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ORIGINAL PAPER

Changes in bacterial diversity of activated sludge exposed to titanium dioxide nanoparticles

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Abstract The rapid growth of the use of nanomaterials in different modern industrial branches makes the study of the impact of nanoparticles on the human health and environment an urgent matter. For instance, it has been reported that titanium dioxide nanoparticles (TiO_2 NPs) can be found in wastewater treatment plants. Previous studies have found contrasting effects of these nanoparticles over the activated sludge process, including negative effects on the oxygen uptake. The non-utilization of oxygen reflects that aerobic bacteria were inhibited or decayed. The aim of this work was to study how TiO_2 NPs affect the bacterial diversity and metabolic processes on an activated sludge. First, respirometry assays of 8 h were carried out at different concentrations of TiO_2 NPs (0.5–2.0 mg/mL) to measure the oxygen uptake by the activated sludge. The bacterial diversity of these assays was determined by sequencing the amplified V3–V4 region of the 16S rRNA gene using Illumina MiSeq. According to the respirometry assays, the aerobic processes were inhibited in a range from $18.5 \pm 4.8\%$ to $37.5 \pm 2.0\%$ for concentrations of 0.5–2.0 mg/mL TiO_2 NPs. The oxygen uptake rate was affected mainly after 4.5 h for concentrations higher than 1.0 mg/mL of these nanoparticles. Results indicated that, in the presence of TiO_2 NPs, the bacterial community of activated sludge was altered mainly in the genera related to nitrogen removal (nitrogen assimilation, nitrification and denitrification). The metabolic pathways prediction suggested that genes related to biofilm formation were more sensitive than genes directly related to nitrification–

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denitrification and N-assimilation processes. These results indicated that TiO_2 NPs might modify the bacteria diversity in the activated sludge according to their concentration and time of exposition, which in turn impact in the performance of the wastewater treatment processes.

Keywords TiO_2 NPs · Aerobic process · Oxygen uptake rate · Predictive metagenomic profile · Illumina sequencing · Ecotoxicology

Introduction

The rapid growth on nanotechnology applications has increased the concerns about the potential effects of nanomaterials (NMs) on the human health and the environment. The incorporation of NMs in cosmetics, sunscreen, food, paints and cleaning agents contributes to their release into the environment (Gottschalk et al. 2013; Keller and Lazareva 2013; Sun et al. 2014). Titanium dioxide nanoparticles (TiO_2 NPs) are widely incorporated in some of these products due to their versatile optical, physical and chemical properties (Lu et al. 2015). Hence, the arrival of TiO_2 NPs to wastewater treatment facilities is imminent.

The activated sludge process is the most applied technology for wastewater treatment. It is estimated that over 70,000 wastewater treatment plants (WWTP) in the world are applying this technology or one of its modifications, e.g. step feed aeration, extended aeration, oxidation ditch, among others (Seviour and Nielsen 2010). An activated sludge is a flocculent suspension of microorganisms stirred by aeration and/or mixing (Jenkins 2014). The microbial activity of these microorganisms is used for oxidation of soluble and particulate organic matter with oxygen or nitrate/nitrite. The question here is how the TiO_2 NPs impact the microorganisms present in activated sludge and how this affects the performance of activated sludge bioreactors.

Although several studies have demonstrated that metal based NPs induce toxicological effects to prokaryotic (Concha-Guerrero et al. 2014; Ulloa-Ogaz et al. 2017) and eukaryotic (Athie-García et al. 2018) microorganisms, neutral and negative results about the effect of TiO_2 NPs over microorganisms of activated sludge bioreactors have been reported. Gartiser et al. (2014) observed that the organic matter removal and the oxygen uptake were affected by 1 mg/mL of TiO_2 NPs, while Zhou et al. (2015) arrived at the same conclusions using 0.1 mg/mL; conversely, García et al. (2012) and Qiu et al. (2016) found no negative effects in the organic matter removal by using similar concentrations. The discrepancies between these studies are dependent on the wastewater characteristics, such as organic matter content, ionic strength, pH, mono-divalent ionic ratio, source of activated sludge, among others (Zhou et al. 2015; Cervantes-Avilés et al. 2017a, b). These factors, including bioprocess conditions as well as the type and concentration of NPs, also influence the autotrophic and heterotrophic bacteria abundance in the activated sludge. For example, Chen et al. (2013) reported that silver NPs (Ag NPs) affected both the activity and abundance of polyphosphate accumulating microorganisms (PAO, heterotrophic bacteria) in short term experiments of 1–3 days. Similarly, the zinc oxide NPs (ZnO NPs) had no influence over microbial community diversity related to organic matter removal (Zhang et al. 2016), but altered the nitrifying (heterotrophic) bacteria populations after 96 days during long term experiment (Zhang et al. 2017).

In turn, it has been reported that the effects of TiO_2 NPs on microbial community at long term include the decrease in the removal efficiency of major contaminants as nitrogen (Ma et al. 2015). For TiO_2 NPs, previous studies have reported a decrease on the enzymatic activities related to nitrifying bacteria after 70 days (Zheng et al. 2011), as well as a shift on the microbial community structure when bulk-TiO_2 and TiO_2 NPs were present in a sequential batch reactor (Ma et al. 2015). Similar changes were found for soil bacterial communities when TiO_2 NPs were spiked into biosolids (Shah et al. 2014), and for aquatic bacterial communities when TiO_2 NPs were added to inland water from Lake Michigan and Chicago River (Binh et al. 2014). Since wastewater treatment process is also a dynamic system, it is of paramount importance to understand the modification of bacterial communities in response to TiO_2 NPs exposition at conventional activated sludge conditions.

The toxicity of TiO_2 NPs over the microorganisms of activated sludge has been noted by performing denaturing gradient gel electrophoresis (DGGE) profiles (Li et al. 2014; Qiu et al. 2016). Li et al. (2014)

reported that TiO_2 NPs significantly reduced the microbial diversity, which could induce a low total nitrogen removal, assuming some effects on the nitrification and denitrification processes. Qiu et al. (2016) confirmed variations in the microbial diversity over time when activated sludge is exposed to TiO_2 NPs, and they also reported a decrease in protein content, which may indicate the inhibitory effects of TiO_2 NPs. Li et al. (2017) performed a high-performance sequencing and found that the microbial richness and diversity presented variations with the increase of TiO_2 NPs (0–60 mg/L) in the reactors, which indicates that toxicity is related with the NPs concentration. This toxic effect was related to an increased in the production of reactive oxygen species (ROS) and lactate dehydrogenase (LDH) that can destroy the membrane integrity of the microorganisms. A recent study by Li et al. (2020) indicated that toxicity of TiO_2 NPs over ammonia-oxidizing-microorganisms (AOMs) and nitrite-reducing-bacteria (NRB) is also related to the crystalline phases of TiO_2, which is higher for rutile than for anatase. Although changes in the bacterial diversity of activated sludge exposed TiO_2 NPs and some effects in the nitrogen removal have been already reported, it is necessary to know the impacts of these NPs in other common metabolic pathways such as carbon assimilation, the development of biofilm, among others.

Understanding the organization of bacterial communities in response to pollutants is a key question in microbial ecology and microbial ecotoxicology (Cravo-Laureau and Duran 2014; Cravo-Laureau et al. 2017). We hypothesized that bacterial community in activated sludge would be modified with exposition to different concentration of TiO_2 NPs, which in turn would affect the wastewater treatment performance. The aim of this work was to study the effect of TiO_2 NPs on bacterial diversity of wastewater treatment process using a microcosm to simulate the aerobic reactor. After the exposition period at different concentrations of TiO_2 NPs, the bacterial diversity was determined by 16S rRNA gene Illumina MiSeq sequencing. A predictive analysis of the functional structure of the microbial communities was also performed.

Materials and methods

Characterization of TiO_2 NPs

Since TiO_2 NPs were received as powder from I&D nanotechnology (Mexico), a suspension was prepared for characterization. The suspension contained 0.5 mg of TiO_2 NPs per mL and was dispersed as recommended by Taurozzi et al. (2012). Briefly, a metal free tube of 50 mL containing the suspension was place in an ultrasonic bath during 1 h at 200 W and a frequency of 40 kHz. This stock suspension was characterized in order to determine the morphology and primary size by scanning electron microscopy (SEM) imaging (Jeol JSM 7401F). Primary size was determined by analyzing the images collected during SEM observation with the open software *ImageJ*. The elemental composition of the suspension was determined through the energy dispersive spectroscopy (EDS) analysis during SEM imaging. Characterization also included the determination of the phase for TiO_2 NPs by X-ray diffraction (XRD) in a diffractometer X Pert Pro (PANalytical). Obtained spectrum of XRD was compared with the patterns reported in database for anatase, rutile and brookite. Finally, the localized surface plasmon resonance (LSPR) of the TiO_2 NPs was determined via scanning of the ultra-violet and visible regions of the electromagnetic spectrum (UV 1800, Shimadzu).

Exposure of activated sludge to TiO_2 NPs

The exposure of activated sludge to TiO_2 NPs was performed in closed batch reactors (BOD Trak II, HACH). The batch reactors for exposure experiments contained a total volume of 95 mL, which were divided in three components: (1) activated sludge seed (40 mL), (2) synthetic wastewater (40 mL) and (3) suspension of TiO_2 NPs or ultrapure water in the case of the control (15 mL). Activated sludge inoculum of all experiments contained 3.06 ± 0.10 g/L of volatile suspended solids (VSS). Physicochemical characteristics of the inoculum included a temperature of 24 ± 1.3 °C, conductivity of 1814 µS/cm and presented a sludge volumetric index (SVI) of 117 mL/g. This inoculum was collected from a pilot plant located in the lab. This plant was operated 63 days before experiments and fed with the same synthetic wastewater than exposure experiments. The synthetic

wastewater was prepared as in previous studies (Cervantes-Avilés et al. 2016), considering the major constituents of wastewater, such as carbon, nitrogen and phosphorous. Briefly, 1902 mg of $C_6H_{12}O_6$, 344 mg of NH_4Cl, and 72 mg of K_2HPO_4 were dissolved in 0.9 L as major constituents. Then, an aliquot of 0.1 L of trace nutrients solution was added to major constituents solution to reach a final concentration of 71 mg/L of NaCl, 44 mg/L of $MgSO_4{\cdot}7H_2O$, 19 mg/L of $CaCl_2{\cdot}2H_2O$, 0.18 mg/L of $MnCl_2{\cdot}4H_2O$, 0.29 mg/L of H_3BO_3, 0.28 mg/L of $C_{10}H_{16}N_2O_8$, and 0.28 mg/L of $FeCl_3{\cdot}6H_2O$.

The exposure experiments were considering concentrations of 0.5, 1.0, 1.5 and 2.0 mg/mL of TiO_2 NPs in wastewater. At these concentrations of TiO_2 NPs, (spiked or cumulative concentrations in the reactors), have been observed effects over the nitrifying bacteria (Zheng et al. 2011) and in the dissolved organic carbon elimination (Gartiser et al. 2014). Moreover, high concentrations of Ti (µg/L to mg/L), presumable as TiO_2, have been measured in influent wastewater and activated sludge of WWTPs (Polesel et al. 2018; Huang et al. 2020). Before addition of NPs to the reactors, the suspensions were dispersed in an ultrasonic bath during 1 h. The experimental concentrations of NPs and the controls (without addition of NPs) were tested per triplicate. The exposure time in the reactors was 8 h due to this time is the typical hydraulic retention time (HRT) of activated sludge process. The experimental conditions included the control of temperature of the reactors at 20 °C, and the isolation of the reactors from light to avoid photolysis caused by TiO_2 NPs. Data about the oxygen transference between gas phase to liquid phased where recorded every 20 min during the exposure time and used to calculate the oxygen uptake rate (OUR) via analysis of respirometry of static phases. Biochemical oxygen demand (BOD) was calculated as the oxygen uptake accumulated during the exposure time (8 h).

Bacterial diversity

The bacterial diversity was determined in samples of activated sludge exposed to TiO_2 NPs after 8 h. Since all concentrations of TiO_2 NPs were evaluated per triplicate in the batch reactors, a sample of activated sludge (0.2 g) from each replicate was collected and mixed. Then, the total DNA of the mixtures was extracted using the Soil DNA Isolation Kit (Mo Bio™ Laboratories, USA) with slight modifications as previously described (Stauffert et al. 2013). In order to amplify a single fragment that cover the V3–V4 region of the 16S rRNA gene two primers were used. The forward primer was 357F 5′-barcode-CTC CTA CGG GAG GCA GCA G-3′ (Turner et al. 1999) and the reverse primer was CD(R) 5′-barcode-CTT GTG CGG GCC CCC GTC AAT TC-3′ (Rudi et al. 1997). After purifying and pooling the amplicons by the primers, amplicons were sequenced on an Illumina MiSeq platform in the Institute of Interdisciplinary Research on Environment and Materials (IPREM) at the Universitè de Pau et des Pays de l'Adour (UPPA).

Data were processed following the procedure described by Brito et al. (2019). Briefly, raw Illumina fastq files were assembled (R1 + R2), quality-filtered, trimmed in 415 bp, dereplicated, filtered for chimeras and clustered by using USEARCH v11 package (Edgar 2010). A total of 80,256 raw sequences were obtained, resulting in 35,655 unique after filtering (90.8% being singletons). Sequences with less than five events were removed and the resulting sequences were clustered in 586 operational taxonomic units (OTUs) in a 97% identity base (genetic distance < 0.03). The obtained OTUs were then associated to the complete pool of sequences. Taxonomy was checked to the genera level with Ribosomal Database Project (RDP 16S rRNA database, training set v16). Finally, the raw sequences of this project were deposited in the Sequence Read Archive (SRA) database of NCBI under SRA accession code PRJNA644188.

Coverage was calculated by using both Turing's estimator (Eq. 1) (Good 1953) and Chao's estimator (Eq. 2) (Chao and Shen 2010; Chao and Jost, 2012). where n_1 is the number of singletons, n_2 the number of doubletons and n the total number of filtered and trimmed sequences. Both estimators gave similar results for all the samples. Different diversity indices were calculated, such as the Shannon entropy (Eq. 3) (Shannon and Weaver 1949), where p_i is the relative frequency of OTU i, and S is the total number of OTUs; the Shannon effective number of "species" (Shannon diversity index, Eq. 4), $e^{H'}$; the equability (evenness) and the Simpson's diversity index (Eq. 5) (Simpson 1949; Blackwood et al. 2007).

$$C = 1 - \frac{n_1}{n} \quad (1)$$

$$C = 1 - \frac{n_1}{n}\frac{(n-1)n_1}{(n-1)n_1 + 2n_2} \quad (2)$$

$$H' = -\sum_{i=1}^{S} p_i \ln(p_i) \quad (3)$$

$$J' = \frac{H'}{\ln(S)} \quad (4)$$

$$1 - D = 1 - \sum_{i=1}^{S} p_i^2 \quad (5)$$

Predictive analysis of microbial community functional gene structure

The tool Tax4Fun2 was applied to predict the functional structure of the microbial communities, and to obtain some additional information about the main metabolic pathways related to N removal and biofilm formation from the activated sludge exposed to TiO_2 NPs. The procedure was done according to Aßhauer et al. (2015). Briefly, the OTUs were labeled by using NCBI Blast (U.S. National Library of Medicine). Then, OTUs profile was transformed to a metabolic profile based on Kyoto Encyclopedia of Genes and Genomes (KEGG), which included the relative abundance calculation. The classified KEEG ortholog functional genes (KO) were linked to empirical metabolic pathways. Then, the KOs were screened manually for enzymes related to nitrification and denitrification which are process developed by the activated sludge.

Statistical analysis

Exposure experiments of activated sludge to TiO_2 NPs were carried out in triplicate. Data of OUR and BOD were statistically analyzed by applying one-way ANOVA. The statistical differences between TiO_2 NPs concentrations groups and control were analyzed by applying the Tukey's multiple comparison test. P-values < 0.05 were considered statistically significant.

Results

Characterization of TiO_2 NPs

SEM micrographs revealed that TiO_2 NPs were agglomerated even in ultrapure water. NPs exhibited spherical shape with mean ratio between 5 and 8 nm (Fig. 1a). EDS confirmed the presence of Ti and O in NPs (Fig. 1b). The observed peaks at 1.486 and 1.557 correspond to Al-Kα and Al-Kβ from the aluminum mount. The peaks of XRD spectrum can be assigned to anatase phase with tetragonal crystal structure (Fig. 1c). According to the UV–vis scanning, the LSPR was at 297 nm (Fig. 1d), which is the typical peak for TiO_2 NPs (Uboldi et al. 2016).

Oxygen uptake rate (OUR)

The OURs of activated sludge during treatment of wastewater in presence or absence of TiO_2 NPs are shown in Fig. 2a. Although the control showed variations with peaks, the OURs were within the range of those reported for activated sludge (Brdjanovic 2015). In presence of TiO_2 NPs, OURs were lower than control for all tested TiO_2 NPs concentrations (0.5–2.0 mg/mL), indicating a negative impact of TiO_2 NPs on the aerobic microbial activity. For TiO_2 ranging from 0.5 to 1.0 mg/mL, OURs were between 1 and 2 mg O_2/g VSS·h during all experiment. For TiO_2 concentrations higher than 1.0 mg/mL the OUR decreased after 4.5 h. The total decrease on the oxygen consumption by microorganisms was observed in the biological demand of oxygen after 8 h (Fig. 2b), which decreased as the concentration of TiO_2 NPs increased. When oxygen uptake was compared to the control non-spiked with TiO_2 NPs, inhibition of the aerobic processes for wastewater treatment of the activated sludge was statistically lower than control (all in %): 18.5 ± 4.8, 25.8 ± 4.3, 30.8 ± 3.1 and 37.5 ± 2.0, for 0.5, 1.0, 1.5 and 2.0 mg/mL of TiO_2 NPs, respectively. It is important to consider that concentrations of TiO_2 NPs spiked in these experiments can be higher than those currently present in activated sludge reactors. Therefore, the results should be contextualized as acute exposure to high load of TiO_2 NPs.

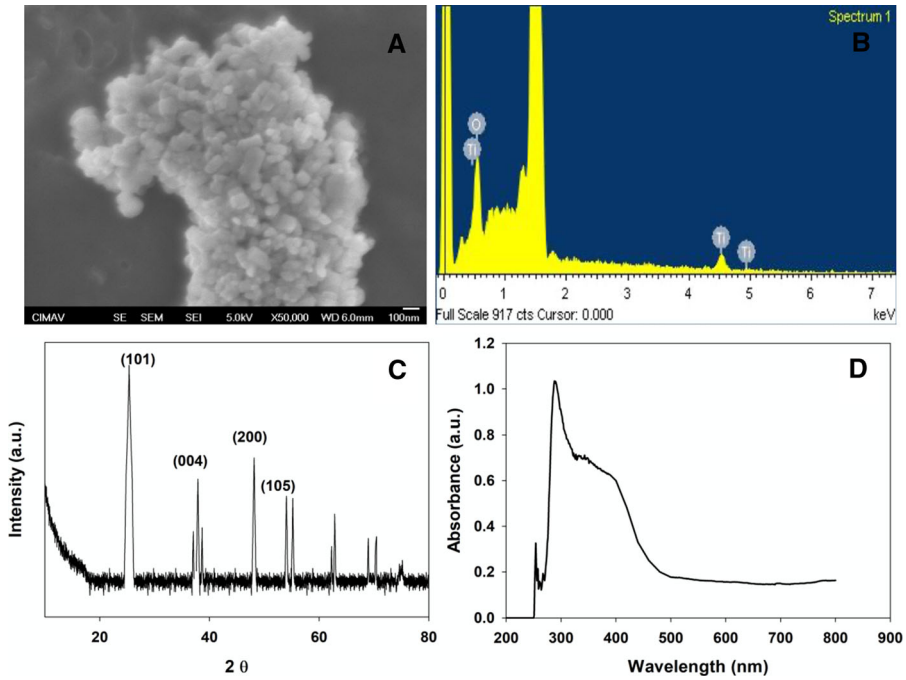

Fig. 1 Characterization of TiO_2 nanoparticles in terms of **a** morphology and size by scanning electron microscopy (SEM), **b** elemental composition by energy dispersive spectroscopy (EDS), **c** X-ray diffraction (XRD) spectrum and **d** localized surface plasmon resonance (LSPR) ultraviolet–visible spectrum. (Color figure online)

Bacterial diversity indices

The indices related to bacterial diversity when exposed to different concentrations of TiO_2 NPs are shown in Table 1. The richness of OTUs varied little among treatments: from control to 1.5 mg/mL of TiO_2 NPs we found 166–168 OTUs, while for 2.0 mg/mL of TiO_2 NPs the number of OTUs was slightly smaller, 162. The variation for the last treatment can also be noted in the abundance of sequences (reads before trimming, Table 1). This variation in the reads associated to OTUs may represent a real impact, although small, of decreasing the bacterial community richness as the concentration of TiO_2 NPs increased. The Shannon-Weiner index (H'), which considers the predominance of certain populations, was similar for all treatments (between 3.6 and 3.8), suggesting that the composition of bacterial communities and the abundance of their members were similar for all tested NP concentrations. Such observation was also supported by Evenness index (J') and a moderate estimation for Simpson's index. As a result, the intrinsic factors of the activated sludge process such as the change of substrate (Griffin and Wells 2017), different COD/N ratios or dissolved oxygen concentrations (Xu et al. 2019), changes in the sludge retention time (Karlikanovaite-Balikci et al. 2019), temperature fluctuations (Johnston et al. 2019), among others, did not affect the shifts in the bacterial diversity of the activated sludge, which was only observed slightly in the test with the highest concentration of TiO_2 NPs.

Bacterial diversity in activated sludge after exposition to TiO_2 NPs

Considering all treatments, 41 OTUs presented relative abundances over 0.5%, which distribution varied according to the treatment (Fig. 3). The control and 0.5 mg/mL TiO_2 NPs treatments formed a group distinct to the other treatments, which was consistent with the Shannon Evenness and Simpson's diversity indices.

Pondering the OTU relative abundances, five main groups were identified (Fig. 3). The *Nakamurella* genus (OTU 1), the predominant population (22.5 ± 4%) in all treatments, formed the first group. The second group includes the other three most

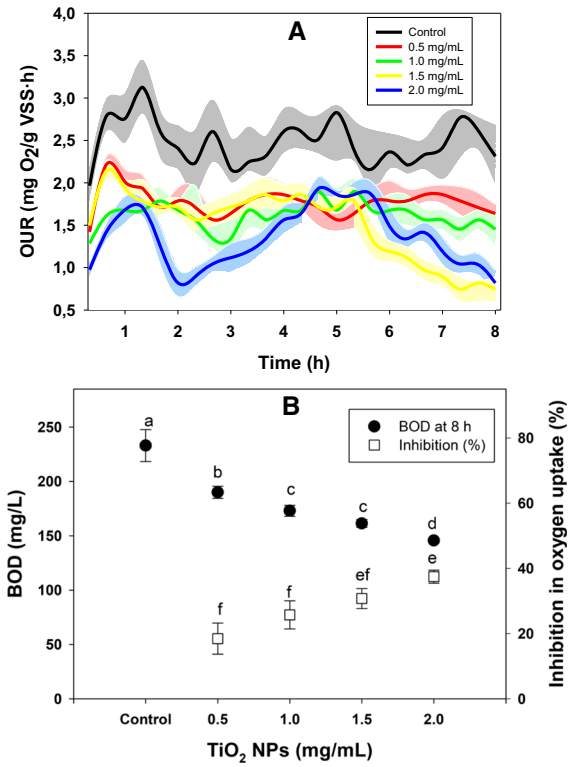

Fig. 2 Oxygen consumed by activated sludge in presence of TiO_2 nanoparticles (NPs): **a** Oxygen uptake rate (OUR) during 8 h, and **b** Biological oxygen demand (BOD) and inhibition in the oxygen consumption by activated sludge when exposed to 0.5, 1.0, 1.5 and 2.0 mg/mL of TiO_2 NPs. Error bars correspond to standard deviation (n = 3). Lowercase letters in (**b**) represent significant differences between mean values at each concentration, as determined by one-way ANOVA and Tukey's multiple comparison test (p-value < 0.05). (Color figure online)

abundant OTUs (about 6–8%), which were affiliated to *Flavobacterium, Thermomonas* and *Zoogloea* genera. Members affiliated to the genera of these two groups are related to C, N and P removal (Whang and Park 2006; Tice et al. 2010; Zhang et al. 2015). The *Nakamurella* genus has been associated with polysaccharide storage as Glycogen-accumulating non-polyphosphate microorganisms (GAOs) (Tice et al. 2010). They are strictly aerobic bacteria competing with PAOs bacteria responsible of phosphorous removal in the aerobic wastewater treatment (Whang and Park 2006). The genus *Thermomonas* includes species able to uptake carbohydrates and carry out nitrification by transporting nitrite/nitrate (Zhang et al. 2015), and they are commonly found in the WWTP. Similarly, members of the *Zoogloea* genus have been described to participate in the denitrification process through aerobic granules assembled during the wastewater treatment (Weissbrodt et al. 2012). Members of the *Flavobacterium* genus possess genes involved in polysaccharide decomposition and polysaccharide utilization (Hahnke et al. 2015). These populations, playing probably a key role in the basic processes in WWTP (Ma et al. 2015), were marginally affected by the probed concentrations of TiO_2 NPs.

The third group contains nine OTUs (5–10, 13, 14 and 16), affiliated to *Amaricoccus*, *Arcobacter*, *Ferruginibacter, Ohtaekwangia*, *Rhodoferax*, *Sphingomonas* and other *Flavobacterium* and *Zoogloea* genera. They have in common the use of many sources of carbon under aerobic or anoxic conditions. For example, *Sphingomonas* and *Amaricoccus*, strict aerobes, are known to assimilate D-glucose, L-arabinose, and D-mannose (Maszenan et al. 1997; Margesin et al. 2012), while *Ohtaekwangia*, facultative anaerobes, assimilate organic acids such as succinic acid (Yoon et al. 2011). The genus *Arcobacter*, similarly to the *Flavobacterium,* is related to nitrate reduction (Zhang et al. 2006; Collado et al. 2011). They are known for

Table 1 Diversity and related indices in samples of activated sludge exposed to TiO_2 NPs

Parameter	TiO_2 NPs concentration (mg/mL)				
	0	0.5	1.0	1.5	2.0
Reads before trimming	13,495	11,617	15,177	11,322	9903
Coverage (%)	39.02	39.36	39.76	37.31	37.39
OTUs	166	166	167	168	162
Reads associated to OTUs	9603	8637	11,215	8027	7128
Shannon–Weiner (H')	3.59	3.57	3.79	3.72	3.74
Shannon diversity index ($e^{H'}$)	36.2	35.4	44.4	41.4	42.3
Shannon evenness (J')	0.70	0.70	0.74	0.73	0.74
Simpson's index (1 − D)	0.91	0.91	0.94	0.93	0.94

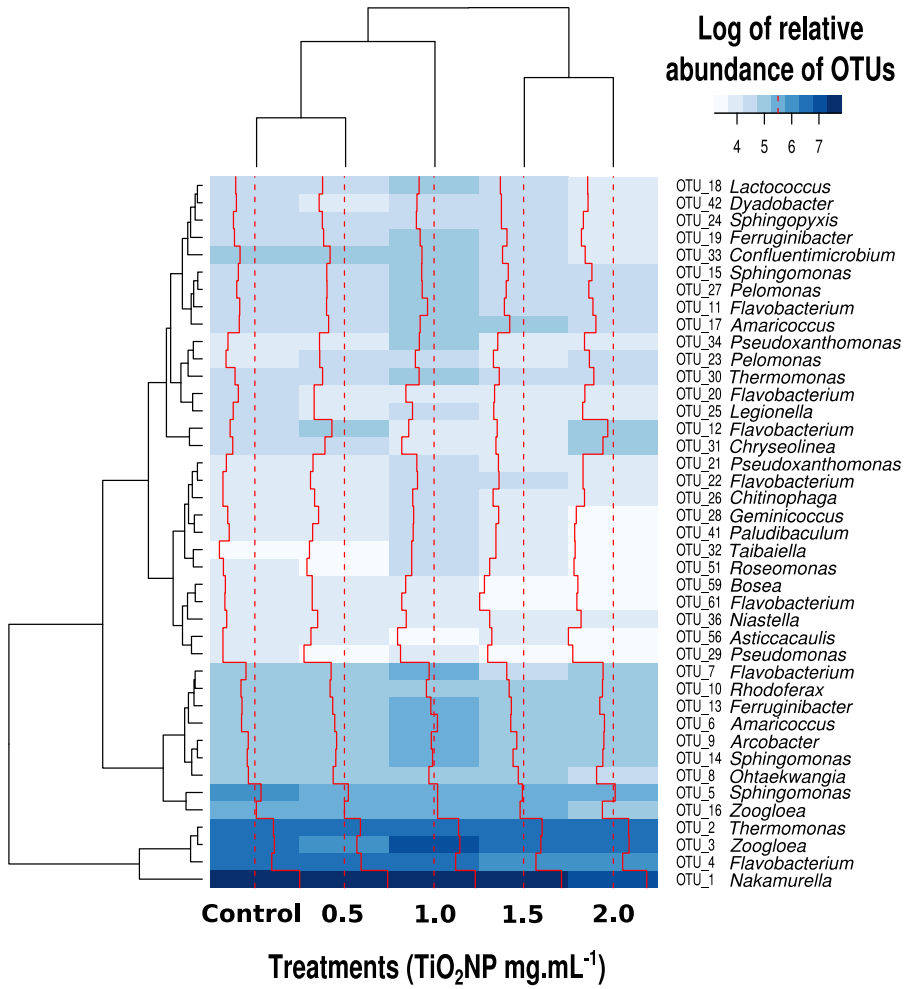

Fig. 3 Distribution of the 41 most abundant OTUs (> 0.5% relative abundance) in the Control and treatments with 0.5, 1.0, 1.5 and 2.0 mg/mL of TiO_2 NPs, hierarchically clustered both according to the OTUs (left tree) and samples (top tree). The intensity in the blue scale is related to the logarithm of the abundance according to the top right palette. The red dotted line represents the diversity for each TiO_2 NPs concentration. The red line histograms along columns represent the abundance of OTU sequences, again in a log scale for each sample. (Color figure online)

their ability to perform the denitrification in wastewater treatment (Wang et al. 2012).

The fourth and fifth group of OTUs had the lowest relative abundance and included bacteria able to hydrolyze and assimilate many organic sources belonging to *Chryseolinea* (Kim et al. 2013), *Legionella* (Wang et al. 2012), *Roseivivax* (Wang et al. 2012), *Niastell* (Zhang et al. 2010), and *Labilithrix* (Yamamoto et al. 2014) genera. Other OTUs of these both groups belong to genera known to degrade complex organic compounds such as cyclic organic compounds, such as OTU affiliated to *Taibaiella* and *Confluentimicrobium* genera (Son et al. 2014), and also to *Chitinophaga* genera that was shown to be able to degrade cellulose (Winogradsky 1929; Zhou et al. 2016). Some OTUs of the fourth and fifth groups are related to genera involved in nitrogen removal processes, including the nitrification such as *Sphingopyxis* genus (Subhash et al. 2014), and the nitrate reduction such as *Geminicoccus* (Foesel et al. 2007), *Rhizobacter* (Stackebrandt et al. 2009), and *Undibacterium* (Eder et al. 2011) genera.

Prediction of metabolic profile of activated sludge exposed to TiO_2 NPs

The predictive analysis of functional structure from 16S rRNA gene sequence data is a promising tool to predict the possible metabolic capacities of microbial communities (Kavamura et al. 2018; Sharma et al. 2018; Cordier 2020). However, it must be emphasized that these results should be used with caution (Sun et al. 2020): they provide an overview about some processes as a basis for future validation works. For example, testing the hypothesis that the TiO_2 NPs are (or are not) interfering on the specific processes of nitrogen transformation in wastewater systems. Although alterations in the nitrogen removal processes have been already reported (Li et al. 2014; Cervantes-Avilés et al. 2017b), it is possible to carry out q-PCR technique targeting the gene encoding for ammonia mono-oxygenase (amoA) or nitrite reductase (nirK and nirS) aiming to determine the abundance of ammonia oxidizers or denitrifying bacteria in order to test the hypothesis (Geets et al. 2007; Li et al. 2020). Recently, Li et al. (2020) used this approach (qPCR) to study the effect of chronic use of TiO_2 NPs over the WWTP and verified a correlation between the increase in the concentration of these nanoparticles and: (i) the increase in cells mortality, (ii) the decrease in the abundance of genes related to nitrification and ammonium oxidation, and (iii) the decrease in the secretion of exopolysaccharides (EPS).

The Tax4Fun2 analyses revealed predicted metabolic pathways (Fig. 4) dominated by cellular metabolism (57.4%) and environmental information processing (22.3%), followed by cellular processes (10.0%), genetic information processing (7.7%), human diseases (2.1%) and organismal systems (0.9%). Tests with triplicates revealed that the uncertainty in these numbers is around 0.12%. The high percentage of the cellular metabolism pathways (57%) was expected because they are related to survival activities such as biosynthesis, oxidation and phosphorylation, photosynthetic carbon fixation, cofactors metabolism, nitrogen metabolism, xenobiotics biodegradation. The considerable percentage of pathways related to environmental information processing, includes genes related to the membrane transport and signals for cell recognition known as signal transduction. The other pathways are related to the cell growth and death, mobility, transport and catabolism, quorum sensing (QS) and biofilm formation.

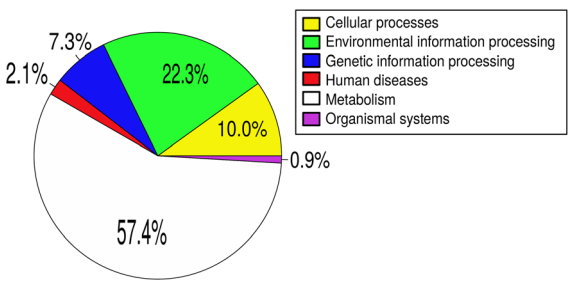

Fig. 4 Distribution of the associated metabolic pathways as predicted by using Tax4Fun2 and KEEG ortholog functional genes (KO's). (Color figure online)

Considering the importance of N removal to the WWTP, we focused on the analysis of 20 KO's related to N metabolism pathways (Table 2), including assimilatory nitrate reduction (K00360, K00367, K00372 and K00366), dissimilatory nitrate reduction (K00370, K00371, K00374, K02567, K02568, K00362, K00363), denitrification (K00370, K00371, K00374, K02567, K02568, K00368, K15864, K04561, K02305 and K00376) and nitrogen fixation (K00531, K02586, K02588 and K02591). Only small differences were observed between the TiO_2 NPs treatments and the control, suggesting that TiO_2 NPs had minor effects on nitrogen routes. However, a tendency observed before can also be verified here:

Table 2 Distribution of the KO functions according to the N associated pathways (in percentages)

KO	TiO_2 NPs concentration (mg/mL)				
	Control	0.5	1.0	1.5	2.0
ANR1	18.01	17.96	18.88	18.64	19.35
ANR2	5.74	5.78	5.14	5.43	5.20
DNR1 = D1	37.86	37.89	35.56	36.22	35.85
DNR2	30.03	29.98	29.77	29.80	29.88
D2	2.24	2.29	2.91	2.64	2.56
D3	3.21	3.32	4.30	3.80	3.87
D4	0.28	0.29	0.57	0.39	0.39
NF	2.62	2.49	2.87	3.09	2.89

The codes for the routes are: (ANR1 + ANR2) for assimilatory nitrate reduction, (DNR1 + DNR2) for dissimilatory nitrate reduction, (D1 + D2 + D3 + D4) for denitrification and (NF) for nitrogen fixation (see Fig. 5 for details)

while the 0.5 mg/mL of TiO_2 NPs treatment was almost indistinguishable from control in terms of the importance of the pathways, the other three treatments were relatively different. In fact, for pathways ANR2, DNR1 = D1 and DNR2 (first part of assimilatory and dissimilatory nitrate reduction) the abundance of KO's decreased in this direction (percentages changed from higher to lower than the average), while for pathways ANR1, D2, D3, D4 and NF (second part of assimilatory nitrate reduction, denitrification and nitrogen fixation) they became more important (percentages changed from lower to higher the average) with the increase in the NPs concentration. For better viewing the pathways, the averages of the relative abundances of KO's were calculated for each step (Fig. 5). Noteworthy, the main pathways including both dissimilatory and assimilatory nitrate reduction were the most abundant KO's (90.6%).

We have also focused on the prediction of pathways involved in biofilm formation, encouraged by the importance they have in WWTPs (Wang et al. 2019). The formation of a biofilm is complex: it involves processes such as the QS systems, the formation of flagellar structures, and the ability to produce EPS itself. Although biofilms are widely studied, the existence of a universal biofilm gene-expression pattern is still uncertain (Beloin and Ghigo 2005). Our analysis revealed the presence of 184 KO's associated to biofilm formation, 158 of which (86%) presenting an increase of the abundance with the increase in the TiO_2 NPs concentration.

Discussion

The presence of TiO_2 NPs in activated sludge due to accumulation of loads in the influent may alter the conventional wastewater treatment. The oxygen uptake results indicated that the aerobic activity of microorganisms present in the conventional activated sludge is affected by factors such as the TiO_2 NP concentration and exposure time (e.g. HRT). This cause-effect was also observed and reported for ZnO NPs (Zhang et al. 2017). However, compared to other NPs, TiO_2 NPs are not soluble at typical pH of the WWTP (6.5–7.5). Hence, mechanisms for inhibition of the oxygen uptake probably do include physical damages of the aerobic microorganisms. Although the microorganisms were damaged, the OUR experiments with TiO_2 NPs remained between 1 and 2 mg O_2/g VSS·h, indicating that a fraction of the bacterial community was able to tolerate the presence of these NPs, which persisted after TiO_2 exposition. Since in this work a synthetic media was used as substrate, this tolerance should be confirmed by using real wastewater in further exposition experiments.

The bacterial diversity indices indicated that bacterial populations in the control reactor were more similar to populations of reactors exposed to the lowest concentration of NPs (0.5 mg/mL of TiO_2), and less similar to populations in reactors exposed to higher concentrations of NPs. This means that activated sludge can tolerate high loads of TiO_2 NPs, but bacterial diversity can have a slight change. These results may be considered as indicative since the coverage obtained by our sampling was estimated to

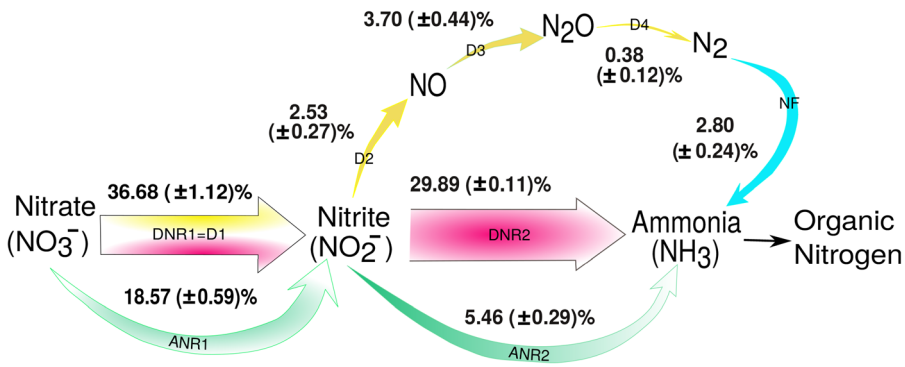

Fig. 5 The predicted metabolic pathways for nitrogen cycle. The percentages (averages with their respective standard deviations) are indicated for each route: (ANR1 + ANR2) for assimilatory nitrate reduction (Green), (DNR1 + DNR2) for dissimilatory nitrate reduction (Pink), (D1 + D2 + D3 + D4) for denitrification (Yellow) and (NF) for nitrogen fixation (Blue). (Color figure online)

be relatively poor (around 39% individually and 47% when considered together). Moreover, the bacterial diversity estimated by Simpson's index of the control group had similarity to that reported in previous studies using TiO_2 NPs (Li et al. 2020). Based on that study, the effect that TiO_2 NPs may cause in the bacterial diversity of activated sludge can be related to the concentration when the process is exposed to concentrations lower than 60 mg/L (Li et al. 2020).

The most abundant OTUs in five groups identified were affiliated to *Flavobacterium* and *Sphingomonas* genera, which members have been related to the degradation of organic matter and the utilization of polysaccharides under aerobic conditions. These results showing different distribution of the OTUs according to the treatment, particularly those affiliated to *Flavobacterium* and *Sphingomonas,* reflect that the organic matter removal process and the reduction of nitrates were probably disturbed when activated sludge was exposed to TiO_2 NPs, especially at 1.5 and 2.0 mg/mL.

The limitation in the reduction of nitrates was in line with the predictive metabolic analysis, which indicated that the first part of nitrate reduction to nitrite presented lower values than the average and with the increase in the NPs concentration. However, the second part nitrate reduction, denitrification and nitrogen fixation may keep the performance in presence of TiO_2 NPs. Besides the potential alterations in the nitrogen removal in activated sludge process caused by TiO_2 NPs, the biofilm formation and the EPS excretion can be altered. The over production of EPS has been reported in aerobic treatments exposed to ZnO NPs (He et al. 2017), to CuO NPs (Hou et al. 2015), to TiO_2 NPs (Qian et al. 2017) and even in anaerobic treatments exposed to TiO_2 NPs (Mathur et al. 2017; Cervantes-Avilés et al. 2018). Hence, this work supports that the presence of TiO_2 NPs, at least in the probed range of concentrations, can stimulate the formation of biofilms by inducing an overexpression of genes associated to specific enzymatic processes related to it. Therefore, the evaluation of the enzyme activity related to the biofilm and flocs formation such as aminopeptidase (Hassard et al. 2018; Zhao et al. 2018) and phosphatase (Hassard et al. 2018; Huang et al. 2018), could be performed in further studies.

Conclusions

The bacterial diversity profile of activated sludge exposed to TiO_2 NPs was modified mainly in the genera related to organic matter and nitrogen removal (nitrogen assimilation, nitrification and denitrification). These observations indicated that TiO_2 NPs may impact the functional structure and the capacity to remove organic matter and nitrogen by activated sludge. The analysis of predicted metabolic profiles supports the hypothesis that the TiO_2 NPs can stimulate the overexpression of genes associated to the excretion of polysaccharides and proteins.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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