Focus Issue Paper

Long-term exposure of bacterial and protozoan communities to TiO$_2$ nanoparticles in an aerobic-sequencing batch reactor

Chitpisud Supha, Yuphada Boonto, Manee Jindakaraked, Jirapat Ananpattarachai and Puangrat Kajitvichyanukul

Center of Excellence on Environmental Research and Innovation, Faculty of Engineering, Naresuan University, Phitsanulok, 65000, Thailand

E-mail: puangrat@nu.ac.th

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Abstract
Titanium dioxide (TiO$_2$) nanopowders at different concentrations (0–50 mg L$^{-1}$) were injected into an aerobic-sequencing batch reactor (SBR) to investigate the effects of long-term exposure to nanoparticles on bacterial and protozoan communities. The detection of nanoparticles in the bioflocs was analyzed by scanning electron microscopy, transmission electron microscopy, and energy-dispersive x-ray spectroscopy. The SBR wastewater experiments were conducted under the influence of ultraviolet light with photocatalytic TiO$_2$. The intrusion of TiO$_2$ nanoparticles was found both on the surface and inside of the bioflocs. The change of microbial population in terms of mixed liquor-suspended solids and the sludge volume index was monitored. The TiO$_2$ nanoparticles tentatively exerted an adverse effect on the microbial population, causing the reduction of microorganisms (both bacteria and protozoa) in the SBR. The respiration inhibition rate of the bacteria was increased, and the viability of the microbial population was reduced at the high concentration (50 mg L$^{-1}$) of TiO$_2$. The decreasing number of protozoa in the presence of TiO$_2$ nanoparticles during 20 days of treatment with 0.5 and 1.0 mg L$^{-1}$ TiO$_2$ is clearly demonstrated. The measured chemical oxygen demand (COD) in the effluent tends to increase with a long-term operation. The increase of COD in the system suggests a decrease in the efficiency of the wastewater treatment plant. However, the SBR can effectively remove the TiO$_2$ nanoparticles (up to 50 mg L$^{-1}$) from the effluent.

Keywords: TiO$_2$, antibacterial effects, wastewater, nanoparticles, microbial, inhibition

1. Introduction
Nanotechnology is becoming an attractive discipline that draws many scientists, researchers, and engineers to explore, investigate, and create many innovations. Nanoparticles include metal oxide nanopowders that are mainly titanium dioxide (TiO$_2$), zinc oxide (ZnO), silica (SiO$_2$), alumina (Al$_2$O$_3$), and iron oxides (Fe$_3$O$_4$, Fe$_2$O$_3$). These nanomaterials have some superior physicochemical properties than the bulk materials due to their nanoscale size. This small size is critical for the enhanced physical phenomena leading to different properties in chemical and biological reactions. As the advanced exploration of nanotechnology continues, a great
number of consumer products containing nanoparticles have reached the markets. The nanoparticles from sunscreen, toothpaste, detergents, and other products are finally entering sewage systems. These nanoparticles exhibit antibacterial properties [1–3], disrupt microbial activities in activated sludge, and affect the efficiency of wastewater treatment plants [4, 5]. Among the many nanoparticles, titanium dioxide (TiO₂) is one of the most widely used nanomaterials and is present in many personal products. The TiO₂ nanoparticles may exert a negative impact on aquatic ecosystems, which are related to human health [6, 7]. When the TiO₂ nanoparticles enter wastewater treatment plants, many adverse effects may occur. The physicochemical stability and biological stability of the activated sludge bioflocs are possibly threatened by exposure to these nanoparticles. TiO₂ nanoparticles have been reported to show toxicity towards bacteria [8] under ultraviolet (UV) irradiation [9, 10] due to the generation of reactive oxygen species (ROS) [11, 12]. These ROS can disrupt cellular membranes, causing damage to bacterial cells [10, 13]. In addition, the nanoscale size may contribute to the toxicity of TiO₂ nanoparticles, as well as the inhibitory effects of TiO₂ nanoparticles that can be observed in the absence of light [8].

The likely concentration of TiO₂ nanoparticles in the effluent of wastewater treatment is reported to be around 0.01–0.2 mg L⁻¹ [4, 14]. Kiser et al also showed that 96% of the TiO₂ was possibly removed from the wastewater treatment plant when the TiO₂ concentration was below 2 mg L⁻¹ [4]. Therefore, the TiO₂ concentration in wastewater would be around 0.1–2.0 mg L⁻¹. Although many previous works have reported the effect of TiO₂ nanoparticles on the bacteria, more research studies are needed to better understand the impact of these nanoparticles on other microbial populations and the stability of bioflocs in the system. In addition, the changes in the microbial population in the system and the stability of bioflocs that tentatively exert an effect on the treatability performance of wastewater treatment plants should be investigated.

In the present investigation, the effect of TiO₂ nanoparticles on biological wastewater treatment was examined in activated sludge. The low concentrations of TiO₂ at 0.05 and 1.0 mg L⁻¹ were selected to represent the likely concentration of the real wastewater to be treated. The concentrations of TiO₂ at 10, 30, and 50 mg L⁻¹ were also investigated to demonstrate the impact of high loading rates of nanoparticles on water treatment plants. The changes in the microbial community, the biofloc, and the treatability performance of the wastewater treatment plant were the principal investigated topics. The COD of the effluent was measured to indicate the treatability performance of the investigated aerobic-sequencing batch reactor (SBR). This parameter is the indirect measurement of the amount of pollution that cannot be oxidized biologically in the wastewater. All results were incorporated to demonstrate the impact of TiO₂ nanoparticles on the microbial community, as well as its effect on the effluent that indicated the performance of the wastewater treatment plant.

2. Materials and methods

2.1. Materials

Titanium tetraisopropoxide (TTiP), C₆H₁₂O₆, NH₂HCO₃ and KH₂PO₄ were purchased from Aldrich Chemicals. Ethanol (EtOH), HNO₃, H₂SO₄, NaOH, MgSO₄•7H₂O, CaCl₂•2H₂O, MnCl₂•4H₂O, ZnSO₄•7H₂O, CuCl₂•2H₂O, Na₂MoO₄•2H₂O, FeSO₄•7H₂O, and KI were obtained from Merck Chemicals. All of the reagents were of analytical grade. Ultrapure water from Millipore, Billerica, was used for the wastewater experiment, and 18 MΩ deionized water (H₂O) was used to prepare the solutions.

2.2. TiO₂ synthesis and suspension

A modified sol–gel method was used to synthesize TiO₂ with a molar ratio of 1:20:1:1 for TTiP:EtOH:HNO₃:H₂O as described by Ananpattarachai et al [15]. First, TTiP was dissolved in EtOH, and the solution was stirred for 30 min. In the second solution, EtOH was mixed with H₂O that contained HNO₃. Precipitation readily occurred after both portions were mixed. The homogeneous transparent solution was then kept for 30 min at 4 °C with stirring before undergoing the drying process. After drying at 100 °C for 90 min, the powder was collected and calcined at 500 °C in an electric furnace. The TiO₂ nanoparticles were collected after they were cooled to room temperature. The TiO₂ nanoparticles obtained from this procedure were previously reported for photocatalytic degradation of 2-chlorophenol [15].

The particle size distributions and the hydrodynamic radii of the nano-TiO₂ particles were determined by dynamic light scattering (DLS) at a 90-degree angle by using a Zetasizer Nano series instrument (Malvern, UK). The concentrations of Ti in the bioflocs and effluent were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) [16]. The TiO₂ extraction method has been described previously [17]. For the wastewater experiment, a stock solution of 1.0 g L⁻¹ TiO₂ was prepared by dispersing 0.1 g of TiO₂ nanoparticles in ultrapure water with sonication for 30 min (50 W L⁻¹ at 40 kHz). The test solutions of TiO₂ nanoparticles were prepared immediately prior to use by diluting this stock solution with synthetic wastewater.

2.3. Activated sludge wastewater treatment plant

The synthetic wastewater contained 400.0 mg L⁻¹ of C₆H₁₂O₆, 40.0 mg L⁻¹ of NH₂HCO₃, and 8.0 mg L⁻¹ of KH₂PO₄. It also contained 70.0 mg L⁻¹ MgSO₄•7H₂O, 20.0 mg L⁻¹ CaCl₂•2H₂O, 17.5 mg L⁻¹ FeSO₄•7H₂O, 0.15 mg L⁻¹ MnCl₂•4H₂O, 0.15 mg L⁻¹ ZnSO₄•7H₂O, 0.07 mg L⁻¹ CuCl₂•2H₂O, 0.03 mg L⁻¹ Na₂MoO₄•2H₂O, and 0.03 mg L⁻¹ KI. The experiments were conducted in six lab-scale aerobic SBRs (five parallel samples were spiked with different concentrations of TiO₂ nanoparticles, and one reactor was used as the control). A 10-watt UV lamp (TUV T8, Phillips) was used as the light source for each reactor. The SBR reactors were made of glass with a working volume of 10 L. The UV lamp was placed on top of the reactor without
any baffle between the lamp and the wastewater. Activated sludge from a domestic wastewater treatment plant (Phitsanulok, Thailand) was used as the inoculum. The SBRs were operated with a cycle time of 24.0 h at room temperature. Before being doped with TiO2 nanoparticles, the six SBRs were in stable operation for one month and were monitored for the related parameters. The TiO2-containing reactors were run for another month to achieve a quasi-steady state. The SBR was operated with a food-to-mass ratio of 0.25. The mixed liquor- suspended solids (MLSS), sludge volume index (SVI), influent COD, and effluent COD were measured in every cycle following the standard methods [18]. The residual Ti in biofloccs and effluent was also analyzed routinely by atomic absorption spectroscopy (Varian, Model AA220Z) as described in the standard methods [18].

2.4. Wastewater and sludge characteristics

Wastewater and sludge characteristics were analyzed in duplicates following the established standard methods (APHA 1999). For bioflocc morphology analysis by scanning electron microscopy (SEM) and energy-dispersive x-ray spectroscopy (EDS), bioflocs were sampled from the SBR. They were washed with phosphate buffer solution (PBS) and soaked overnight in 2.5 wt% glutaraldehyde. The treated sample was washed again with PBS and dehydrated with an ethanol gradient (25, 50, 75, 90 and 99%) [18]. The samples were resuspended in 99% ethanol before dropped onto the SEM specimen mount holder and dried in a desiccator. The dried samples were coated with platinum for SEM analysis using a JOEL JSM-5310 microscope operated at 15 kV.

For transmission electron microscopy (TEM) analysis, samples were obtained from the sludge with 1.0 mg L\(^{-1}\) of TiO2 concentration. The sample was fixed in 2% glutaraldehyde and 0.05 M sodium cacodylate buffer (pH 7.2) for 2 h and washed three times with 0.05 M sodium cacodylate buffer. The samples were dried in a desiccator before analysis using a Hitachi H-8100 TEM operated at 200 kV.

2.5. Microbial community analysis

The protozoan community was monitored as described previously [19]. A 1.0 mL aliquot of sample was injected into a culture tube (16 by 150 mm). Two drops of brilliant green dye (2 g of brilliant green dye and 2 mL of glacial acetic acid diluted to 100 mL with distilled water) were added, and the contents were mixed and allowed to stand for at least 4 h. Then, 9 mL of 30% glycerol solution was added, resulting in a 1:20 dilution of the original protozoa contents. The diluted sample was pipetted into a Sedgewick–Rafter counting chamber. Protozoa in the samples were counted at a magnification of ×100 with a counting grid 0.5 mm\(^2\). By using a calibrated microscope stage, 50 grids, evenly spaced over the entire chamber surface, were counted. The chamber was then rotated by 180 degrees, a second 50-grid count was made, and these two counts were averaged [19].

The bacterial enumeration was performed by the drop plate method [20] for heterogeneous plate count. The samples (100 μL) were taken at time intervals during wastewater treatment. A series of 10-fold dilutions of the samples were performed. Ten μL of each dilution was plated on R2A agar (Voigt Global Distribution, Inc., Kansas, USA) in triplicate. Plates were incubated at 31 °C for 24 h and held at room temperature for another seven days. Counting was performed after seven days for the total number of bacteria. The lower detection limit was 10\(^2\) CFU/mL (CFU = colony-forming unit). All assays were expressed as mean ± standard deviation. An analysis of variance (ANOVA) was used to test the significance of the results, and \(p < 0.05\) was considered to be statistically significant.

The bacterial activity was assessed by the oxygen uptake rate (OUR) measurements. Aliquots (60 mL) of the mixed liquor were taken from the aeration zone for the OUR measurement by extant respirometry, following the procedure described previously [21]. The OUR was also used to estimate the inhibition rate using the following equation:

\[
\text{inhibition rate \%} = \frac{(\text{OUR of control} – \text{OUR of dosed reactor}) \times 100}{\text{OUR of control}}
\]

Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) fingerprinting was performed to examine the bacterial communities. For PCR-DGGE analysis, 10 mL of mixed liquor was centrifuged for 5 min at 1000 g, and the supernatant was decanted, frozen and stored at −20 °C. The DNA extraction was performed with a QIAamp DNA Mini Kit (Qiagen, Valencia, California, USA) to obtain the fingerprint patterns and the band similarities. To minimize variations in DNA extraction, templates used for PCR amplification were prepared by mixing the DNA extracted in triplicate for each sample. The resultant image was analyzed using the Quantity One 4.6.2 program (Bio-Rad, USA).

3. Results and discussion

3.1. Characterization of TiO\(_2\) nanoparticles and ROS generation

Figures 1(a) and (b) show SEM and TEM images of TiO\(_2\) nanoparticles. From TEM analysis, TiO\(_2\) nanoparticles (with 97% purity) have a crystal size of 10–20 nm and consist of 66% anatase and 34% rutile phases. The average hydrodynamic diameter of the particles from DLS measurement (in ultrapure water medium) increased with the concentration of TiO\(_2\) nanoparticles; it was 85 ± 9 nm, 125 ± 18 nm, 525 ± 21 nm, 755 ± 28 nm and 1038 ± 23 nm for TiO\(_2\) concentrations of 0.05 mg L\(^{-1}\), 1 mg L\(^{-1}\), 10 mg L\(^{-1}\), 30 mg L\(^{-1}\) and 50 mg L\(^{-1}\), respectively.

In this present work, the TiO\(_2\) nanoparticles were loaded to the wastewater, and the treatment plant was irradiated by the UV light to study the impact of long-term exposure of irradiated TiO\(_2\) nanoparticles on bacterial and protozoan communities. These investigated nanoparticles have been proved to possess photocatalytic properties capable of
degradation of 2-chlorophenol with 80 and 14% removal efficiency under UV and visible light, respectively [15]. The impact of UV light without TiO$_2$ nanoparticles on the microorganisms in the wastewater treatment plant was also conducted as the control experiment. As reported previously in several studies [11, 12, 22], light absorption by the photocatalytic TiO$_2$ can generate ROS, including hydroxyl radicals (OH$^-$), superoxide radicals (O$_2$$^-$), and hydrogen peroxide (H$_2$O$_2$). These radicals are considered to be the active components promoting the bactericidal effect [11, 12]. Their ability to disrupt cellular membranes and damage bacterial cells has been reported [10, 13]. Thus, the schematic scenarios for the treatment plants with and without TiO$_2$ (or control experiment) are shown in figure 2. The impacts of the TiO$_2$ nanoparticles under the UV light on the bioflocs and change of microorganisms are discussed in the following section.

3.2. Sorption of TiO$_2$ by bioflocs and change of mixed liquor-suspended solids in the SBR

The time profile of Ti adsorption on bioflocs of activated sludge is shown in figure 3(a). The term $q_t$ represents the maximum sorption capacity of Ti on bioflocs in the unit of mg of Ti/g of MLSS. At a lower initial TiO$_2$ concentration at 0.05 mg L$^{-1}$, the adsorption of Ti on the activated sludge ($q_t$) reached equilibrium sooner than the higher initial TiO$_2$ concentration at 1.0 mg L$^{-1}$. This behavior may due to the relative abundance of sorption sites available. The maximum sorption capacity was 0.382 and 0.834 mg g$^{-1}$ for 0.05 and 1.0 mg L$^{-1}$ TiO$_2$ concentration, respectively. The sorption of TiO$_2$ by bioflocs may be the major mechanism that causes the agglomeration of TiO$_2$ nanoparticles in the bioflocs and results in the decrease in microorganisms in the treatment plant. To elucidate the impact of nanoparticles on the quantity of microorganisms in the system, the MLSS were assessed. This testing measures the concentration of suspended solids during the aeration stage of the activated sludge process. It consists mostly of microorganisms and non-biodegradable suspended matter. The SVI, which is the tendency indicator of activated sludge solids to become concentrated during the sedimentation or thickening process, was also determined. Change of MLSS (or SVI) is an important parameter indicating the performance of the wastewater treatment plant. The MLSS changes that occurred in the investigated SBR with and without TiO$_2$ are shown in figure 3(b). It was found that, without TiO$_2$, MLSS was stable at 3400–3500 mg L$^{-1}$. However, with the TiO$_2$ addition, the MLSS decreased sharply. The concentrations of MLSS with 1, 10, 30, and 50 mg L$^{-1}$ for 12 h in SBR were 3143, 3010, 2945, and 2796 mg L$^{-1}$ on average. Figure 3(c) shows the change of SVI with time for SBR with and without TiO$_2$. The SVI for the SBR without TiO$_2$ nanoparticles was steady at 200 mL g$^{-1}$. With TiO$_2$ nanoparticles, the average SVI values were 165 and 170 mL g$^{-1}$ for 30 and 50 mL g$^{-1}$ of TiO$_2$ concentration, respectively, during 12 days of activated sludge activity in the SBR system. Results from changes of MLSS and SVI shows that TiO$_2$ nanoparticles tentatively exerted an adverse effect.
on the microbial population, causing the reduction of MLSS in the system. In addition, the decrease of the SVI value indicates the higher agglomerate of sludge or bioflocs in the system. Both changes of MLSS and SVI were possibly related to the change in the microbial community in the SBR system.

3.3. SEM and TEM of bioflocs with and without TiO2 nanoparticles

Analytical SEM was employed for imaging the detail of the surface of the sludge or bioflocs with and without the TiO2 nanoparticles as shown in figures 4(a)–(c). The surface morphology of the bioflocs without TiO2 nanoparticles was relatively smooth compared to other bioflocs in the presence of TiO2. Morphology of bioflocs with TiO2 revealed that the TiO2 nanoparticles were found both on the surface and inside the bioflocs. Energy-dispersive spectroscopy (EDS) data reveal the presence of Ti in the bioflocs. Apparently, with the increasing concentration of TiO2 in the SBR system, the Ti contents in bioflocs were also increased. The SEM images and EDS data clearly show that the activated sludge bioflocs were surrounded by the TiO2 nanoparticles. The TEM analysis with electron diffraction was also performed to detect the nanoparticles in the bioflocs with 1.0 mg L⁻¹ TiO2 concentration as shown in figure 5. Figure 5(a) shows the optical microscopy image of the dead protozoa on the TEM copper grid. The nanoparticles were detected in different locations inside the cell by TEM analysis (b–d). The electron diffraction patterns of TiO2 from the red circle area in TEM analysis also demonstrated the existence of agglomerates of small nanoparticles in the cell. One previous study [23] reported that a high concentration of TiO2 can cause greater interruption to the cell than with low concentration. Our data demonstrated that the TiO2 nanoparticles interact with wastewater bioflocs and may be taken up by the protozoa. This result is possibly due to the bioflocs having accumulated much of the TiO2 particles presented in the influent over a long period by the activated sludge flocs.

3.4. Effect of TiO2 on changes in the bacterial community

The impact of TiO2 on the bacterial community was studied by the culturability of heterotrophic bacteria in activated sludge. The heterotrophic plate count after 24 h (figure 6(a)) indicated the fast-growing heterotrophic bacteria in activated sludge samples, and that after 7 days plating (figure 6(b)) indicated the total number of viable bacteria in the samples. Initially, the fast-growing and total bacteria in sludge samples were around 4.44 × 10⁵ and 8.45 × 10⁵ CFU mL⁻¹, respectively. During the 25 days of wastewater treatment in the control experiment without TiO2 nanoparticles, the number of the fast-growing heterotrophic bacteria was stabilized at around 3.4 × 10⁵ CFU mL⁻¹. The number of bacteria in sludge samples with 50 mg L⁻¹ TiO2 nanoparticles was similar during 25 days of treatment, revealing that the nanoparticles had no significant impact on the heterotrophic cell culturability of the sludge samples. However, the effect of TiO2 nanoparticles on the total number of viable bacteria after seven days plating was clearly seen (figure 5(b)) with significant statistical differences. The 50 mg L⁻¹ TiO2 caused a loss of about 1.5log units in the total number of heterotrophic bacteria. The heterotrophic plate count started to decrease from around two days of the wastewater treatment. It was reported that this contact time may be associated with the time taken for nanoparticles to diffuse into the activated sludge flocs. Tiede et al [24] and Sun et al [25] reported that 6–8 h contact time was needed for Ag nanoparticles to partition into the sewage sludge. In this TiO2 study, two days contact time was required for the partitioning of TiO2 nanoparticles into the sludge.

A PCR-DGGE analysis was also used in this work to obtain the information on the bacterial community. Although PCR-DGGE is a culture-independent molecular fingerprinting technique to observe microbial diversity and community
Figure 4. SEM images (left) and EDS spectra of bioflocs (right, x-scale is in keV). (a) without TiO$_2$, (b) with 30 mg L$^{-1}$ of TiO$_2$ and (c) with 50 mg L$^{-1}$ of TiO$_2$. 
Figure 5. Optical microscopy image of protozoa on the TEM copper grid (a) and TEM images with electron diffraction patterns of TiO$_2$ nanoparticles from different locations inside cell (b)–(d).
bacteria in activated sludge samples. Table 1. Respiration rate (OUR) and inhibition rate of bioflocs in activated sludge (using ANOVA and statistical significant at p<0.05).

| Concentration of TiO2 (mg L⁻¹) | Respiration rate (mg O2 L⁻¹h⁻¹) | Inhibition rate (%) | p value |
|--------------------------------|---------------------------------|--------------------|---------|
| Control                        | 6.8 ± 0.6                       | 0.0 ± 0.5          | 0.512   |
| 0.05                           | 5.6 ± 0.2                       | 18.5 ± 0.7         | 0.534   |
| 1                              | 4.0 ± 0.5                       | 41.2 ± 0.7         | 0.510   |
| 10                             | 3.3 ± 0.5                       | 51.9 ± 0.4         | 0.632   |
| 30                             | 2.7 ± 0.4                       | 60.3 ± 0.2         | 0.587   |
| 50                             | 2.0 ± 0.2                       | 70.5 ± 0.3         | 0.531   |

Figure 6. Effect of 50 mg L⁻¹ TiO2 on heterotrophic activated sludge bacteria in activated sludge samples.

3.5. Effect of TiO2 on changes in the protozoa community

Apart from bacteria, protozoa are the next most important group of microbes in the wastewater. In this work, we also investigated the protozoa community. Protozoa also play a significant role in the efficient functioning of wastewater treatment plants. They are efficient at gathering microbes as food, and this improves the wastewater treatment performance, resulting in a lower organic load in the effluent of the treated wastewater. Currently, whereas many studies examined the bacterial community, relatively few have monitored the protozoan community. In this work, changes of major protozoa species found in the wastewater treatment plant were monitored and counted according to the previous study [18]. Changes in crawling ciliates, rotifer, free-swimming ciliates, and stalk ciliates are shown in figures 7–10, respectively. The statistical differences in the concentration of each protozoan with the application of the different amount of TiO2 concentration were observed.

During the earlier stage of our wastewater treatment, the dominant protozoa species were crawling ciliates (figure 7) and rotifers (figure 8). The crawling ciliates increased during the first five days before then decreasing with time. The pattern of changes in the population of these species during the wastewater treatment was similar both with and without TiO2 nanoparticles. However, it is obvious that the number of
crawling ciliates was reduced with increasing TiO\textsubscript{2} concentration.

For rotifers, this group was frequently found for the whole period of treating wastewater. Without TiO\textsubscript{2} in the system, this species was detected in the range of 600–1200 cell/mL. However, in the presence of 0.5 mg L\textsuperscript{−1} of TiO\textsubscript{2}, the rotifer count was in the range of 200–900 cell mL\textsuperscript{−1}. The rotifer count was at the relatively low level in the range of 30–400 cell/mL with 1.0 mg L\textsuperscript{−1} of TiO\textsubscript{2}. The rotifer numbers reduced sharply with a high concentration of TiO\textsubscript{2} in the wastewater treatment plant. TiO\textsubscript{2} nanoparticles also exerted an adverse effect on rotifer numbers as seen in figure 8. In the presence of 0.05 and 1.0 mg L\textsuperscript{−1} TiO\textsubscript{2}, the number of rotifers was drastically reduced, with the residual rotifer count less than 50% of that in the control (without TiO\textsubscript{2}) wastewater treatment plant. This adverse effect on the protozoan community is also shown in the monitoring of stalk ciliates (figure 10). With the 0.05 and 1.0 mg L\textsuperscript{−1} TiO\textsubscript{2} in the wastewater, the stalk ciliates almost disappeared from the system. In overall results, it can be clearly seen that TiO\textsubscript{2} nanoparticles had an adverse impact on the microbial community. Numbers of all the investigated species of protozoa were reduced in the presence of TiO\textsubscript{2}. The higher the concentration of TiO\textsubscript{2}, the more adverse effect on the protozoan community was evident. Presumably, the TiO\textsubscript{2} nanoparticles were readily ingested by the protozoa, as some previous work showed that there were a number of small particles inside the protozoa body [33]. The ciliated protozoa (crawling, free-swimming, and stalking ciliates) can ingest the nanoparticles via a cytostome. After capture, the nanoparticles may be contained in a spherical bubble or phagosome (food vacuole). It was reported that during the maturation steps of the phagosome, the hydrolytic enzymes participate in the phagolysosomal degradation of ingested particles [34]. The enzymes break down food into suitable units for metabolism. As the pH in the lumen of food vacuoles becomes acidic after vacuole formation [35], a metal oxide particle can undergo solution under these conditions. Mortimer et al [33] reported that T. thermophila, one type of ciliate, is capable of functioning after ingesting a large quantity of CuO nanoparticles. For rotifers, the disc cilia can create water currents to move food and particles into the mouth. The nanoparticles may pass down the esophagus into the mastax and the large brownish stomach where food is digested, and the indigestible remains pass into the short intestine [36]. The toxicity of TiO\textsubscript{2} nanoparticles on these protozoa may occur from the generation of ROS in the same manner as with bacterial toxicity. The decreasing number of protozoa in the presence of TiO\textsubscript{2} nanoparticles is clearly demonstrated in our work.

3.6. Treatability performance of SBR from COD and TiO\textsubscript{2} removal

The COD removal efficiency was determined from the effluent of the SBR with and without TiO\textsubscript{2} as shown in figure 11. As the TiO\textsubscript{2} nanoparticles exerted an adverse effect on the microbial community, the decreasing of treatment percentage of the wastewater treatment plant, as measured by...
COD removal, was expected. The COD in the influent was 70 mg L\(^{-1}\). The SBR without TiO\(_2\) addition can eliminate the pollutant efficiently with the residual COD as of 5 mg/L in the effluent. From table 2, the SBR with 0.05, 1 and 10 mg L\(^{-1}\) TiO\(_2\) provided an effluent with COD concentrations of 13, 15 and 23 mg L\(^{-1}\) after 30 days treatment duration, respectively. There is no statistical difference in the COD removal efficiency of the SBR with a TiO\(_2\) concentration less than 1 mg L\(^{-1}\). However, the impact of TiO\(_2\) loading is clearly seen when TiO\(_2\) concentration was greater than 10 mg L\(^{-1}\). The lowest percentage of COD removal (48.9%) was observed when the TiO\(_2\) concentration was the highest (50 mg L\(^{-1}\)). These results indicated that the high concentration of TiO\(_2\) in the influent may cause the failure of pollutant removal in wastewater treatment plants.

The impact of TiO\(_2\) nanoparticles on the efficiency of nutrient removal was also investigated. The concentrations of NH\(_4\)^+-N ions in the effluent using SBRs with 0.05, 2, and 10 mg L\(^{-1}\) TiO\(_2\) were 11, 13, and 10.8 mg L\(^{-1}\), which are approximately at the same level of NH\(_4\)^+-N (11.5 mg L\(^{-1}\)) from the control reactor. Thus, our results suggest a negligible effect of TiO\(_2\) on nitrification in the SBR. The concentration of NO\(_3\)^--N and NO\(_2\)^--N in effluent from the SBR with 10 mg L\(^{-1}\) TiO\(_2\) was also comparable with the control reactor. In the control experiment, the effluent NO\(_3\)^--N and NO\(_2\)^--N concentrations were about 1.2 mg L\(^{-1}\) and 3.0 mg L\(^{-1}\), respectively. With the 10 mg L\(^{-1}\) TiO\(_2\), the nitrite concentration in the effluent was 1.0 mg L\(^{-1}\), while the nitrate rose to 3.2 mg L\(^{-1}\). The phosphorus concentrations in the effluent using SBRs with 10 mg L\(^{-1}\) TiO\(_2\) and the control experiment were 0.78 and 0.81 mg L\(^{-1}\), respectively. These nitrogen and phosphorus concentrations are only slightly different from the control experiment with the application of 10 mg L\(^{-1}\) of TiO\(_2\) to the SBR. Although the nutrient depletion in the present work was not clearly seen, this effect has been observed with the application of a higher concentration of TiO\(_2\) to the wastewater treatment. Zheng et al [27] found that 50 mg L\(^{-1}\) TiO\(_2\) exerted the significant decrease of total nitrogen removal efficiency after long-term exposure (70 days), whereas biological phosphorus removal was unaffected. Li et al [37] also reported that a high concentration of 200 mg L\(^{-1}\) TiO\(_2\) could cause a decreasing of nutrient concentration in the biological wastewater treatment plant. At a high concentration of TiO\(_2\) nanoparticles, abundant adsorption sites on the surface of TiO\(_2\) nanoparticles were accessible for the nutrient and trace metals. The available dissolved nutrients for the microorganisms may decrease due to the adsorption of these chemicals on the solid surface of nanoparticles. Deficiencies in nitrogen, phosphorus, or trace elements can cause the negative effects on growth and reproduction of healthy cells. The decrease of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) in the presence of 50 mg L\(^{-1}\) TiO\(_2\) was reported previously [27]. In addition, the decrease of many protozoa species was reported in this work. Thus, the nutrient depletion that occurred in the biological wastewater treatment can lead to the indirect toxicity to the microorganisms in the system.

Regarding the efficiency of TiO\(_2\) removal using the SBR, a considerable removal of TiO\(_2\) nanoparticles by the activated sludge in the SBR configuration was observed during 30 days wastewater treatment. With the initial concentration in the range of 0.05–50 mg L\(^{-1}\), more than 90% of the TiO\(_2\) was removed. However, the TiO\(_2\) removal percentage varied depending upon the initial concentrations of TiO\(_2\). For example, 95% of the 1 mg L\(^{-1}\) TiO\(_2\) nanoparticles were easily removed, while the removal percentage decreased to 90–91% with 30–50 mg L\(^{-1}\) TiO\(_2\). Our results are in a good agreement with Kiser et al [4] who showed that 96% of the TiO\(_2\) was removed from the wastewater treatment plant when the TiO\(_2\) concentration was below 2 mg L\(^{-1}\). This information suggested that the TiO\(_2\) could be trapped in the bioflocs and, consequently, only a small amount of TiO\(_2\) escapes with the effluent.

4. Conclusions

The effect of TiO\(_2\) nanoparticles on biological wastewater treatment in an SBR has been investigated. The change in the microbial community, the biofloc in term of mixed liquor suspended solids, and the sludge volume index was monitored. The treatability performance in terms of chemical oxygen demand and TiO\(_2\) removal of the wastewater treatment plant were measured and reported. Results suggested that TiO\(_2\) nanoparticles tentatively exerted an adverse effect on the microbial population, causing the reduction of MLSS and SVI in the system. This information was confirmed by the detection of TiO\(_2\) in the biofloc component by SEM and EDS. The intrusion of TiO\(_2\) nanoparticles was found both on the surface and inside of the bioflocs. The reduction of total viable bacteria measured by heterotrophic plate counts was significant with the application of 50 mg L\(^{-1}\) TiO\(_2\) after two days of wastewater treatment. The respiration inhibition rate was increased, and the viability of the microbial population was reduced with the high concentration of TiO\(_2\).

Numbers of all investigated species of protozoa were diminished in the presence of TiO\(_2\). The higher concentration of TiO\(_2\), the more adverse effect on the protozoan community was evident. The TiO\(_2\) nanoparticles were ingested by the ciliates and rotifer protozoa as demonstrated by optical
microscopy. The decreasing number of protozoa in a presence of TiO₂ nanoparticles during 20 days of treatment is clearly described. The increase of COD in the system suggests a reduction in the efficiency of the wastewater treatment plant. The performance percentage based on COD removal during treatment of wastewater was reduced with a high concentration of TiO₂. For TiO₂ removal by SBR, more than 90% of the TiO₂ with the concentration in the range of 0.05–50 mg L⁻¹ was removed. The TiO₂ could be trapped in the bioflocs, and consequently, only a small amount of TiO₂ escapes with the treated water.

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**Table 2. COD and TiO₂ removal using activated sludge.**

| Concentration of TiO₂ (mg L⁻¹) | COD in effluent (mg L⁻¹) | TiO₂ in effluent (mg L⁻¹) | % removal | % removal |
|-------------------------------|--------------------------|--------------------------|-----------|-----------|
| Control                       | 5                        | 92.9                     | 0.00      | 0.0       |
| 0.05                          | 13.2                     | 81.1                     | 0.00      | 100.0     |
| 1                             | 15.1                     | 78.4                     | 0.05      | 95.0      |
| 10                            | 23.3                     | 66.7                     | 0.70      | 93.0      |
| 30                            | 30.5                     | 56.4                     | 2.85      | 90.5      |
| 50                            | 35.8                     | 48.9                     | 4.50      | 91.0      |
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