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Potential effects of suspended TiO_2 nanoparticles on activated sludge floc properties in membrane bioreactors

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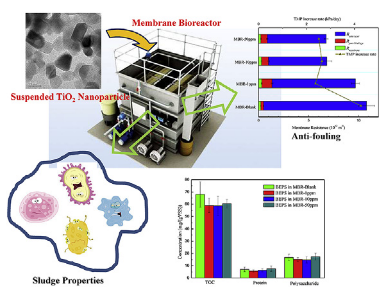
HIGHLIGHTS

- Suspended TiO_2 NPs inhibited floc viability, causing protein secretion into aqua.
- TiO_2 NPs caused over 50% floc size decrease due to over SMP and unstable structure.
- *Thiotrichaceae* replaced *Comamonadaceae* as dominate species with suspended TiO_2 NPs.

GRAPHICAL ABSTRACT



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ABSTRACT

With the rapid development and application of consumer products containing nanoparticles (NPs), especially titanium dioxide (TiO_2) NPs, the potential effects of suspended NPs on wastewater treatment has been a concern over the recent years. This study investigated the potential effects of suspended TiO_2 NPs on activated sludge flocculation properties in a membrane bioreactor (MBR). Results showed that suspended TiO_2 NPs inhibited the viability of activated sludge flocs, and led to bacterial protein secretion for bacterial protection, causing an overall protein increase of soluble microbial products. Suspended TiO_2 NPs also destabilized the activated sludge floc structure and reduced flocculation capacity of flocs, causing an over production of organic matter and resulting in a floc size decrease of over 50%. Suspended TiO_2 NPs also caused a change in the phylogenetic distribution of bacterial community. Whereby, the dominant species in activated sludge was replaced from *Comamonadaceae* to *Thiotrichaceae* in 50 mg/L suspended TiO_2 NPs.

1. Introduction

Rapid development and application of commercial products containing nanoparticles (NPs), especially titanium dioxide nanoparticles (TiO_2 NPs), have resulted in increased quantities of NPs in our sewage and the environment (called "suspended NPs") (Li et al., 2016; Yu et al., 2016b; Abudayyak et al., 2017). Major sources of suspended TiO_2 NPs include personal care products and paint.

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Personal care products containing TiO_2 NPs do not penetrate human skin, and is readily removed into the wastewater stream with washing (Johnson et al., 2011). Furthermore, suspended TiO_2 NPs can easily aggregate in aquatic environments, especially with organic matters (Chowdhury et al., 2013; Ilina et al., 2017), further promoting the accumulation of suspended TiO_2 NP in the wastewater stream with organic pollutants. Large quantities of TiO_2 NPs have also been detected in storm water urban runoff, possibly contributed by the exterior paint from buildings (Kaegi et al., 2008; Kim et al., 2012; Tou et al., 2017). In spite of this, Wastewater Treatment Plants (WWTPs) are considered a major point-source for releasing TiO_2 NPs into surface water, soil, and air, via discharge of treated effluent, biosolids (wasted sludge), and aeration caused aerosols (Kiser et al., 2010). This has raised broad concerns of the possible negative impacts of NPs on human and ecosystem health, as demonstrated in many toxicological studies (Cao et al., 2017; Slavin et al., 2017). Although the risks of suspended TiO_2 NPs have been considered (Salieri et al., 2019), the potential effects of suspended TiO_2 NPs are still unclear. Nevertheless, NPs, are regarded as a promising technology in wastewater treatment (Sharmila et al., 2015).

Up-to-date, many studies have investigated the potential effects of TiO_2 NPs on the performance of conventional activated sludge based WWTPs (Yu et al., 2015, 2016a; Li et al., 2016; Wang and Chen, 2016). However, with increased utilities in the wastewater treatment sector (Sharmila et al., 2015), it is ever pressing to get a holistic understanding of the adverse effect TiO_2 NPs has on the properties of activated sludge based membrane bioreactors (AS MBRs), a common wastewater treatment method around the world (Do et al., 2009, 2012; Kumar et al., 2015). Within AS MBRs, the use of TiO_2 NP coated “low-fouling” membranes or functionalized membranes are being used more frequently (Kim and Van der Bruggen, 2010; Li et al., 2017a). Recently, a study detailing the effects of suspended TiO_2 NPs on the cake layer formed over membrane surfaces, revealed that suspended TiO_2 NPs accelerated membrane pore blockage and postponed cake layer formation (Zhou et al., 2014). However this study only discussed membrane fouling with suspended TiO_2 NPs, with only a few results detailing its effect on activated sludge flocs properties. Other studies have reported the potential effect of suspended TiO_2 NPs on wastewater treatment performance. In sequencing batch reactors (SBRs), Zheng et al. (2011) revealed that suspended TiO_2 NPs inhibited total nitrogen removal of activated sludge, and Li et al. (2017b) further showed that suspended TiO_2 NPs reduced microbial enzymatic activities and inhibited removal of organic matter, nitrogen and phosphorus. While sludge properties with other NPs have been carried out (Cervantes-Aviles et al., 2016; Cervantes-Aviles and Cuevas-Rodriguez, 2017) studies pertaining the effect of suspended TiO_2 NPs on activated sludge floc properties are less common. Additionally, activated sludge properties in MBRs differ to that in SBR due to various operational models. Therefore, even though it is clear that properties of activated sludge play significant roles in wastewater treatment, how suspended TiO_2 NPs change the properties of activated sludge flocs is still unknown.

This study aimed to gain insights into the potential effects of suspended TiO_2 NPs on activated sludge floc properties in MBRs, including viability, floc particle size, functional groups, soluble microbial products (SMP), and bound EPS (BEPS). Four submerged MBRs were operated in parallel with different concentration levels (in ppm) of TiO_2 NPs to obtain a better understanding of the potential effects of TiO_2 NPs. It was noted that suspended TiO_2 NPs concentrations in activated sludge were reported at ppb levels (Mueller and Nowack, 2008; Kiser et al., 2009). However, this might be due to shorter sludge retention times (SRT, typically 5–20 days) compared to MBRs with much longer SRT, therefore MBRs should contain a higher concentration of suspended TiO_2 NPs. Based on previous studies (Zheng et al., 2011; Zhou et al., 2014), 1 (potential concentration) and 10 mg/L (extreme concentration) suspended TiO_2 NPs were chosen in this study, a further 50 mg/L suspended TiO_2 NPs was also selected as assurance of its potential effects. In addition, as previous literature has shown, bacterial community and its phylogenetic analysis have been reported to directly affect wastewater treatment performance and fouling of MBR (Xia et al., 2010; Li et al., 2016; Loh et al., 2018; Wen et al., 2018). Consequently, bacterial community and its phylogenetic analysis of activated sludge were also measured and applied in the study to comprehensively understand the potential effects of suspended TiO_2 NPs on activated sludge floc properties.

2. Materials and methods

2.1. Suspended TiO_2 and the influent of MBR

Suspended TiO_2 NPs were bought from Sigma-Aldrich. The structure of TiO_2 NPs was visualized through a transmission electron microscopy (TEM) image using a Tecnai F20 (Philips Electron Optics, Netherlands) with a 200 kV accelerating voltage (Fig. 1). The primary size of TiO_2 NPs in stock suspension was in the range of 20 nm, which was similar to the description of Sigma-Aldrich. TiO_2 NPs stock suspension (100 mg/L) was prepared by dispersing 100 mg of TiO_2 NPs to 1 L of Milli-Q water, followed by 1 h ultrasonication (25 °C, 300 W, 40 kHz) according to Zhou et al. (2014).

The influent of MBR was synthetized based on a modification of previous literature (Miura et al., 2007; Al-Amri et al., 2010) with tap water, containing 420 mg/L glucose, 420 mg/L corn starch, 102.75 mg/L NH_4Cl and 22 mg/L KH_2PO_4 as well as trace nutrients such as $CaCl_2$ (8 mg/L), $MgSO_4 \cdot 7H_2O$ (9 mg/L), $MnSO_4 \cdot H_2O$ (3.66 mg/L) and $FeSO_4 \cdot 7H_2O$ (0.55 mg/L). $NaHCO_3$ was used as a buffer to adjust the influent pH to around 7.0.

2.2. MBR operation and monitoring

Four submerged MBRs (Table 1) were operated in parallel. Each reactor had a working volume of 3.0 L (20 cm × 10 cm × 25 cm of

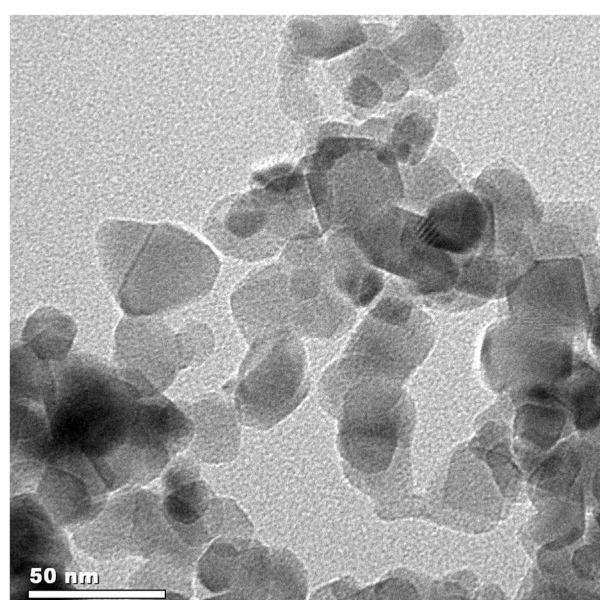

Fig. 1. The transmission electron microscopy (TEM) image of TiO_2 NPs suspension.

Table 1
Operating condition of the four MBRs.[a].

	MBR-Blank	MBR-1 ppm	MBR-10 ppm	MBR-50 ppm
TiO_2 NPs concentration (mg/L)[b]	–	1	10	50
HRT (h)	8	8	8	8
SRT (day)	30	30	30	30
MLSS (mg/L)[a]	5000 ± 270	5100 ± 420	5000 ± 380	5200 ± 340
VSS (mg/L)[a]	4100 ± 320	4000 ± 570	3900 ± 650	4200 ± 330
COD removal efficiency (%)	94 ± 2.1	94 ± 4.7	94 ± 1.9	94 ± 3.2
NH_4^+–N removal efficiency (%)	98 ± 1.6	98 ± 1.5	98 ± 0.87	98 ± 0.98
TN removal efficiency (%)	36 ± 3.0	30 ± 1.6	25 ± 3.2	29 ± 1.3
TP removal efficiency (%)	26 ± 2.1	15 ± 3.6	18 ± 1.7	17 ± 2.7

[a] All the values represent mean ± SD (MLSS and VSS were measured every 2 days, n = 40; removal efficiency of each item was performed every day, n = 80).
[b] TiO_2 NPs concentration means the final concentration of suspended TiO_2 NPs in the mixed liquid of each MBR.

length × width × height; Fig. S1 (Supporting Information, SI)). A hollow fiber microfiltration membrane module (Polyvinylidene fluoride (PVDF), 200 cm^2 total surface area and 0.1 μm pore size, fabricated by Li-tree Company, Suzhou, China), was equipped in each MBR. All MBRs were operated under a hydraulic retention time (HRT) of 8 h and an SRT of 30 d at room temperature (23–26 °C). Coarse bubble aeration was supplied through a perforated pipe under the membrane module to provide oxygen for microbial activity. When the *trans*-membrane pressure (TMP) reached 40 kPa, an indication for fouling, the membrane module was removed from the MBR and washed by physical (tap water washing) and chemical cleaning (2% NaOCl and 1% citric acid immersion for 4 h, respectively) for regeneration. In addition, each MBR was set up in triplicate and covered with aluminum foil to avoid the possible light-induced effects.

Each reactor was seeded with inoculation sludge obtained from Quyang WWTP (Shanghai, China) and fed with the synthetic wastewater. Throughout the experiment, the loading rate of each reactor was kept as 4.5 g COD (chemical oxygen demand)/d and 300 mg NH_4^+–N (ammonia nitrogen)/d. Each newly inoculated MBR was initially operated for over 100 days to achieve acclimatization of the sludge. Each MBR was then fed with suspended TiO_2 NPs stock suspension (100 mg/L) in order to reach the predetermined suspended TiO_2 NPs concentration as shown in Table 1 (TiO_2 NPs were directly injected into MBR during operation and the influent was without TiO_2 NPs). The membrane module was replaced with a new unit and the operational time was recorded when the performance of all MBRs were stable, each MBR was then operated for over 80 days. Because the concentration of suspended TiO_2 NPs in MBRs might slowly decrease due to the discharge of effluent or sludge, TiO_2 NPs stock suspension (100 mg/L) were supplemented every day to maintain the initial suspended TiO_2 NPs concentration according to the total concentration of TiO_2 in MBRs. MBR-Blank was operated as the control unit (i.e., without TiO_2 NPs).

2.3. Extraction and measurement of SMP and BEPS

Extraction of soluble microbial products (SMP), bound EPS (BEPS) were performed according to a modified thermal extraction method (Zhou et al., 2014). Total organic carbon (TOC) of SMP and BEPS were measured using a TOC analyzer (TOC-VPN, Shimadzu, Japan). The polysaccharides and protein contents of the SMP and BEPS were measured by the phenol-sulfuric acid method and Branford method, respectively (Xia et al., 2012b). MW distributions of SMP and BEPS were determined by using a gel chromatography analyzer, equipped with a TSK G4000SW type gel column (TOSOH Corporation, Japan) and a liquid chromatography spectrometer (LC-10ATVP, SHIMADZU, Japan). Three-dimensional excitation-emission matrix (EEM) fluorescence spectroscopy of SMP and BEPS were measured by a fluorescence spectrophotometer (FluoroMax-4, HORIBA, Japan), detailed in SI.

2.4. Analytical methods

Determination of ammonia nitrogen (NH_4^+-N), chemical oxygen demand (COD), total nitrogen (TN), mixed liquor suspended solid (MLSS), mixed liquor volatile suspended solids (MLVSS), and metal ions were conducted in accordance with the Standard Methods (APHA, 1998). The concentrations of TiO_2 NPs and other metal in MBRs were analyzed according to previous papers (Kiser et al., 2009; Zheng et al., 2011), as detailed in SI. A focused beam reflectance measurement (Eyetech particle size and shape analyzer, Ankersmid, Holland) was used to identify particle size distribution (PSD) of activated sludge with mean size (the average size of particles) and Dx (detailed in Table 2, which was mainly to show the size distribution of particles). After 24 h-freeze-drying pretreatment, activated sludge was determined with a Fourier transform infrared spectrometer (FTIR) (Nicolet 5700, Thermo Electron Corporation, USA) for functional groups. Total membrane resistance could be classified into original membrane resistance, pore blockage resistance and cake layer resistance, as measured previously (Zhou et al., 2014). Dissolved oxygen (DO) concentrations and pH values were detected by a DO/pH meter (HQ4d, HACH, USA). *Zeta* potential of activated sludge flocs was measured with *Zeta sizer Nano Z* (Malvern, UK). To evaluate the toxicity on activated sludge viability, oxygen utilization rates (OUR) of activated sludge were measured according to previous papers (Cervantes-Aviles et al., 2017; Li et al., 2017b).

2.5. 16s rRNA gene-cloning and phylogenetic analysis

The bacterial community of activated sludge in each MBR was identified through a cloning library of 16s rRNA. Genomic DNA of activated sludge was extracted with a FastDNA Spin Kit (MP Biomedicals, LLC, France). The complete 16s rRNA genes from extracted

Table 2
PSD of activated sludge flocs (Length, D [1,0]).

	Mean size (μm)	D10 (μm)	D50 (μm)	D90 (μm)
MBR-Blank	28 ± 2.6	2.4 ± 0.84	12 ± 1.4	76 ± 3.1
MBR-1 ppm	11 ± 1.6	0.72 ± 0.16	6.7 ± 1.0	29 ± 2.4
MBR-10 ppm	11 ± 2.1	0.56 ± 0.34	5.9 ± 0.94	20 ± 2.0
MBR-50 ppm	13 ± 1.6	0.71 ± 0.11	6.6 ± 0.88	36 ± 2.1

Mean size represents the average value of particle size. Dx, mainly to show the size distribution of particles, is the diameter at which x % of a sample's mass is comprised of smaller particles. The D50 is also known as the “mass median diameter” as it divides the sample equally by mass.
n = 12, PSD of activated sludge flocs was performed in triplicate every 7 days.
Zeta potential of activated sludge flocs in MBR-Blank, MBR-1 ppm, MBR-10 ppm, and MBR-50 ppm were −28.5 mV, −35.8 mV, −36.2 mV and −36.7 mV.

DNA were amplified with the bacterial universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) for building the cloning library (Xia et al., 2010). Triplicate PCR (polymerase chain reaction) products were pooled to minimize bias. The procedure of cloning library was according to papers (Xia et al., 2010, 2012a). For each sample, approximately 100 positive clones were selected randomly from different locations in the culture dish for sequencing (Ma et al., 2013; Chen et al., 2014). Chimeric sequences were identified with Bellerophon (where uploaded sequences to the Bellerophon website "http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl" identified if a sequence was chimeric or not). All the sequences were compared to known sequences for phylogenetic analysis. Operational taxonomic units (OTUs) were defined as groups in which the sequence similarity was more than 97%. Phylogenetic trees were constructed by neighbor-joining method with the Clustal X software package. Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies.

The 16s rRNA gene sequences from this study have been submitted to National Center for Biotechnology Information (NCBI) public databases (GenBank) under accession numbers KJ486338 to KJ486457.

3. Results

3.1. Reactor performance and activated sludge viability

Essential operating parameters of the MBRs during the 80-day experimental period are summarized in Table 1. With identical HRT (8 h) and SRT (30 days), all MBRs accumulated approximately similar concentrations of MLSS and MLVSS. In addition, the quality of effluent from all four MBRs regarding COD and NH_4^+–N were comparable to their respective average removal efficiencies between approximately 96% and 98%. However, removal of TN and TP both decreased with addition of suspended TiO_2 NPs. Fig. S2 also reveals a decrease in OUR of activated sludge with an increase in suspended TiO_2 NPs.

3.2. PSD of activate flocs

Table 2 shows the PSD of activated sludge flocs in each MBR. With each increase in concentration of suspended TiO_2 NPs, the mean size of activated sludge flocs was halved, compared to those in MBR-Blank. Mass median diameter (D50) in the MBR with suspended TiO_2 NPs also ranged from 5.5 to 6.5 μm. The result of D90 indicated that suspended TiO_2 NPs promoted an increase in the percentage of small particles in activated sludge flocs.

3.3. FTIR of activated sludge

The four MBRs had a similar FTIR spectrum of activated sludge (Fig. S3). The spectrum shows a broad adsorption peak at 3435 cm^{-1}, which is attributed to O–H bond stretching in hydroxyl functional groups, and a peak at 2925 cm^{-1}, which is ascribed to the C–H bonds stretching (Kumar et al., 2006). Two additional peaks (1641 and 1548 cm^{-1}) are also observed in the spectra and are unique to the protein secondary structure: amide I, C=O stretching and amide II, mostly N–H bending. Additionally, there is a peak at 1043.5 cm^{-1}, indicating the characteristics of polysaccharide or polysaccharide-like substance (Croue et al., 2003).

3.4. SMP and BEPS of activated sludge

Fig. 2 shows that the constituents of SMP and BEPS varied between MBRs. The TOC attributed to SMPs was almost identical across all MBRs (Fig. 2(a)). Conversely, with higher TiO_2 NPs concentrations, the extracellular protein concentrations within SMPs increased while the extracellular polysaccharide concentrations within SMPs decreased. The protein/polysaccharide ratios in MBR-Blank, MBR-1 ppm, MBR-10 ppm and MBR-50 ppm were 0.56, 1.9, 2.0 and 6.5, respectively.

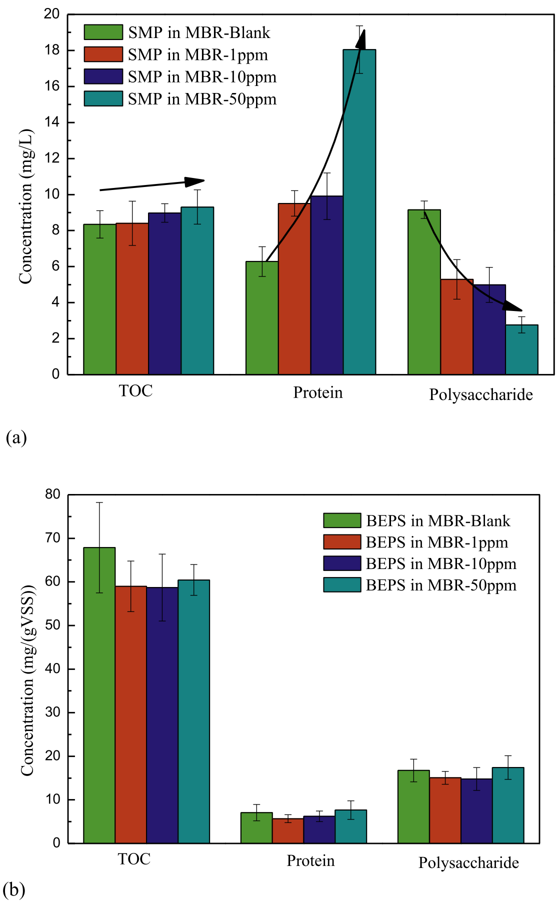

Fig. 2. Constituents of (a) SMP and (b) BEPS in each MBR (n = 30: Each measurement was performed in triplicate every 7 days.).

It was observed that two peaks can be clearly identified from the EEM fluorescence spectra of SMP (Fig. 3) in four MBRs. The first main peak is observed at the excitation/emission wavelengths (Ex/Em) of 275–280/320–330 nm (Peak A), which has been reported as a protein-like-substance peak (Zhou et al., 2014). The second main peak identified at the Ex/Em of 230–250/430–460 nm (Peak B) is related to the humic acid-like-substances peak (Liu et al., 2011). The fluorescence of protein-like-substances and humic acid-like-substances were the dominant part of the SMPs fluorescence. Fig. 3 also shows that with the suspended TiO_2 NPs concentration increasing to 50 mg/L, the fluorescence intensity (FI) of protein-like-substances (Peak A) increased but the FI of humic acid-like substances (Peak B) decreased. In addition, EEM fluorescence spectra of BEPS clearly shows a tryptophan peak (Peak A) and tyrosine peak (Peak C; Ex/Em = 220–230/320-340 nm), with two hydrophobic amino acids (Yamashita and Tanoue, 2003; Sheng and Yu, 2006; Zhou et al., 2014).

MW distribution of SMP and BEPS (Fig. 4) is classified into 5 ranges: <1 kDa, 1–100 kDa, 100–500 kDa, 500–1000 kDa and >1000 kDa. It is evident that the exposure to suspended TiO_2 NPs had no measurable effects on the 1–100 kDa portion of SMPs. Additionally, with the presence of suspended TiO_2 NPs, the

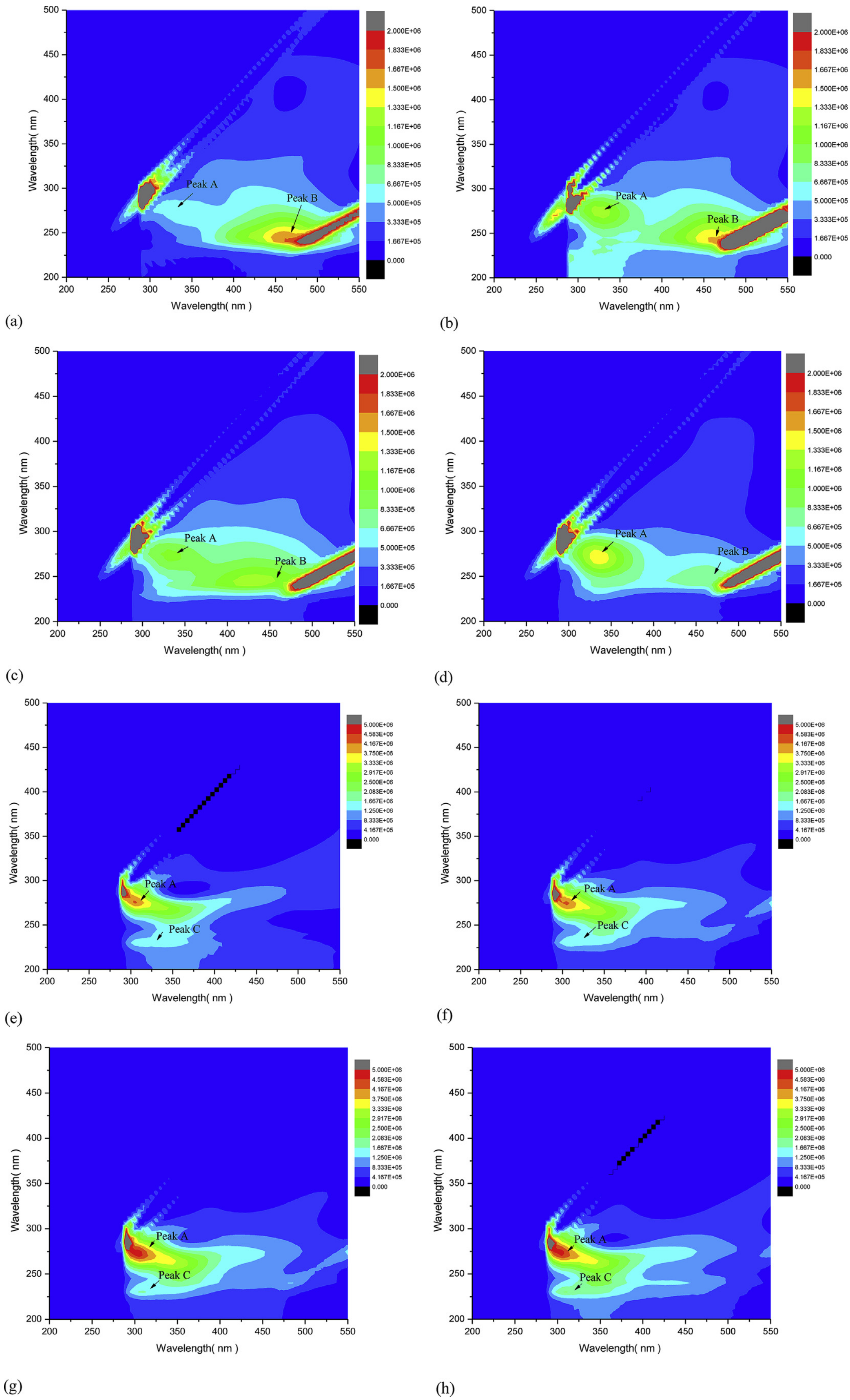

Fig. 3. EEM fluorescence spectra of SMP in (a) MBR-Blank, (b) MBR-1 ppm, (c) MBR-10 ppm and (d) MBR-50 ppm and BEPS in (e) MBR-Blank, (f) MBR-1 ppm, (g) MBR-10 ppm and (h) MBR-50 ppm. (EEM analysis of SMP was carried out every 7 days. The above EEM fluorescence spectra of SMP in each MBR were the representative ones in all samples).

dominance of SMP changed from MW < 1 kDa to MW > 100 kDa.

3.5. *Bacterial community of activated sludge*

A 16s rDNA gene clone library was constructed to explore bacterial population and identify dominant species in each MBR. Each clone library included 100 randomly selected clones. All clones were grouped into different OTUs on the basis of more than 97% sequence similarity within an OTU (Xia et al., 2010). Respectively 31, 31, 35 and 23 OTUs were obtained from the MBR-Blank, MBR-1 ppm, MBR-10 ppm and MBR-50 ppm reactors. A phylogenetic tree of activated sludge flocs in each MBR is shown in Fig. S5. Table 3 shows the phylogenetic distribution of the OTUs in each MBR (classified to family-level, Table S1). *Comamonadaceae* accounted for 30%, 61% and 41% of the bacterial community in the activated sludge of MBR-Blank, MBR-1 ppm and MBR-10 ppm. However, *Thiotrichaceae* (42%), which belongs to *Gamaproteobacteria*, replaced *Comamonadaceae* (5%) as the predominant bacteria in MBR-50 ppm. *Sphaerotilus* sp. was also found in the activated sludge.

4. Discussion

4.1. *Potential effects of suspended TiO_2 NPs on activated sludge viability*

As can be seen by the reactor performance results, exposing the activated sludge to high concentrations of suspended TiO_2 NPs, caused adverse effects to the functionalities of the microorganisms residing within the sludge flocs. Furthermore, results of OUR also demonstrated a potential decrease in viability of activated sludge when exposed to suspended TiO_2 NPs. This is attributed to the suspended TiO_2 NPs ability to easily aggregate on cell membranes, inhibiting bacterial viability (Cervantes-Aviles et al., 2017). Li et al. (2017b) further identified that the viability decrease was also due to the fact that suspended TiO_2 NPs could inhibit the respiration and catabolic activity of heterotrophic bacteria. Comparatively in SBRs, results have shown that exposure to suspended TiO_2 NPs does not cause the adverse effects or cytoplasmic leakage of activated sludge flocs as seen in MBRs (Zheng et al., 2011). It was hypothesized that the discrepancy between the resistance of activated sludge in MBR and SBRs to suspended TiO_2 NPs is directly related to the sludge separation mechanisms. Whereby, the membrane module in the MBR system can completely intercept both activated sludge and TiO_2 NPs (Zhou et al., 2014), causing an exposure of activated sludge to a steady concentration of suspended TiO_2 NPs.

The similar FTIR results across all reactors indicate that suspended TiO_2 NPs has no impact on the functional groups of activated sludge flocs, especially on its protein and polysaccharide composition. According to previous work TiO_2 NPs bounded to activated sludge with the bidentate coordination of carboxylate to TiO_2 (Fig. S4) (Kiser et al., 2009; Kim and Van der Bruggen, 2010; Zhou et al., 2014), this was again confirmed in this study by a peak at 670 cm^{-1} indicating the stretching of Ti—O bonds. This is an indication to the ability of bidentate coordination to reduce the surface charge of activated sludge flocs.

4.2. *Potential effects of suspended TiO_2 NPs on activated sludge flocs*

As reflected by the PSD results, suspended TiO_2 NPs partly induced the decrease of the size of activated sludge flocs. A similar phenomenon was observed by Cervantes-Aviles and Cuevas-Rodriguez (2017) where NPs caused a size decrease of activated sludge floc during raw wastewater treatment. Hou et al. (2015)

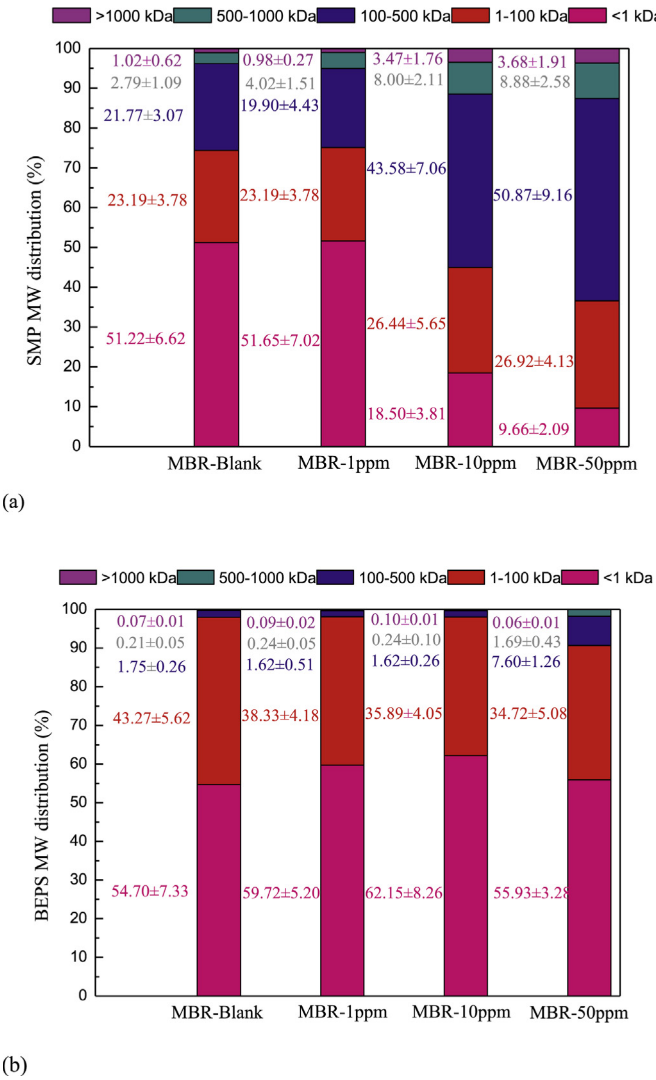

Fig. 4. MW distribution of SMP (a) and BEPS (b) in each MBR (n = 30: Each measurement was performed every 7 days.).

identified that the over production of loosely bound EPS resulted in less flocculation of flocs and a further reduction of flocs stability. Therefore, it can be inferred that an increase of organic matter (both SMP and EPS) with suspended TiO_2 NPs (Fig. 2) are one of the contributors for a size decrease in activated sludge flocs. In addition, Cervantes-Aviles et al. (2016) reported that NPs also caused a morphological interaction to suspended biomass, leading to the physical damage in the flocs' structure, and reducing the activated sludge floc size. Moreover, Cervantes-Aviles and Cuevas-Rodriguez (2017) related their flocs size being exposed to NPs, to the double layer theory. In this theory the parameter known as *Zeta* potential indicates the stability of flocs. Table 2 showed that *Zeta* potential of activated sludge flocs increased as suspended TiO_2 NPs advanced, further confirming an unstable floc structure with suspended TiO_2 NPs. This is as expected, again caused due to the interaction between TiO_2 NPs and organic matter (Sharmila et al., 2015). Consequently, it can be said that an unstable floc structure promoted the size decrease of activated sludge flocs.

4.3. *Potential effects of suspended TiO_2 NPs on SMP and BEPS*

As Fig. 2(a) shows, concentrations of suspended TiO_2 NPs up to

Table 3
Phylogenetic distribution of the OTUs in each MBR (classifying in family, %).

	Family	MBR-Blank	MBR-1 ppm	MBR-10 ppm	MBR-50 ppm
1	*Nitrospiraceae*	2	0	0	0
2	*Acidobacteria*	3	0	0	5
3	*Nitrosopumilaceae*	4	0	0	0
4	*Sphingomonadaceae*	3	0	0	0
5	*Thiotrichaceae*	17	0	0	42
6	*Neisseriaceae*	1	0	0	0
7	*Burkholderiaceae*	1	2	0	0
8	*Rhodocyclaceae*	18	13	6	7
9	*Comamonadaceae*	30	61	41	5
10	*Lactobacillaceae*	0	1	0	0
11	*Streptococcaceae*	0	0	5	0
12	*Rhizobiaceae*	0	1	0	0
13	*Flexibacteraceae*	0	0	4	0
14	*Sphingobacteriaceae*	0	0	2	5
15	*Polyangiaceae*	3	0	0	0
16	*Planctomycetales*	1	1	0	6
17	*Xanthomonadaceae*	0	1	0	14
18	*Bosea*	0	1	0	0
19	*Aeromonadaceae*	0	1	1	0
20	*Chitinophagaceae*	0	4	2	0
21	*Euglenophyceae*	0	1	0	0
22	*Chitinophagaceae*	0	0	1	0
23	*Hydrogenophilaceae*	0	0	1	0
24	Other	17	13	37	16

50 mg/L did not cause excessive production of SMPs. However, the protein/polysaccharide ratios indicate that higher TiO_2 NPs concentrations caused a shift of the major composition of SMP from polysaccharide to protein. Sheng et al. (2010) reported that sludge would accumulate more EPS under toxic conditions as a protective response to toxins, and Cervantes-Aviles et al. (2016) showed that the use of NPs makes it easy to improve the production of protein used for coating. Protein is the primary component secreted from activated sludge flocs to retard the contact of the metals to the bacteria inside activated sludge (Hou et al., 2015; Wang and Chen, 2016; Li et al., 2017b). Thus, due to the presence of suspended TiO_2 NPs, there was an increase of protein in SMP. In addition, section 3.4 showed that both polysaccharide and humic acid in SMP decreased with suspended TiO_2 NPs, as bacteria prioritize protein production for protection. On the other hand, for BEPS, similar concentrations of TOC, protein and polysaccharide demonstrated that suspended TiO_2 NPs had no obvious effects on the constituent of BEPS. This is because SMPs are applied prior to BEPS for coating suspended TiO_2 NPs as a protection for bacteria in activated sludge (Li et al., 2017b). Therefore, BEPS showed little variations with suspended TiO_2 NPs in this study.

It is important to note that, an increase in soluble protein concentrations and decrease in soluble polysaccharide concentrations might catalyze a change in membrane fouling. But according to a previous study (Zhou et al., 2014), suspended TiO_2 NPs should mitigate membrane fouling due to a reconstructed cake layer structure, as suspended TiO_2 NPs changes features of the complexes on the membrane surface for a postponed cake layer fouling.

Finally, as can be seen in Fig. 4, TiO_2 NPs caused a slight variation of molecular weight (MW) distribution of BEPS, whilst the substances that ranged from 1 to 100 kDa and <1 kDa played the dominant role in this distribution. As previously reviewed (Liu and Fang, 2003; Raszka et al., 2006; Ni and Yu, 2012), EPS is classified as SMP- or BEPS-based according to whether EPS is located on the bacterial surface or not. SMP consists of utilization-associated products (UAP) and biomass-associated products (BAP). UAP (low Molecular Weights, MW) is the growth associated products that are produced at a rate proportional to the rate of substrate metabolism, while BAP (high MWs) is the non-growth associated products formed as by-products of endogenous respiration led cell-lysis and produced at a rate proportional to the concentration of biomass (Barker and Stuckey, 1999). The MW increase of SMP indicated that suspended TiO_2 NPs led to high MWs BAP secretion from bacteria, and the BAP were partly protein or protein-like substances based on the SMP constituent results. In addition, BEPS not only works as a mechanism of protection of microbial cells, but also can be potentially used as a nutrition source to support bacterial energy demand (Raszka et al., 2006). Therefore, suspended TiO_2 NPs only caused a slight variation of MW distribution of BEPS, and most of the BEPS were low MWs substances.

4.4. Potential effects of suspended TiO_2 NPs on bacterial community of activated sludge

As Table 3 shows, *Comamonadaceae* was a predominant member in the bacterial communities of MBR-Blank, MBR-1 ppm and MBR-10 ppm. But, with 50 mg/L suspended TiO_2 NPs a variation of bacterial community from *Comamonadaceae* to *Thiotrichaceae* as the predominant bacteria was observed. As a genus of filamentous sulfur-oxidizing bacteria, members in the genus *Thiothrix* (belonging to *Thiotrichaceae*) are either chemoorganotrophs or mixotrophs (Larkin and Strohl, 1983; Howarth et al., 1999). The shift of the predominant microbes possibly indicated that the toxicity of TiO_2 NPs enhanced the growth of *Thiotrichaceae* which have more versatile metabolisms. Compared to the other MBRs with suspended TiO_2 NPs, MBR-Blank had a higher diverse bacterial community, indicating that suspended TiO_2 NPs reduced the microbial diversity of activated sludge.

Another interesting microorganism residing in the MBRs is *Sphaerotilus* sp. which has been widely used in heavy metal bioremediation (Esposito et al., 2001; Pagnanelli et al., 2003). The existence of *Sphaerotilus* sp. in MBR-1 ppm probably indicated that suspended TiO_2 NPs even at low concentration (1 mg/L) possesses toxic effects to the microbial communities in the MBRs. Consequently, TiO_2 NPs caused the structure variation of the bacterial community of activated sludge, and TiO_2 NPs of 50 mg/L especially led to the replacement of the dominant species from *Comamonadaceae* to *Thiotrichaceae*.

Regarding MLSS and MLVSS (Table 1), it seems that the suspended TiO_2 NPs did not change the bacterial mass. Although, suspended TiO_2 NPs does reduce viability of activated sludge flocs, and significantly inhibits the respiration and catabolic activity of heterotrophic bacteria (Cervantes-Aviles et al., 2017; Li et al., 2017b).

5. Conclusion

Potential effects of suspended TiO_2 NPs on activated sludge flocs properties in MBR were studied. Suspended TiO_2 NPs inhibited viability of activated sludge flocs, and caused further bacterial protein secretion into aqua for bacterial protection, presented as an increase of protein in SMP. Suspended TiO_2 NPs also destabilized activated sludge floc structure and reduced flocculation capacity of flocs, causing over production of organic matter, and decreasing the floc size (over 50%). Suspended TiO_2 NPs also changed phylogenetic distribution of bacterial community, and the dominant species in activated sludge was replaced from *Comamonadaceae* to *Thiotrichaceae* with 50 mg/L suspended TiO_2 NPs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.02.042.

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