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Effects of ZnO nanoparticle exposure on wastewater treatment and soluble microbial products (SMPs) in an anoxic-aerobic membrane bioreactor

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Abstract

The effect of zinc oxide nanoparticles (ZnO NPs) on the performance of an anoxic-aerobic submerged membrane bioreactor (MBR), and the characterization of the soluble microbial products (SMPs) produced in the presence of ZnO NPs was evaluated. Continuous operation over 144 days showed that ZnO NPs at concentrations of 10 and 50 mg/L exerted a negative impact on chemical oxygen demand (COD) and nitrogen removal, although ZnO NPs were efficiently removed in the MBR (>92%). 10 and 50 mg/L ZnO NPs decreased COD removal substantially from $93.1 \pm 0.6\%$ to $90.1 \pm 0.8\%$ (< 0.05) and $86.3 \pm 2.3\%$ (< 0.05), respectively. Similarly, with 10 and 50 mg/L ZnO NPs, the decreased in $\text{NH}_4\text{-N}$ removal was 8.1% and 21.1%, respectively. Exposure to 1, 10 and 50 mg/L ZnO NPs increased SMP concentrations by 12.8%, 42.4% and 51.5%, respectively. High performance size exclusion chromatograph (HP-SEC) analysis revealed that the presence of ZnO NPs caused a significant increase in high-molecular weight (MW) (583 kDa) SMPs at 1 and 10 mg/L ZnO NP concentration. A substantial decrease in the concentration of high-MW compounds in the MBR effluent was observed at the end of the experiment. Excitation emission matrix (EEM) fluorescence contours revealed that SMPs were dominated by amino acid-, tryptophan protein-, polyaromatic-, and polycarboxylate-type substances. The presence of ZnO NPs enhanced the production of amino acid-like (7.5-25.1%) and tryptophan protein-like compounds (31.7-38.1%), compared to the control (6.0-20.2% for amino acid-like compounds; and 28.5-36.7% for tryptophan protein-like compounds). In contrast, the fulvic and humic acid-like compounds decreased with exposure to ZnO NPs. This work may help better understanding the effect of nanoparticle exposure on wastewater treatment performance and SMP characteristics.

Keywords: ZnO nanoparticles; membrane bioreactors; soluble microbial products; wastewater treatment

1. Introduction

Rapid manufacture and application of nanomaterials have prompted increased concern regarding the potentially adverse effects nanoparticles (NPs) have on the aquatic environment and human health (Boxall et al., 2007; Brar et al., 2010). Zinc oxide (ZnO) NPs are regarded as one of the most toxic engineered NPs, and have demonstrated broad scientific and technological application in electronic sensors, solar cells, coatings, pigments and optics, due to their novel and unique physicochemical properties (Li et al., 2008; Westerhoff et al., 2013).

The expanding production of ZnO NPs increases the potential for their unintentional release into the environment (Brar et al., 2010; Ma et al., 2013). Wastewater treatment plants (WWTPs) are considered as the last barriers prior to the environmental release of engineered NPs (Ganesh et al., 2010). Previous studies have demonstrated that significant amount of ZnO NPs can be adoptively removed from wastewater during treatment (Hou et al., 2014; Puay et al., 2015). However, variable physicochemical properties of ZnO NPs (e.g., particle size, surface area, morphology etc.) and the environmental water chemistry (e.g., pH, ionic strength, redox conditions, natural organic matter) may elicit their different mode of action, such as behaviour, transformation and toxicity in biological treatment processes (Ma et al., 2013). Moreover, ZnO NPs can decrease the abundance and diversity of the microbial community in activated sludge system (CAS), leading to the negative influence on the efficiency of biological nutrient removal (Hou et al., 2014; Tan et al., 2015).

Membrane bioreactors (MBRs) has been widely used for the treatment of municipal and industrial wastewater (Meng et al., 2009). Compared to conventional CAS, MBR technology may be more efficient in removing engineered NPs, because of their unique properties such as long sludge retention times (SRTs), high mixed liquor concentration, and high biomass diversity that favours the optimum biodegradation of organic pollutants (Wang et al., 2014; Tan et al., 2015). However, it has been reported that beyond the potential microbial inhibition, the efficient absorption of ZnO NPs on sludge within

MBRs is undesirable, and could impair the quality of biosolids through decrease in microbial populations and diversity, resulting in the deterioration of treatment performance (Tan et al., 2015). Moreover, the extended MBR operation time may strengthen the accumulation of NPs and help with the enrichment of NP-resistant bacteria, thus long-term studies are necessary to provide crucial information on the behaviour of ZnO NPs and their potential inhibitory impact on the performance of MBRs (Mei et al., 2014; Wang et al., 2014). Although previous studies have been carried out on the impact of ZnO NPs on the system performance in sequencing batch reactors (Zheng et al., 2011; Puay et al., 2015), anaerobic digesters (Mu and Chen, 2011; Lombi et al., 2012), simulated rotating biological contactor (Hou et al., 2014), to date very little information is available on the use of MBRs to eliminate environmental NPs. Furthermore, there has been inconsistency in the information on the effects of ZnO NPs on pollutant removal in MBRs. Wang et al. (2014) reported that no obvious inhibiting effects on carbonization, nitrification, denitrification and phosphorus removal in MBRs exposed to ZnO NPs during 185 days' operation, while Tan et al. (2015) showed that ZnO NPs at both 1.0 and 10.0 mg/L caused moderate deterioration in the removal of COD, nitrogen and phosphorus during continuous MBR operation over 242 days.

Soluble microbial products (SMPs) represent soluble extracellular polymeric substances (EPS), which are mixture of polymers that result from substrate metabolism (Barker and Stuckey, 1999; Jarusutthirak and Amy, 2006). Previous studies have revealed considerable variability in the production of SMPs in response to environmental stresses imposed on the microorganisms, such as nutrient deficiency (Aquino and Stuckey, 2003; Wang and Zhang, 2010), low pH (Wang and Zhang, 2010), low temperature (Wang and Zhang, 2010), and the presence of toxic compounds (Avella et al., 2010; Han et al., 2013; Wu et al., 2015). EPS/SMP are usually considered as an important barrier protecting microorganisms against harsh environmental stress (Otero-González et al., 2014), and previous studies revealed that ZnO NPs had a substantial impact on SMP production (Mei et al., 2014; Tan et al., 2015). Although EPS can enhance the ability of activated sludge to more effectively adsorb and aggregate engineered NPs, to date, the roles of EPS play in mitigating toxicity and improving the resistance of microbial community in a submerged MBR under exposure to ZnO NPs are still not well understood. It is also not clear how variable EPS and SMP secretion is under ZnO NP exposure, and what the characteristics of such compounds are in response to the changed environment. Presently there is a dearth of knowledge concerning the changes in SMP

concentrations, properties and compositions, which may reveal the response and resistance of microorganism to ZnO NP toxicity.

Previous modelling studies suggested a ZnO NPs concentration of 0.34-1.42 $\mu\text{g/L}$ in treated wastewater in Europe in 2008 (Gottachalk et al., 2009), which was increased to 1.7-21 $\mu\text{g/L}$ in 2012 (Sun et al., 2014). An environmentally relevant concentration of ZnO NPs in wastewater would be around 24-300 $\mu\text{g/L}$, considering the removal efficiency (around 93%) used in the model (Sun et al., 2014). Taking into account the rapid and continuous increase in the production volumes, the environmental concentration of ZnO NPs is expected to inevitably increase (Tan et al., 2015) and it may range from the high end of these modelling studies to an order of magnitude greater (Keller and Lazareva, 2014). Thus, the potential effects of ZnO NPs at concentrations ranging from 1-50 mg/L were investigated in this study. The objective of this study was to assess the long-term effect of ZnO NPs on pollutant removal in an anoxic-aerobic submerged MBR over 144 d in the presence ZnO NPs. The MW distribution of SMPs, and their characteristics and composition under exposure to ZnO NPs was also assessed using high performance size exclusion chromatograph (HP-SEC) and three-dimensional fluorescence excitation emission matrix (EEM).

2. Materials and methods

2.1. Set-up and operation of MBRs

Two identical lab-scale MBR systems, i.e., MBR-C (control) and MBR-T (treatment), consisting of an anoxic compartment (3 L) and an aeration compartment (7 L), were operated in parallel (Figure S1). A hollow fibre ultrafiltration (UF) membrane (ZeeWeed 500, GE Singapore), made of polyvinylidene fluoride, was submerged inside the aerobic compartment. Effective membrane surface area was 565 cm^2 , with a nominal pore size of 0.04 μm . To control the MBR process, 3 min of filtration followed by 1 min of relaxation was achieved using fully automated SCADA software (IFIX).

The MBRs were inoculated with biomass obtained from Ulu Pandan Wastewater Reclamation Plant (WRP), Singapore. Synthetic wastewater was used in this study to simulate domestic sewage, and fed

from two 70-L glass tanks (maintained at 4°C). The mixed liquor suspended solid (MLSS) in the aeration tank was around 3-6 g/L, with an average sludge retention time (SRT) of 25 d for each MBR. The hydraulic retention time (HRT) was approximately 10 h, and a constant permeate flux of 13 - 15 L/m² h was maintained, with level sensors controlling the feed, membrane flux and HRT. Both MBRs contained air diffusers on the bottom of the tank to maintain the dissolved oxygen (DO) concentration at 3-4 mg/L for biological oxidation, and membrane scouring. The transmembrane pressure (TMP) was monitored continuously using a digital pressure gauge (Ashcroft), and parameters such as membrane flux, pH, DO, and temperature were recorded using a data logger. The two MBRs were operated continuously for a period of 144 d after the systems reached steady-state.

In this experiment, synthetic wastewater was introduced into the MBRs by peristaltic pump. The concentrations of chemical compositions were (in mg/L): 356 sucrose (C₁₂H₂₂O₁₁), 22 KH₂PO₄, 153 NH₄Cl, 34 CaCl₂, 640 NaHCO₃, 30 CuSO₄·5H₂O, 20 MgSO₄·7H₂O, 20 FeSO₄·7H₂O, 58 CoCl₂·6H₂O, 150 H₃BO₄, 20 EDTA (C₁₀H₁₆N₂O₈), and 30 KI,

2.2. Characterization of engineered ZnO NPs

The ZnO NPs (50% w/w) were purchased from Sigma-Aldrich (Singapore) and stock solutions (100 mg/L) were prepared. To avoid agglomeration or aggregation, ultrasonic treatment (30°C, 100 W, 40 kHz) for 30 min and shaking for 2 h was carried out to increase their dispersion before the use of the suspension of ZnO NPs. The morphology of the ZnO NPs was examined using transmission electron microscopy (TEM) (JEOL JEM-3010, Japan). A TEM image of the ZnO NPs (50 mg/L) used for this study is available in the Supporting Information (Figure S2a). Analysis of particle size was performed using a Zetasizer Nano Z90 (Malvern Instruments, UK) and the primary particle size of ZnO NPs was 33±8 nm (Figure S2b).

2.3. Assessment of water quality

Influent, anoxic and aerobic mixed liquor, and membrane effluent were collected twice a week from the MBRs for measurement of the conventional parameters. Chemical oxygen demand (COD), MLSS, mixed liquor volatile suspended solids (MLVSS), ammonium ($\text{NH}_4\text{-N}$) and phosphate (PO_4^{3-}) were determined in accordance with Standard Methods (APHA, 2005).

2.4. Dissolved zinc and zinc accumulation

The soluble Zn fraction in the liquid sample was determined after passing through 0.02 μm syringe filters (Whatman) (Zhao et al., 2013). 0.5 mL of the supernatant was added to 4.5 mL of Milli-Q water (containing 2% HNO_3) (Zheng et al., 2011). Acid digestion of the sample wastewater was conducted in accordance to Standard Method (APHA, 1999) (Zheng et al., 2011). Zn in the biosolids was determined after acid digestion of sludge samples using an Anton Paar Microwave Reaction System (Multiwave 3000, Alpha Analytical USA) following the USEPA 3051 method. The samples after microwave treatment were filtered using a 0.22 μm nylon membrane (Whatman) after centrifugation. The supernatants were topped off to 10 mL for further concentration measurement. The concentrations of Zn for the ZnO NPs in both liquid and biosolids were detected using MP-AES (4100, Agilent Technologies) with a detection limit of 1 $\mu\text{g/L}$.

2.5. Transmission and scanning electron microscopy

ZnO NPs samples were examined using transmission electron microscopy (TEM) to gain the information on the impact of ZnO NPs on the particle size and morphology. TEM grids were prepared by placing a drop of suspension (mixed liquor) on a holey carbon grid and drawing the suspension through the TEM grid using a paper tissue. The TEM grids were washed afterwards in a drop of distilled water to remove the dissolved compounds (Kaegi et al., 2013). TEM images were obtained on a JEOL 3010 instrument at 200 kV.

SEM images were used to assess the changes in morphology of the activated sludge induced by the aggregated ZnO NPs (Zheng et al., 2011). To prepare the SEM image, mixed liquor was first washed three times with 0.1 M phosphate buffer solution (PBS) (pH 7.7) and fixed in 0.1 M phosphate buffer (7.4) containing 2.5% glutaraldehyde at 4 °C for 4 h. After rinsing twice with 0.1 M BPS (pH 7.4), the pellets were dehydrated in ethanol serials (50%, 70%, 80% 90% and 100%, 15 min per step), and then dried in air. The samples were then coated with platinum and examined using a Hitachi S-4800 ultra-high resolution field emission SEM instrument at 15 kV; energy-dispersive X-ray spectroscopy (EDS) was used to confirm the presence of Zn in the samples.

2.6. Determination of protein and polysaccharide concentration

The extraction of SMPs and bound EPS followed the procedure described by Mei et al. (2014), and classic UV-Vis methods were used. The MLSS were harvested following centrifugation at 6,000 g for 10 min; the resulting supernatant represented the SMPs. The dewatered sludge pellet was washed with 0.9% NaCl solution (w/w) twice prior to extraction; the mixed liquor was then sonicated at 20k Hz for 2 min, and centrifuged at 8,000 g for 10 min. The supernatant was regarded as loosely bound EPS (LB-EPS); the residual sludge pellet left in the centrifuge tube was suspended in a 0.9% (w/w) NaCl solution, heated at 60 °C for 1 h, and centrifuged at 11,000 g for 10 min. The supernatant was regarded as the tightly bound EPS (TB-EPS). The phenol-sulfuric acid method (Dubois et al., 1956) and the Lowry method (Lowry et al., 1951) were used to determine the concentrations of polysaccharides and proteins, respectively.

2.7. SMP molecular weight (MW) distribution

A 10 mL sample was first centrifuged at 10,000 rpm for 10 min and then filtered through a 0.22 µm PTFE syringe filter (SLFG013NK, Millipore, Millex-FG). A high performance size exclusion chromatograph (HP-SEC) (Agilent Technologies, 1260 LC system) equipped with the PL Aquagel-OH 8 µm MIXED-M column was used for the MW distribution analysis; Milli-Q water was used as the mobile phase at a flow rate of 1 mL/min. Polyethylene glycols (PEGs) and polyethylene oxide standards with

molecular weights of 500 kDa, 70 kDa, 4 kDa, 600 Da and 106 Da were used to calibrate the column. MW was calculated according to the calibration curve and a linear relationship derived between the log of MW (Da) and retention time (Rt: min) as shown in Eq. (1):

$$\text{Log (MW)} = 9.8823 - 0.6748 (\text{Rt}) \quad \text{Eq. (1)}$$

2.8. Three-dimensional fluorescence excitation emission matrix (EEM)

Three-dimensional EEM fluorescence spectra were measured in replicate using a luminescence spectrometry (Perkin Elmer LS55 Fluorescence Spectrometer), and the results were similar. The spectrometer used a high energy pulsed Xenon source for excitation, with slits at 10 nm for both excitation and emission. The EEM fluorescence spectra were obtained from 220 nm to 600 nm at 10 nm steps; for each wavelength the emission was detected from 300 nm to 550 nm at 10 nm steps, with a scan speed of 1000 nm/min. FL Winlab Version 4.00.03 (Perkin Elmer) was employed for analysis of EEM data, which were plotted as elliptically shaped contours. The X-axis represented the emission spectra from 280 nm to 550 nm, while the Y-axis indicated the excitation wavelength from 220 nm to 400 nm.

2.9. Statistical analysis

All tests were conducted in triplicate and the results are presented as mean $\pm$ standard deviation (SD) for each concentration. Tests to determine statistical differences between treatments were carried out by comparing the critical value through ANOVA one-way analysis of variance (SPSS Statistics V17.0). Comparisons were considered significantly different at $p < 0.05$.

3. Results and discussions

3.1. Effects of ZnO NPs on MBR performance

Prior to ZnO NP exposure, the COD removal efficiency in the MBR-C was $93.4 \pm 0.9\%$ (Figure 1a).

As expected, 1 mg/L ZnO-NPs displayed no significant ($p > 0.05$) changes in removal efficiency (93.1 $\pm$ 0.6%), implying that ZnO NPs at current environmentally relevant concentrations were unlikely to affect COD removal in activated sludge processes. However, 10 and 50 mg/L ZnO NPs decreased COD removal substantially to 90.1 $\pm$ 0.8% (< 0.05) and 86.3 $\pm$ 2.3% (< 0.05), respectively (Figure 1b). With NH_4^+ -N removal in the MBR-C, it was relatively constant at 86.1 $\pm$ 3.6% (Figure 1c). In the presence of 1 mg/L ZnO NPs, the comparatively stable NH_4^+ -N removal of 85.0 $\pm$ 2.5% indicated the ZnO NP concentration was within the tolerance limit of activated sludge and the metabolism of microorganisms was maintained in a steady state. However, with 10 and 50 mg L ZnO NPs, removal decreased significantly to 78.2 $\pm$ 5.8% ($p < 0.05$) and 65.2 $\pm$ 5.7% ($p < 0.05$), respectively (Figure 1d). In terms of PO_4^{3-} -P removal, the overall removal efficiency in MBR-C remained constant at 46.8 $\pm$ 5.1% (Figure 1e) without ZnO NPs. However, 1 and 10 mg/L ZnO NPs caused small but significant ($p < 0.05$) decreases in PO_4^{3-} -P removal efficiencies of 41.7 $\pm$ 2.5% ($p < 0.05$) and 45.3 $\pm$ 4.3% ($p > 0.05$), respectively, while when exposed to 50 mg/L the removal efficiencies increased to 58.5 $\pm$ 6.2% ($p < 0.05$), respectively (Figure 1f).

Our finding is consistent with Tan et al. (2015) who reported that a significant ($p < 0.05$) decreases in NH_4^+ -N removal (from 99.3% to 97.2%) and in COD removal (from 98.5% to 96.3%) was observed when ZnO NPs concentration was increased to 10 mg/L, while 1.0 mg/L ZnO NPs did not significantly impact NH_4^+ -N and COD removal. Similar results were observed by Hou et al. (2013) who revealed that continuous input of ZnO NPs at concentrations up to 5 mg/L did not reduce COD removal, but reduced NH_4^+ -N removal in simulated wastewater treatment processes. Inhibition of COD and NH_4^+ -N removal at a high ZnO NP concentration indicates that ZnO NPs at high concentrations may adversely affect activated sludge processes. The authors also indicated that the respiration of nitrifying microorganisms was inhibited by 14.2% and 23.7% at dosing concentration of 1 and 5 mg/L, respectively. The inhibition was mainly due to the toxicity of ZnO NPs imposed on activated sludge, which is usually a combination of both nanoparticles and dissolved Zn^{2+} from particle dissolution, or their aggregates to the organisms (Zheng et al., 2011; Tan et al., 2015). Previous studies suggested that the toxicity of ZnO NPs may arise from the released Zn^{2+} due to particle dissolution (Puay et al., 2015; Liu et al., 2011). Zheng et al. (2011) showed that the effect of 0.12 mg/L of Zn^{2+} on activated sludge activities was not detectable, while higher concentrations (>0.45 mg/L) of Zn^{2+} were inhibitory to biological nitrogen and phosphorus removal. In

this study, the measure concentration of Zn^{2+} in the effluent was 0.42-0.90 mg/L and 1.65-2.79 mg/L in the presence of 10 and 50 mg/L ZnO NP, respectively (Figure 3a), implying that is likely that the adverse and toxic effects of ZnO NPs may be attribute to the release of Zn^{2+} .

3.2. Effects of ZnO NPs on sludge characteristics

A steady decrease in biomass concentration occurred after the exposure to ZnO NPs, although the MLVSS/MLSS concentrations remained relatively constant in MBR-C (Figure S3). Upon addition of 1 mg/L ZnO-NPs, the average MLSS and MLVSS concentrations decreased to 2.7 ± 0.3 g/L ($p < 0.05$) and 2.5 ± 0.2 g/L ($p < 0.05$), respectively. A remarkable decrease in MLSS was further observed to 13.7% and 29.9% after continuous exposure to 10 and 50 mg/L ZnO-NPs, respectively, resulting in a deterioration of flocculation. Good flocculating ability of sludge flocs can aggregate microbial cells and particles in the wastewater, leading to superior settleability and low turbidity in effluent (Chen et al., 2014a; Hou et al., 2015). This finding corroborates a previous study by Tan et al. (2015) who found that activated sludge exposed to 10 mg/L ZnO NPs caused a more stable suspension and poorer flocculation, indicating that there was a great loss in the cell viability. Hou et al. (2015) implied that CuO NPs at high concentration of 50 mg/L may diffuse to deeper positions in the sludge flocs, thus, the protective capability of EPS/SMP to impede the access of the CuO NPs to the activated sludge become limited.

The morphological changes of the activated sludge induced by the aggregated ZnO NPs were further revealed by SEM (Figure 2a). It can be seen that the NPs at 10 and 50 mg/L formed small aggregates on the loose surface of the sludge, which may involve adherence of singles or clusters due to attractive forces or chemical binding. Elemental analysis was further employed to demonstrate the accumulation of ZnO NPs on the sludge with EDX profile (Figure S4), where the weight percentage increased in Zn as the ZnO-NPs concentration increases, from 2.56% (10 mg/L ZnO NPs) to 14.59% (50 mg/L ZnO NPs). Moreover, the inorganics Ca, Mg, Fe, Cl, Na, Cu, Pt, K, and P were detected with EDX (Figure 2b), and demonstrated that Ca (4.57%), Fe (6.81%), Cl (0.57%), Pt (4.40%) and P (3.71%) concentration in the MBR-T were higher than those in the MBR-C. Ca, Mg, Al and Fe often precipitate as carbonates and impact on fouling layer formation (Wang et al., 2014).

Engineered NPs may influence specific physicochemical properties of the activated sludge, e.g., flocculating ability, hydrophobicity, settleability, surface charge and bacterial EPS, which are important factors for ensuring the normal operation of WWTPs (Chen et al., 2014a). At low concentrations, the dissolved Zn ions from ZnO NPs could serve as bridges between the negatively charged functional groups on the surface of bacteria to help with cell aggregation. However, at higher concentrations the increased cell surface charge weakened the strength of the bridge between EPS and the cations, resulting in the deterioration of the flocculating ability of activated sludge (Chen et al., 2014a). Moreover, the toxicity exerted at higher concentrations of ZnO NPs might be another reason for the deteriorated flocculating ability. Therefore, the accumulation of ZnO-NPs may affect the performance of the MBR system, as poorer flocculation ability would result in more dispersed sludge particles.

3.3. Fate and behaviour of ZnO NPs in the MBR

A high Zn removal efficiency (> 92%) was observed in the MBR system throughout the experiment. Previous studies have indicated that the main mechanisms for NP removal from wastewater is believed to be biosorption onto biomass (Puay et al., 2015; Tan et al., 2015). The Zn accumulation in the sludge increased from 0.8-9.1 mg/g-MLSS (1 mg/L ZnO NPs) to 17.5-112 mg/g-MLSS (10 mg/L ZnO NPs), and finally to 154 -595 mg/g-MLSS at 50 mg/L (Figure S5). This finding reinforces the results obtained from previous investigations (Lombi et al., 2012; Kaegi et al., 2013) that engineered NPs had a strong affinity for the biomass rather than dispersing in the effluent. In addition, many ZnO NPs tend to aggregate in aqueous solutions containing salts and some organic constituents (Kiser et al., 2010; Ma et al., 2013). The aggregation of ZnO NPs in the nutrient solution was also observed in the present study (Figure S2a). It worth noting that the particle size distribution of 15-47 nm with a mean size of 33 ± 8 nm was observed in the wastewater utilized (Figure S2b). Taking into account 40 nm pore size of membrane used in the present study, it would be expected to remove ZnO NP completely based on size exclusion. These findings also enhance the hypothesis that the main removal pathway for ZnO NPs from wastewater is aggregation and adsorption onto activated sludge.

The Zn levels in the influent, aerobic supernatant and MBR effluent are shown in Figure 3a, and the mass balance for total Zn in the MBR reactor is shown in Figure 3b. The difference between influent and aerobic supernatant concentration could be considered similar to that of the removal efficiency of ZnO NPs in a CAS, and the remainder due to membrane rejection (Tan et al., 2015). The dissolution profile revealed that with 1 and 10 mg/L NPs, the Zn^{2+} level in the aerobic supernatant was 0.13 and 2.9 mg/L, and Zn removal by bioadsorption was around 71.4% and 62.3%, respectively, while the overall Zn removal was 93%. This finding is in good agreement with Tan (2015) who found that activated sludge adsorption/settling accounted for 65.9% of the total Zn contents in an MBR with 10 mg/L ZnO NPs. However, at high concentration of 50 mg/L, the average Zn^{2+} level in the aerobic supernatant was 6.9 mg/L and Zn removal by a bioadsorption was 82.6%, while overall Zn removal still stayed high at around 94%.

3.4. Effects of ZnO NPs on the formation and composition of SMP

3.4.1. Effects of ZnO NPs on protein and polysaccharide profile

Proteins and carbohydrates are usually found to be the primary components of SMP in the activated sludge (Kunacheva and Stuckey, 2014; Sheng et al., 2010). As expected, more SMPs were generated when exposed to ZnO NPs, and higher concentrations induced a higher generation of SMPs. At concentrations of 1, 10 and 50 mg/L, the average concentrations of SMPs were 15.9 ± 1.2 , 28.8 ± 3.1 and 43.2 ± 3.7 mg/L, respectively (Figure 4). It was shown that the SMPs increased by 12.8%, 42.4% and 51.5% after exposure to 1, 10 and 50 mg/L ZnO NPs, respectively, compared to the control (14.1 mg/L). SMP formation relies on the major processes including substrate utilization, biomass decay, and EPS hydrolysis (Fenu et al., 2010). At high concentrations, heavy metals may disturb cellular function, damage cell membrane and DNA structure, and even cause significant cell lysis, which would promote a substantial increase in SMP content (Barker and Stuckey, 1999; Han et al., 2013; Wu et al., 2015). Proteins and polysaccharides dominate the constituents of cell walls (Aquino and Stuckey, 2004), therefore, the increase of SMP under the exposure of ZnO NPs was likely result of extensive biomass decay and mass

cell rupture. This finding was consistent with the observations that drastic rises in effluent COD and $\text{NH}_4^+\text{-N}$ occurred at high concentrations of ZnO NPs.

The polysaccharide and protein contents of EPS included loosely bound-EPS (LB-EPS) and tightly bound-EPS (TB-EPS). As seen in Figure 5, the contents of EPS-protein (113.2 mg/g VSS) and EPS-polysaccharides (54.3 mg/g VSS) did not change significantly ($p > 0.05$) upon exposure to 1 mg/L ZnO NPs. However, with an increase in the dosage of the ZnO NPs to 10 mg/L, the production of EPS-protein (141.6 mg/g VSS) and EPS-polysaccharides (66.4 mg/g VSS) increased by 28.4% and 25.5%, respectively (Figure S6). At 50 mg/L ZnO NPs the concentrations of EPS-protein (167.1 mg/g VSS) and EPS-polysaccharides (78.3 mg/g VSS) increased by 42.6 % and 37.5 %, respectively. Therefore, this finding enhances the previous result that the presence of ZnO NPs induced an increased production of EPS, which plays a key role in protecting microorganisms against environmental stress (Han et al., 2013; Hou et al., 2015). On the one hand, engineered NPs might be embedded within the EPS matrix which serves as a protective barrier preventing the NPs contacting the cell surface and damaging the cell (Kiser et al., 2010). On the other hand, EPS production can be enhanced in the presence of heavy metal as they exhibited chelating properties for metal uptake (Barker and Stuckey, 1999). Typical chelating functional groups that are found in EPS/SMP, such as carboxylates, hydroxyls, and phenols, can serve as ligands with metals commonly found in wastewater (Aquino and Stuckey, 2004). However, the increase in EPS was clearly less dramatic under exposure to 50 mg/L ZnO NPs, probably due to the enhanced resistance to the toxicity after long-term exposure to ZnO NPs and the escalated hydrodynamic diameter of the NPs, since large NPs exhibit less toxicity than smaller ones.

3.4.2. Effects of ZnO NPs on molecular weight (MW) distribution of SMPs

Typical HP-SEC chromatograms of SMPs from different treatment unit in MBRs are shown in Figure 6; the SMPs exhibited a bimodal distribution of high- and low-MW compounds. Two representative peaks were identified: Peak A (583 kDa) represent high-MW (> 100 kDa) compounds, while Peak B (11 Da) indicated the the presence of low-MW organic compounds ($\text{MW} < 1$ kDa). A substantial decrease in the intensity of Peak A (583 kDa) was observed in the aerobic stage of MBR-C at first phase (1mg/L NPs)

(Figure 6a), implying that high-MW compounds might have been biodegraded into low-MW fractions due to high biological activity in the aerobic stage. However, over time a significant increase in the intensity of Peak A was observed in the aerobic stage (Figures 6b and c), implying that the increased high-MW compounds might be produced from the cell lysis that occurred during the endogenous stage (Aquino and Stuckey, 2004). In contrast, there was an increase in Peak B (11 Da) in both the aerobic stage and MBR-C effluent. This finding implies that more low-MW fractions were generated in the aerobic stage due to the enzymatic hydrolysis of large molecules into smaller ones, and these compounds could further permeate through the membrane into the MBR effluent.

In the presence of ZnO NPs, a significant increase in high-MW (583 kDa) compounds in the MBR-T was observed, compared to the MBR-C, implying that the presence of ZnO NPs enhanced the accumulation of high-MW fractions. A similar result was noted by Aquino and Stuckey (2004) who found that toxic compounds (Cr and CHCl_3) caused an increased accumulation of high-MW SMPs. Likewise, Avella et al. (2010) found that a cytostatic drug (cyclophosphamide) caused a noticeable increase in SMPs in MBR supernatants. The presence of toxic compounds tends to cause cell lysis and release intracellular high-MW SMP in response to environmental stress (Aquino and Stuckey, 2004). In addition, although many SMP produced during biological treatment can be degraded, high-MW compounds exposed to toxic environment were likely to more slowly degraded resulting an increase of SMP (Chen et al., 2014b). Furthermore, the intensity of Peak A was much lower in the MBR-T effluent compared to the aerobic stage, implying an efficient membrane rejection of high-MW compounds (Figures 6e and f). Avella et al. (2010) investigated the effect of cytostatic drugs on EPS formation in an MBR, and found that membrane rejection kept a significant fraction of the soluble macromolecules within the MBR, presumably to be biodegraded over time. In addition, when ZnO NPs increased to 50 mg/L, the increase in Peak A intensity in MBR-T was not obvious (Figures 6e and f); this inferred that microorganism acclimation to the stressful conditions had taken place. When exposed to metal toxicity, microorganisms can use several methods for self-protection, e.g., proteins can take in the metal within the cells through binding, and certain enzymes can also mitigate the toxicity of the metal by catalysing their detoxification (Bruins et al., 2000). Mirimanoff and Wilkinson (2000) found that bacteria (*Phodococcus opacus*) were able to produce small (<3 kDa) complexing ligands to regulate Zn (II) concentration in the external medium. This implied

that microorganisms were able to regulate the concentration of unwanted or harmful metals by means of efflux systems where specific biomolecules were secreted to bind with those metals.

3.4.3. Effects of ZnO NPs on the characteristics and composition of SMP

Three-dimensional excitation and emission matrix (EEM) fluorescence spectroscopy has been proven to be a useful technique to differentiate between the changes and transformation of organic matters in natural environments (Wang et al., 2009). EEM fluorescence spectra of SMPs in both MBR-C and MBR-T (10 mg/L ZnO NPs) are shown in Figure 7, and give specific information on the chemical composition of SMPs. Four main peaks can be identified (Figure 7a); the first peak was located at the excitation/emission wavelength (Ex/Em) of 222/360 nm (Peak A), while the second peak (Peak B) was at Ex/Em 275/348 nm, which were attributed to amino acid-like and tryptophan protein-like substances, respectively (Chen et al., 2003; Sheng and Yu, 2006). The other two peaks were located at Ex/Em of 242/440 nm (Peak C) and 328/420 nm (Peak D), which were associated with fulvic acid-like and polycarboxylate-type humic acid-like substances (Coble, 1996; Chen et al., 2003).

Specific parameters of the spectra such as peak location can be used for quantitative analysis. Peak locations of SMPs in the different treatment units only showed a slight difference. The location of Peak D in both the aerobic stage and MBR effluent exhibited a blue shift (10-15 nm) along the emission axes in the presence of 10 mg/L ZnO NPs, compared to the anoxic stage. This indicated a decomposition of condensed carboxylate moieties, and the break-up of large molecules into small fragments, such as a reduction in the number of aromatic rings, or conjugated bonds in a chain structure, or the conversion of a linear ring system to a non-linear system by the elimination of particular functional groups (Coble, 1996).

Peak density is another parameter identifying the changes in SMP composition. EEM analysis revealed that the intensity of Peaks A, B and C significantly reduced down the MBR treatment flowsheet, implying a decomposition of amino acid-, tryptophan protein- and fulvic acid like-substances in the anoxic-aerobic processes. This was also believed to be attributable to the greater retention of these organics by the PVDF membranes (Juang et al., 2013). Moreover, no significant change in the intensity of Peak C between the aerobic stage and the MBR effluent was observed, implying the poor removal of

fulvic acid. This finding is consistent with previous studies (Lyko et al., 2008; Wang et al., 2011) which revealed that fulvic acid was minimally rejected by membranes due to low MWs compared with carbohydrates and protein. However, the intensity of Peak D (polycarboxylate humic acid-like substances) increased in the aerobic stage and effluent.

When examining the impact of ZnO NPs on the composition of SMPs, the EEM contour maps indicated an enhancement in the peak intensity of Peaks A, B and C under exposure to 10 mg/L ZnO NPs, implying that ZnO NPs seemed to stimulate the production of amino acid-, tryptophan protein-, and fulvic acid-like substances. This finding is consistent with Wu et al. (2015) who investigated the effect of Ni (II) exposure on SMP formation, and reported an increase in tryptophan protein and amino acid in response to the effect of toxic metals using three-dimensional EEM. Similarly, Wang and Zhang (2010) reported an increase in hydrophilic protein-like substances when 50 mg/L Cr (III) was added. It has been well accepted that protein plays a significant role in microbes' adaptation to the presence of heavy metals, the mechanism of which include sequestering the metal through binding and mitigating the toxicity of metals by enzymatic detoxification (Bruins et al., 2000). This result also indicated that the enhancement of SMP functions is important in the alleviation of toxicity induced by ZnO NPs.

In order to better understand SMP differences between MBR-C and MBR-T, fluorescence regional integration (FRI) analysis was also carried out, and the distribution of the SMPs in two MBRs is illustrated in Figure 8. It can be seen that the SMPs were dominated by fluorescence in Regions I, II, V and VI, representing amino acid-, tryptophan protein-, polyaromatic-, and polycarboxylate-type substances, while the sum of Regions III and IV only accounted for less than 25%. At 1 mg/L an increase in the fluorophores in Region I (7.5-25.1%) and II (31.7-38.1%) was observed in MBR-T compared to MBR-C (6.0-20.2% for Region I and 28.5-36.7% for Region II), implying that more amino acid-like and tryptophan protein-like matter were secreted in response to the ZnO NPs added. It is well known that proteins play a crucial role in the composition, properties and functions of microbial aggregates (Wang et al., 2009). The difference in protein composition should correlate well with the changes in the structure and properties of the microorganisms in response to ZnO NP exposure. In contrast, a decrease in the fluorophores in Region V (7.4-14.1%) and VI (9.4-23.9%) representing polyaromatic- and

polycarboxylate-type substances, was observed in MBR-T compared to MBR-C (8.4-15.8% for Region I and 9.5-25.0% for Region II), and similar phenomena were also observed with 50 mg/L.

4. Conclusions

Although ZnO NPs at an environmentally relevant concentration showed no measurable adverse effects on MBR performance, the long-term exposure of activated sludge to ZnO NPs at 10 and 50 mg/L caused a significant reduction in COD and nitrogen removal. SEM images also demonstrated significant deterioration in sludge settleability with exposure to ZnO NPs at 10 and 50 mg/L. All the methods used to study SMPs (HPLC-SEC, FEEM and UV-Vis) clearly showed that ZnO NPs induced an increase in SMP production. HPLC-SEC indicated that ZnO NPs substantially enhanced high-MW SMP production. FEEM analysis revealed that SMPs were dominated by amino acid-, tryptophan protein-, polyaromatic-, and polycarboxylate-type substances, while ZnO NPs seemed to stimulate the production of amino acid-, tryptophan protein-, and fulvic acid-like substances.

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- 635

Figure

Figure 1. Pollutant removal performance in MBRs. a) COD in MBR-C; (b) COD in MBR-T; c) $\text{NH}_4^+\text{-N}$ in MBR-C; d) $\text{NH}_4^+\text{-N}$ in MBR-T; e) $\text{PO}_4^{3-}\text{-P}$ in MBR-C; and f) $\text{PO}_4^{3-}\text{-P}$ in MBR-T. "C" represents control; and "T" represents treatment.

a)

b)

Figure 2. a) SEM Images of activated sludge in A) MBR-C at 10 mg/L ZnO-NP concentration; B) MBR-T at 10 mg/L ZnO-NP concentration; C) MBR-C at 50 mg/L ZnO-NP concentration; and, D) MBR-T at 50 mg/L ZnO-NP concentration; and b) EDX analysis for inorganic weight distribution in the two MBRs.

a)

b)

Figure 3.a) Zn concentrations in aerobic supernatant and MBR effluent; and b) Total Zn mass balance in the MBR.

Figure 4. Comparison of SMP concentrations in MBR-C and MBR-T. (a) Protein concentration; and (b) Polysaccharide concentration. “C” represents control; and “T” represents treatment.

Figure 5. Comparison of EPS concentrations in MBR-C and MBR-T under exposure to a) 1 mg/L of ZnO NPs; and b) 50 mg/L of ZnO NPs. “C” represents control system; and “T” represents treatment system.

Figure 7. Comparison of FEEM contour maps of SMPs in MBR-C and MBR-T under addition of 1mg/L ZnO NPs at a concentration of 10 mg/L; a) anoxic MBR-C; b) anoxic MBR-T; c) aerobic MBR-C; d) aerobic MBR-T; e) effluent MBR-C; and f) effluent MBR-T. “C” represents control; and “T” represents treatment.

Figure 8. FRI distribution in MBR-C and MBR-T. "C" represents control ; and "T" represents treatment.