Influence of wastewater type in the effects caused by titanium dioxide nanoparticles in the removal of macronutrients by activated sludge

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Abstract
The imminent arrival of nanoparticles (NPs) to the wastewater treatment plants (WWTP) brings concern about their effects, which can be related to the wastewater composition. In this work, the effects of titanium dioxide (TiO₂) NPs in the removal of carbon, nitrogen, and phosphorus by activated sludge bioreactors during the treatment of synthetic, raw, and filtered wastewaters were evaluated. Floc size, compaction of sludge, and morphological interactions between sludge and NPs were also determined. The main effect of TiO₂ NPs was the inhibition of up to 22% in the removal of ammonia nitrogen for all types of wastewaters. This effect is strong dependent on combined factors of TiO₂ NPs concentration and content of organic matter and ammonia in wastewater. The removal of dissolved organic carbon was affected by TiO₂ NPs in lower level (up to 6%) than nitrogen removal for all types of wastewaters. Conversely to adverse effects, the removals of orthophosphate in the presence of TiO₂ NPs were improved by 34%, 16%, and 55% for synthetic, raw, and filtered wastewater, respectively. Compaction of the sludge was also enhanced as the concentrations of NPs increased without alterations in the floc size for all types of wastewaters. Based on TEM and STEM imaging, the main interaction between TiO₂ NPs and the activated sludge flocs was the adsorption of NPs on cell membrane. This means that NPs can be attached to cell membrane during aerobic wastewater treatment, and potentially disrupt this membrane. The effects of TiO₂ NPs on macronutrient removal clearly depended on wastewater characteristics; hence, the use of realistic media is highly encouraged for ecotoxicological experiments involving NPs.

Keywords TiO₂ · Nanoparticles · Elemental mapping · Nanotoxicology · Nitrification

Introduction
TiO₂ nanoparticles (TiO₂ NPs) are present in multiple everyday products, such as food, toothpaste, and sunscreens (Weir et al. 2012; Adam et al. 2018; Polesel et al. 2018). According to the life cycle assessment studies (Keller and Lazareva 2013; Adam et al. 2018), unintentional release of TiO₂ NPs to the environmental matrices, such as air, soil, or water, during or after the use of such products is expected. Therefore, some concerns have raised due to the potential effects of TiO₂ NPs in such matrices. Water streams have been identified as the main transport media of NPs in the environment owing to the connection between treated or non-treated effluents and water bodies (Brar et al. 2010; Kunhikrishnan et al. 2015). Some studies have reported the presence of TiO₂ NPs in wastewater streams (Kiser et al. 2009; Tuoriniemi et al. 2012), and in the sludge of the bioreactors applied for wastewater treatment (Polesel et al. 2018; Huang et al. 2020; Cervantes-Avilés and Keller 2021). This confirms that NPs present in
wastewater are interacting with microorganisms of bioreactors, which can induce some effects on the performance of wastewater treatment processes.

Some effects of TiO₂ NPs during aerobic wastewater treatment are already reported, including toxicity to some microorganisms of the aerobic processes (Li et al. 2017) and inhibition of oxygen consumption (Supha et al. 2015; Zhou et al. 2015). This deficiency in oxygen consumption by aerobic microorganisms could negatively affect removal of carbon, nitrogen, and phosphorus in the aerobic bioreactors. Gartiser et al. (2014) exposed activated sludge to a synthetic wastewater (SWW) containing TiO₂ NPs during a period of 32 days, and found that the cumulative effect of up to 840 mg/L of TiO₂ NPs negatively affected the removal of organic matter.

Studies about the effects of TiO₂ NPs on the removal of ammoniacal nitrogen and phosphorus in activated sludge systems are limited. In the few studies, no impact of TiO₂ NPs on nitrogen and phosphorus removal was reported in activated sludge process exposed to 1, 10, and 50 mg/L of NPs in SWW during 24 days (Supha et al. 2015). In contrast, for higher concentration of TiO₂ NPs and also using SWW, an inhibitory effect on nitrogen removal has been observed, which was attributed to the inhibition of microorganism related to nitrification-denitrification processes (Li et al. 2020). In the experiment of García et al. (2012), a synthetic media, such as ammonium chloride, was also used to evaluate the nitrification process, which was inhibited 5% after 4 h in a system containing suspended biomass. Higher inhibition of the nitrification process was observed in long-term experiments performed by Zheng et al. (2011). In these experiments, 50 mg/L of TiO₂ NPs in SWW were added to activated sludge bioreactors, decreasing the ammoniacal nitrogen removal from 80.3 to 24.4% after 70 days. The loss of nitrification function of activated sludge exposed to a synthetic media was linked to a decrease in nitrifying bacteria (Ma et al. 2015). Moreover, adverse effects of TiO₂ NPs over organic matter and nitrogen removal were mainly related to the stability of the NPs (Zhou et al. 2015), which can change according to wastewater characteristics (Keller et al. 2010; Gartiser et al. 2014; Cervantes-Avilés et al. 2017). Considering that most of the experiments testing effects of TiO₂ NPs on activated sludge were performed with SWW, such effects should be evaluated using real wastewater to determine similarities and differences between wastewater types.

Previous findings suggest that the effects of TiO₂ NPs on organic matter and nitrogen removal depends on several factors, such as the accumulated concentration of TiO₂ NPs in bioreactor and the exposure time (Zheng et al. 2011; García et al. 2012; Li et al. 2020). Moreover, given the influence that physicochemical characteristics of wastewater has on the stability of NPs (Keller et al. 2010; Gartiser et al. 2014), type of wastewater can be also considered as important factor when testing the performance of activated sludge exposed to NPs. Currently, there are not studies that evaluate the influence of the type of wastewater in the effects that NPs can cause over activated sludge. Therefore, the aim of this work was to evaluate the effect of TiO₂ NPs in the removal of macronutrients, such as carbon, nitrogen, and phosphorus, by activated sludge, when the NPs are in three types of domestic wastewaters: synthetic, raw, and filtered. Floc size, compaction of the activated sludge, and the morphological interaction between TiO₂ NPs and microorganisms were also evaluated for all types of wastewaters.

### Materials and methods

#### Materials

Milli-Q water with a resistivity of 18.2 MΩ cm (Merck Millipore) was used in all suspensions of NPs and solutions. TiO₂ NPs were obtained as powder from ID-Nano (Mexico). Characterization of TiO₂ NPs is reported in “Characterization of the TiO₂ NPs” section. SWW was prepared according to previous study (Cervantes-Avilés et al. 2017). Briefly, two main solutions, macronutrient and micronutrient solutions were mixed. Macronutrient solution contained: 1902.20 mg/L C₆H₁₂O₆ (Sigma-Aldrich), 344.25 mg/L NH₄Cl (Fisher Scientific), 72.42 mg/L K₂HPO₄ (Fisher Scientific), 44.21 mg/L MgSO₄·7H₂O (Fisher Scientific), 18.56 mg/L CaCl₂·2H₂O (Fisher Scientific), and 71.40 mg/L NaCl (Sigma-Aldrich). An aliquot of 50 mL of the micronutrient solution was diluted in the macronutrient solution to reach the following concentrations in the SWW: 0.18 mg/L MgCl₂·4H₂O (Fisher Scientific), 0.29 mg/L H₃BO₃ (Fisher Scientific), 0.10 mg/L ZnCl₂ (Fisher Scientific), 0.27 mg/L FeCl₃ (Fisher Scientific), and 0.28 mg/L C₁₀H₁₆N₂O₈ (Sigma-Aldrich). NaOH 1 M (Sigma-Aldrich) and HNO₃ 1 M (Fisher Scientific) were used to adjust to pH 7 in SWW.

Evaluation of macronutrients removal was performed in glass reactors of 2 L, which were covered from light to avoid photolysis induced by TiO₂ NPs. Each reactor was equipped with air diffusers and an air pump that provided air flux at 4 m³·h⁻¹·m⁻³ reactor. Commercial tests (Hach-Lange) were used to measure the concentration of chemical oxygen demand (COD), soluble COD (sCOD), ammoniacal nitrogen (NH₃-N), nitrate-nitrogen (NO₃-N), nitrite-nitrogen (NO₂-N), and orthophosphate (PO₄³⁻). The dissolved total organic carbon (dTOC) was measured in a TOC-L (Shimadzu). Nylon filters (Whatman) with a pore size of 0.45 μm were used to remove the suspended solids before analysis. Glass fiber filters (Whatman) with a pore size of 0.7 μm were used to determine the concentration of total suspended solids (TSS) and volatile suspended solids (VSS) according to the standard methods (APHA 2005).
The sludge samples were prepared for transmission electron microscopy (TEM) imaging by using ethanol absolute (Sigma-Aldrich), glutaraldehyde 10% (Electron Microscopy Sciences, EMS), sodium cacodylate at pH of 7.4 (EMS), osmium tetroxide 10% (EMS), propylene oxide (EMS), and epoxy embedding medium kit EPON812. Copper grids of 200 mesh (EMS) were used for TEM observation.

**Wastewater characterization**

Three types of wastewaters, namely SWW, raw wastewater (RWW), and filtered wastewater (FWW), were tested separately in the reactors. SWW was prepared based on the constituents and procedure reported in materials section. RWW was collected in the septic tank of a house without access to sanitation services in Guanajuato, Mexico. FWW consisted of RWW without TSS; hence, the collected RWW was passed through 0.45-μm nylon filters. The three types of wastewaters were characterized to determine COD, sCOD, BOD, NH₃-N, NO₃-N, NO₂-N, PO₄³⁻, TSS, and VSS (Table 1).

**Characterization of the TiO₂ NPs**

Stock suspension of NPs was prepared with cleaned TiO₂ NPs for characterization. TiO₂ NPs were cleaned to remove impurities by washing three times with absolute ethanol and then dried at 90 °C overnight. Stock suspension of TiO₂ NPs (2 g/L) was prepared in Milli-Q water and then ultrasonicated during 1 h at 40 kHz and 200 W, as recommended to promote dispersibility for ecotoxicological assessment (Taurozzi et al. 2012). The primary size, shape, purity, phase, and UV spectrum were determined in a previous study (Cervantes-Avilés et al. 2018). Briefly, TiO₂ NPs were spherical with mean diameter ranging from 3 to 10 nm and were found in the anatase phase. Energy-dispersive X-ray spectroscopy (EDS) confirmed the purity, detecting Ti and O in the NPs. Finally, the localized surface plasmon resonance was detected at 295 nm. Before spiking the experiments, TiO₂ NPs stock suspension was ultrasonicated again during 1 h at same frequency and intensity.

**Macronutrient removal tests in activated sludge**

The reactors contained three main components: 0.842 L of inoculum of activated sludge, 0.842 L of wastewater, and 0.316 L of TiO₂ NPs suspension or Milli-Q water for controls. Inoculum of activated sludge had a concentration of 3120 ± 210 mg/L of SSV and was collected from a pilot plant fed with SWW and operated in the laboratory during 60 days before the experiment. The inoculum was exposed to final concentrations of 500, 1000, 1500, and 2000 mg/L of TiO₂ NPs. All concentrations of TiO₂ NPs and controls were performed at the same time with three replicates for each type of wastewater. The physicochemical parameters such as sCOD, dTOC, NH₃-N, NO₃-N, NO₂-N, PO₄³⁻, and the sludge volumetric index (SVI) were determined per triplicate at the end of the tests in all systems. The time of exposure of activated sludge to NPs was 8 h, which is the typical hydraulic retention (HRT) time of the conventional secondary treatment at WWTPs. The results of the physicochemical parameters were processed by ANOVA one factor. Dunnett’s test was applied to determine the significant difference between groups. Moreover, statistical analysis was performed for each type of wastewater. Results with p value < 0.05 were considered statistically significant. Variation partitioning analysis (VPA) was performed for data of ammoniacal nitrogen removal and explanatory data.
of TiO₂ NPs concentration and physicochemical characteristics of wastewater. Procedure was based according public script in R language (Borcard et al. 2011).

**Interactions between TiO₂ NPs and activated sludge**

Morphological interactions between flocs of activated sludge and NPs were studied at the end of the macronutrient removal tests by measuring size of the flocs, and by imaging of flocs exposed to TiO₂ NPs through TEM and high-angle annular dark field scanning-transmission electron microscopy (HAADF-STEM). Size of the flocs was determined by static light scattering (SLS, Microtrac S3500), applying a value of 1.81 as the refractive index of the flocs. For TEM imaging, samples of activated sludge (1.5 mL) were collected immediately after macronutrient removal tests and centrifuged at 9000g. Supernatant was replaced by 2.5% glutaraldehyde, and pellets were resuspended and kept at room temperature for 2 h. Then, samples were centrifuged to replace glutaraldehyde with 1% osmium in sodium cacodylate 0.1 M, keeping the samples at room temperature for 1 h. After this period, the samples were dehydrated gradually with ethanol absolute, starting at 10% (v/v) up to ethanol 100%. Finally, samples were embedded in an epoxy resin (EPON812) to form blocks of resin containing the samples. The resin blocks were cut in thin sections, between 60 and 90 nm, by using an ultramicrotome (MTX-RMC). TEM imaging was conducted at 80 kV in a JEOL-1010, while HAADF-STEM imaging was performed at 300 kV in a microscope FEI-Titan 80-300.

**Results and discussion**

**Removal of organic matter in presence of TiO₂ NPs**

The removal of organic matter in the three types of wastewaters was evaluated by relating the initial and final concentrations (C/C₀) of sCOD and dTOC (Figure 1), which serves as indicative of such removal in the presence of TiO₂ NPs. According to these results, the control groups presented removal of sCOD higher than 70% for the three types of wastewater (Figure 1A), which corresponds to the typical removal for activated sludge systems (van Loosdrecht et al. 2016). However, impact of TiO₂ NPs on sCOD removal was different for all types of wastewaters. In the presence of SWW, TiO₂ NPs concentrations did not affect sCOD removal, which was statistically similar for all concentrations to control group. Conversely, in the case of RWW and FWW, removal of sCOD decreased linearly as the concentration of TiO₂ NPs increased, decreasing up to 10% and 14% lower removal for RWW and FWW, respectively. Since experimental conditions were similar for all three wastewaters, the negative effect of TiO₂ NPs over sCOD removal can be attributed to the type of wastewater and concentration of NPs. In this way, real wastewater without suspended solids (FWW) showed the higher potential effect on sCOD removal, while a synthetic medium such as SWW did not present any effect at the experimental concentrations of NPs. The non-influence of the TiO₂ NPs suspended in synthetic substrates on the removal of organic matter during activated sludge experiments has been already reported (García et al. 2012; Qiu et al. 2016). In contrast, TiO₂ NPs in real wastewater have decreased the oxygen uptake during aerobic experiments (Zhou et al. 2015), limiting the oxidation of organic matter and leading to a decrease in the removal of sCOD. Although the removal of sCOD from RWW, which is more realistic matrix, was affected only 10% in the presence of high load of TiO₂ NPs, this removal is sensible to the wastewater characteristics, including the suspended solids content. In this experiment, a difference on the chemical nature of the contained substrates in wastewaters (e.g., glucose for SWW, and proteins, fat, carbohydrates, among other compounds for real wastewaters), can play an important role in the effect of TiO₂ NPs over activated sludge culture.

The removal of dTOC in the control reactors was at least 70% for the three types of wastewaters (Figure 1B). However, organic carbon removal was most affected when more than 1000 mg/L of TiO₂ NPs was contained in RWW, decreasing the removal of dTOC up to 6%. In contrast, removal of organic carbon in experiments using SWW and FWW was not affected at all. This means that the presence of high concentrations of TiO₂ NPs in activated sludge process can have a slight effect on the removal of organic matter, which is also a fraction of the measured sCOD. A minimum adverse effect on organic matter removal was also found by a similar study performed by Gartiser et al. (2014), who fed synthetic wastewater based on peptone and meat extract, and exposed an activated sludge reactor to 840 mg/L of TiO₂ NPs, inducing a deficiency of 7% in the organic matter removal.

Removal of sCOD was more affected than the dTOC removal by the presence of TiO₂ NPs. In the experiments with real wastewaters (RWW and FWW), the most adverse effects were observed on sCOD removal. This means that the oxidizable (bio or chemically) inorganic compounds were not removed by the aerobic microorganisms present in the activated sludge due to the presence of TiO₂ NPs. The poor performance of aerobic microorganisms can be confirmed by previous experiments treating real wastewater in the presence of TiO₂ NPs, which inhibited the oxygen consumption by activated sludge in higher levels than SWW (Cervantes-Avilés et al. 2017). Therefore, since dTOC was not affected considerably in this experiment, the TiO₂ NPs present in real wastewaters could affect the microorganisms responsible for the oxidation of the inorganic compounds in wastewaters, commonly measured as sCOD. Although in this study there was no bacterial diversity analysis, there are a couple of studies...
that reported decrease in viable aerobic bacteria. Ma et al. reported a decrease of aerobic activity in activated sludge due to shifts in microbial community composition due to the presence of TiO$_2$ NPs (Ma et al. 2015). Another study reported a decrease in bacterial diversity of activated sludge exposed to 1 mg/L of TiO$_2$ NPs for 40 days, which was affected from the second day of exposure to NPs (Qiu et al. 2016). The effect of TiO$_2$ NPs in the activated sludge is a decrease in sCOD removal when real wastewaters are used, which could be attributed to the damage of aerobic microorganisms capable of oxidizing inorganic compounds.

Besides the type of wastewater, sCOD removal alterations also depended on the concentration of NPs in the performed experiments. The wastewater characteristics and the concentration of NPs are related to the particle stability in aqueous media (Keller et al. 2010; Zhou et al. 2015). This may result in stable dispersion or formation of aggregates. Some studies have reported that TiO$_2$ NPs may form heteroaggregates (e.g., suspended solids of the wastewater and organic colloids linked to NPs) (Gartiser et al. 2014), which could be deposited in activated sludge flocs and induce a toxic effect on bacteria, such as physical or chemical damage in the cell membrane. The formation of heteroaggregates containing TiO$_2$ NPs, organic colloids, and suspended solids has also been related to the carbon/nitrogen (C/N) ratio in wastewater. The most stable and small heteroaggregates have been found at low C/N ratio is close to 1 (Hotze et al. 2010; Keller et al. 2010). In this work, the C/N ratio for RWW and FWW was 1.27 and 1.22, respectively, while the C/N ratio for the SWW was 8.18. Thus, formation of small and stable heteroaggregates in real wastewaters could potentially explain a damage in aerobic microorganisms. Besides, the presence of TSS may alter the effects of NPs on sCOD and dTOC removal. This means that the type of organic substrate and suspended solids content in wastewater, combined with NPs concentration can play an important role when evaluating toxicity by NPs.

**Ammoniacal nitrogen removal in the presence of TiO$_2$ NPs**

In the control experiments using the three types of wastewaters, the ammoniacal nitrogen removals were 64% for RWW and FWW, and 76% for SWW (Figure 2). These removal percentages are commonly observed in activated sludge reactors treating domestic wastewater. However, when the activated sludge was exposed to TiO$_2$ NPs, the ammoniacal nitrogen removal was negatively affected for the three types of wastewaters, with the only exception of FWW containing 500 mg/L that presented statistically similar removal than control group (Figure 2).

Removal of ammoniacal nitrogen in SWW containing TiO$_2$ NPs was less affected than that for the other types of
wastewaters, decreasing the removals between 5 and 10% for all TiO$_2$ NPs concentrations (Figure 2). Experiments with RWW and FWW indicated that inhibition of ammoniacal nitrogen removal increased as concentration of TiO$_2$ NPs in activated sludge increased. In experiments with RWW, ammoniacal nitrogen removal decreased up to 22% in the presence of 2000 mg/L of TiO$_2$ NPs, while for FWW, the removal decreased up to 12% for the same concentrations of NPs. These results clearly indicate that the effect of TiO$_2$ NPs on ammoniacal nitrogen removal was more severe when real wastewater with suspended solids is used.

Ammoniacal nitrogen removal in activated sludge is mainly via nitrification-denitrification. To assess nitrification (ammoniacal oxidation processes), NO$_2$-N and NO$_3$-N were determined at the end of the macronutrient removal tests (Figures 3A, B, respectively). In the case of SWW, NO$_2$-N formation in presence of TiO$_2$ NPs was similar to the control. However, the NO$_2$-N formation decreased as the concentration of TiO$_2$ NPs increased. Hence, for SWW containing TiO$_2$ NPs, the main effect occurred in nitrate formation, which is the second step of the nitrification process.

Formation of NO$_2$-N and NO$_3$-N from RWW containing TiO$_2$ NPs was higher or similar to control group, e.g., NO$_3$-N production increased 7% in the presence of 2000 mg/L of TiO$_2$ NPs compared to control group. Conversely, in experiments treating FWW with TiO$_2$ NPs, the production of NO$_2$-N and NO$_3$-N by activated sludge was affected for the NPs. This means that TiO$_2$ NPs in wastewater without TSS may damage the aerobic oxidizing bacteria (AOB) of the activated sludge. Li et al. (2020) performed a quantitative polymerase chain reaction (qPCR) test followed by Illumina high-throughput sequencing of the activated sludge exposed to a high cumulative concentration of TiO$_2$ NPs (450–900 mg/L) and found that AOB can be mainly affected by acute exposition to these NPs.

In general, ammoniacal nitrogen removal was affected in the presence of TiO$_2$ NPs regardless of the type of wastewater. However, first and second steps of nitrification in the presence of TiO$_2$ NPs were better performed using RWW than FWW. Considering that the only difference between FWW and RWW is the TSS content, the presence of suspended solids may contribute to the formation of heteroaggregates less toxic for nitrifying bacteria (Nitrobacter, Nitrosospira, and Nitrosomonas). In contrast, for the experiments using the SWW and FWW, deficiencies in the production of nitrite and nitrate were found under the presence of TiO$_2$ NPs, which could be attributed to damage in the nitrifying bacteria. The limited production of nitrite and nitrate in experiments treating SWW and FWW suggests that the overall removal of ammoniacal nitrogen in the presence of NPs could occur due to the adsorption of nitrogen onto TiO$_2$ NPs.

To explore influencing factors in ammoniacal nitrogen removal, VPA was performed as function of TiO$_2$ NPs concentration and physicochemical parameters of all three types of wastewaters (Figure 4). During this analysis, collinearity > 10 was found for almost all parameters of wastewaters, except for BOD$_5$ and NH$_3$-N, which were then used for VPA. Combined effect of NPs concentration and content of both BOD$_5$ and NH$_3$-N in each type of wastewater were dominant factors (0.61) in ammoniacal nitrogen removal, followed by residuals influence (e.g., experimental conditions or others non-monitored parameters) and concentration effect. This analysis confirms that effect of different concentrations of TiO$_2$ NP on nitrogen removal is strong dependent on organic matter and ammonia content.

**Orthophosphate removal in presence of TiO$_2$ NPs**

The orthophosphate removal in the activated sludge process containing TiO$_2$ NPs was enhanced for all three types of wastewaters (Figure 5). In experiments with SWW, the highest PO$_4^{3-}$ removal was observed in the presence of 1000 mg/L of TiO$_2$ NPs, with 34% higher removal than the control group. However, the most noticeable improvement
was observed for experiments with real wastewater, which increased the removal of \( \text{PO}_4^{3-} \) as the concentration of TiO\(_2\) NPs also increased. The percentages of enhancement when compared to their respective control groups were 16% and 55% for RWW and FWW, respectively. This suggests a direct action of NPs over \( \text{PO}_4^{3-} \), such as adsorption or precipitation.

Phosphorus removal during activated sludge process is developed by polyphosphate-accumulating organisms (PAO), which capture the chains of polyphosphates under aerobic conditions. Since TiO\(_2\) NPs may affect the bacterial performance, the possible explanation of phosphate removal could be adsorption and co-precipitation of \( \text{PO}_4^{3-} \) adsorbed by TiO\(_2\) NPs. The ability of TiO\(_2\) NPs for adsorbing compounds has been already reported (Cervantes-Avilés et al. 2017), including those measured as sCOD such as the polyphosphate chains present in activated sludge reactors. The adsorption of phosphate in NPs surface is related to the pH of the aqueous medium and pH between 7 and 8; the typical of activated sludge is the most favored (Rathnayake et al. 2014). Moreover, the type of substrates in wastewater containing phosphate could also be an influencing factor (Neale et al. 2015; Zhang et al. 2019); hence, further evaluation of NPs for phosphorus removal or recovery should be addressed. Since the pH of the experiments in the present study was between 7 and 7.8, and the sources of \( \text{PO}_4^{3-} \) in real
wastewater are commonly detergents, the TiO₂ NPs could be an adsorbent agent during wastewater treatment.

The removal of orthophosphate via TiO₂ NPs could be considered as a positive effect that is due to potential adsorption. However, the affinity between TiO₂ NPs and orthophosphate should be proven through measuring orthophosphate adsorption capacity by TiO₂ NPs, and other parameters that complement the characterization of NPs, such as zeta potential and specific surface area. Considering that TiO₂ NPs are widely investigated for water treatment due to their photocatalytic activity, and their potential contribution to the extracellular electron transfer (Wang et al. 2016), the ability of TiO₂ NPs to remove orthophosphate from wastewater could promote their use during biological wastewater treatment.

**Effect of TiO₂ NPs in the floc size and SVI of activated sludge**

The distribution of floc size of activated sludge in the three wastewater types is observed in Figure 6. For all different types of wastewaters, the flocs size of activated sludge was not affected by the presence of TiO₂ NPs in the reactors. However, the average values of SVI in the presence of TiO₂ NPs were 50% lower than control SVI values for all types of wastewaters (Figure 7), improving the compaction of activated sludge.

The activated sludge compaction during settling was dependent on the NPs content. This was observed in the SVI values, which were inversely proportional to the concentration of TiO₂ NPs regardless of the type of wastewater used (Figure 7). These findings suggest that the flocs exposed to TiO₂ NPs kept the same size but had higher

![Fig. 7 Sludge volume index (SVI) of activated sludge exposed to TiO₂ NPs during the treatment of synthetic (SWW), raw wastewater (RWW), and filtered wastewater (FWW)](image-url)

**Fig. 7** Sludge volume index (SVI) of activated sludge exposed to TiO₂ NPs during the treatment of synthetic (SWW), raw wastewater (RWW), and filtered wastewater (FWW)

![Fig. 8 Transmission electron microscopy (TEM) imaging of microorganisms present in aerobic wastewater treatment exposed to TiO₂ NPs. A Control treating RWW. B SWW + 1000 mg/L of TiO₂ NPs. C RWW + 1000 mg/L of TiO₂ NPs. D FWW + 2000 mg/L of TiO₂ NPs](image-url)

**Fig. 8** Transmission electron microscopy (TEM) imaging of microorganisms present in aerobic wastewater treatment exposed to TiO₂ NPs. A Control treating RWW. B SWW + 1000 mg/L of TiO₂ NPs. C RWW + 1000 mg/L of TiO₂ NPs. D FWW + 2000 mg/L of TiO₂ NPs
Morphological interactions between TiO$_2$ NPs and activated sludge

The way TiO$_2$ NPs interacted with microorganisms was elucidated by electron microscopy. TEM imaging was performed for embedded samples of activated sludge exposed to TiO$_2$ NPs, treating all three types of wastewaters. During TEM observation, electrodense and particulate material (black color) was observed attached to the membrane of microorganisms (Figure 8), presumably TiO$_2$ NPs. This electrodense material presented aggregates smaller than 500 nm, and in some of the samples were surrounded by organic material (gray color). These aggregates are consistent with the description of heteroaggregates proposed by Sani-Kast et al. (2015) and Dale et al. (2015). The integrity of the cell membrane of microorganisms in the control and SWW imaging is similar (Figure 7A, B), without internalized electrodense material. A similar pattern was observed for the sample of the experiment treating FWW (Figure 7D). However, in the case of the experiment treating RWW (Figure 7C), electrodense material as crystals and abundant cellular detritus was observed. This indicates that, in addition to the TiO$_2$ NPs supplied in the experiments, the RWW already contains water-insoluble metallic material. Although internalization of some NPs such as ZnO (Sirelkhatim et al. 2015; Cervantes-Avilés et al. 2016) and CuO (Perreault et al. 2012) has previously been considered as a pattern that leads to apoptosis of cells, the internalization of electrodense material in cells was not observed for the samples analyzed. Electrodense material was frequently observed attached to the cell membrane. To confirm that electrodense particulate material correspond to TiO$_2$ NPs, elemental mapping was performed in the sample of activated sludge treating RWW.

Electrodense material was confirmed to correspond to Ti (Figure 9). Overlapping the layers of Ti-K, Ti-L, and O-K, can be concluded that Ti corresponds to TiO$_2$ at the nanoscale. Distribution of Ti in image captured in HAADF-STEM confirmed that this material is attached to the cell membrane (Figure 9). Therefore, one of the mechanisms by which TiO$_2$ NPs could harm microorganisms is through damage to the cell membrane, such as disruption. Some studies about the toxicity of TiO$_2$ NPs in isolated microorganisms reported the chemical activity of these particles on the phospholipid membrane of cells (Ma and Lin 2013; Neale et al. 2015). Since our experiments were performed without exposition to UV light, the main mechanism of damage from TiO$_2$ NPs to microorganisms of activated sludge occurs potentially through physical interactions conducting to the cell membrane rupture. This would lead to lysis of microorganisms present in activated sludge, affecting the processes involved in the biological macronutrient removal from wastewater.

Conclusions

In this work, the effects of the type of wastewater such as synthetic, raw, and filtered, in the removal of macronutrients by the activated sludge process exposed to TiO$_2$ NPs were evaluated. The most representative impact of TiO$_2$ NPs was the inhibition of up to 22% of ammoniacal nitrogen removal for the highest load of TiO$_2$ NPs. Although this inhibition was related to the combined effect of NPs concentration and content of BOD$_5$ and NH$_3$-N, there is evidence of alterations of nitrification process when real wastewater is used. Organic matter removal was not substantially affected; however, oxidation of the inorganic compounds in real wastewaters, commonly measured as sCOD can be affected by the presence of TiO$_2$ NPs. Phosphate removal and compaction of the sludge are positive effects when TiO$_2$ NPs are in activated sludge reactors, which can be explored carefully to avoid overload of NPs in activated sludge reactors.

The use of real wastewater in ecotoxicological experiments of this and other NPs is highly encouraged due to effects of NPs were limited for synthetic substrate. Moreover, the most adverse effects were detected for real matrices of wastewater. Furthermore, although activated sludge still removes macronutrients in the presence of high load of TiO$_2$ NPs, the removal mechanisms could be physicochemical rather than biological.

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**Data availability** The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**

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**Consent for publication** Not applicable.

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