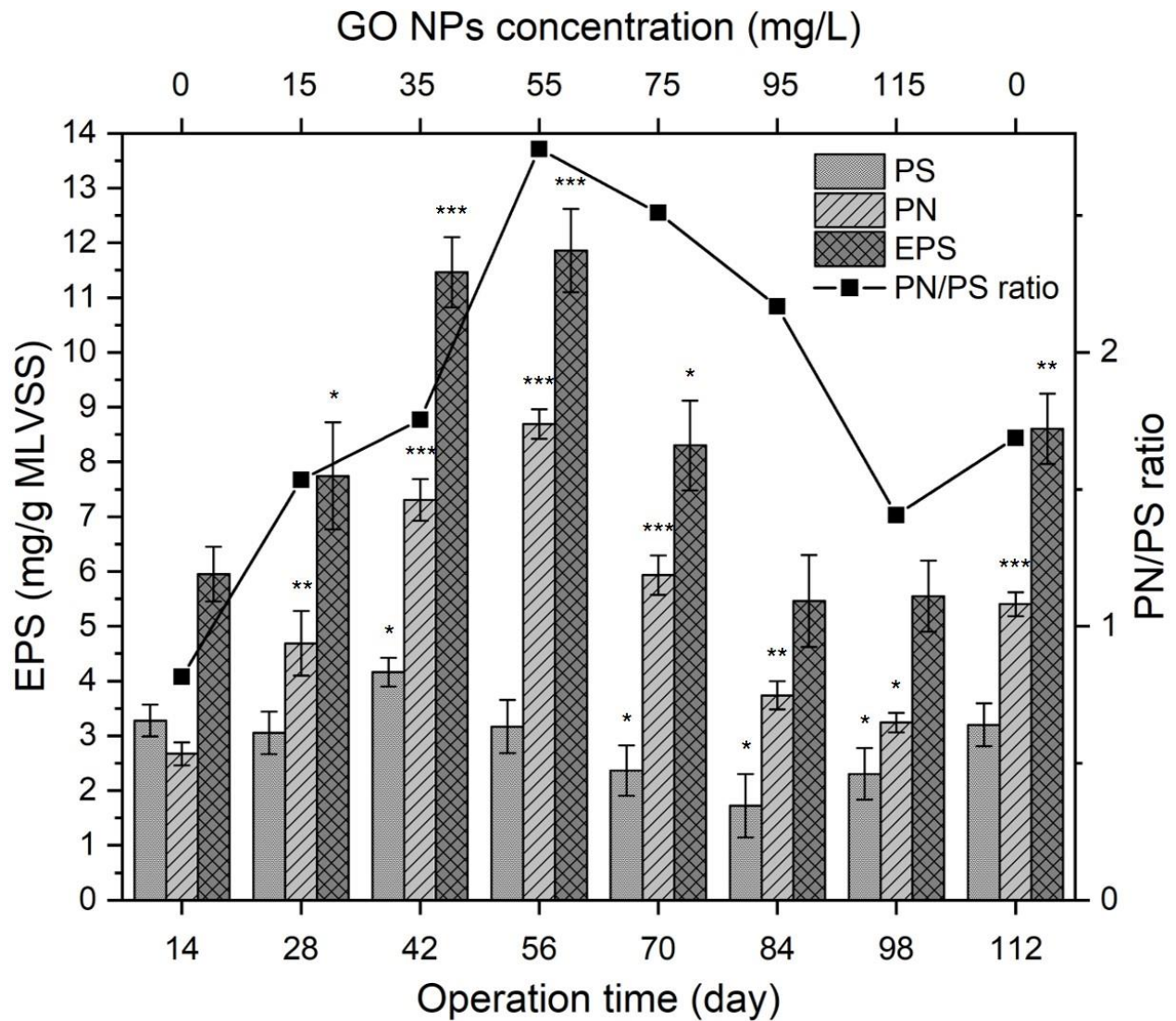


Fig. 4. The impacts of GO NPs on content of polysaccharide (PS), protein (PN), and extracellular polymeric substance (EPS) in the initial phase on day 14 (0 mg/L GO NPs), on days 28, 42, 56, 70, 84, and 95 (15, 35, 55, 75, 95, and 115 mg/L GO NPs), and finally, after the termination of adding GO NPs on day 112. The stars show statistical differences (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$) compared to the control (on day 14 and without GO NPs) (one-way ANOVA).

4. Conclusions

The effect of GO NPs on nutrient removal, microbial activity, and EPS production of AGS in the SBR was evaluated by increasing the NP concentrations stepwise. During the experiment, the concentrations of nitrite- and nitrate-nitrogen remained stable, which is consistent with the results of SNRR and SNIRR of AGS. The effluent NH₄-N and COD contents increased considerably, and the SOUR and SAOR of the sludge decreased when the GO concentration was above 55 mg/L. This may suggest that the particles influenced only the aerobic organic matter-degrading and ammonia-oxidizing microorganisms, which are mainly on the outer surface of the granulated sludge. The removal rate of TP decreased from 95% to 57% and a strong significant decrease in SPUR was observed ($p < 0.001$) with the addition of GO NPs at 115 mg/L concentration. This may also indicate that the GO NPs had a more significant inhibitory effect on aerobic processes. The amount of EPS increased from 5.95 to 11.86 mg/g MLVSS when the GO concentration in the influent was increased to 55 mg/L; however, the EPS content decreased drastically by further increasing the GO concentration. Finally, our study demonstrated that the AGS SBR has an excellent recovery ability as the bioreactor performance improved significantly after 14 days when the influent SWW did not contain GO particles. To our best knowledge, this is the first study wherein the long-term exposure of GO NPs was investigated by increasing the concentrations of particle stepwise. Hence, the present results may provide appropriate information about the AGS biological wastewater treatment process. However, further molecular biology studies (for example next generation sequencing) are crucial to better understand the long-term effect of GO NPs on AGS.

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