Desalination and Water Treatment
www.deswater.com
doi: 10.5004/dwt.2019.24973

172 (2019) 15–20
December

Investigation of inhibition effects of different sol-gel based TiO_2 nanoparticles on activated sludge and toxicity to *Daphnia magna*

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Received 20 May 2019; Accepted 28 September 2019

ABSTRACT

The application of nanotechnology in textile products is progressively increasing as it is proving to be a useful tool in improving the performance of textiles. Titanium dioxide (TiO_2) nanoparticles (NPs) have been the most studied ones to develop self-cleaning and anti-bacterial nano textiles. However, the adverse effects of NPs and the risk of the possible release of NPs into the wastewater process and then in receiving waters, thus ecotoxicity studies need to be performed for the safeguard of the environment. Monitoring oxygen uptake rate (OUR) has been demonstrated to be are a liable method to show any inhibition response of the activated sludge. Hence this study was performed to investigate the ecotoxicity and inhibition effects of NPs to *Daphnia magna* and activated sludge comparatively. Activated sludge inhibition tests were conducted to define the EC_{50} levels after 30 and 180 min of reaction time for TiO_2 NPs, Ag-doped TiO_2 NPs, and Cu-doped TiO_2 NPs while *Daphnia magna* immobilization test was carried out for TiO_2 NPs prepared by Degussa P25 and sol-gel method. The Ag-doped TiO_2 NPs exhibited a toxic effect on the activated sludge 10 times higher than the TiO_2 NPs.TiO_2 NPs prepared by the sol-gel method caused 100% immobilization to *Daphnia magna* when exposed to 1 h settled sample after 48 h, and this severe toxicity repeated in the 2 h settled sample too. As a whole, the results obtained indicate that the technology should be accurately evaluated for the safety of the environment and human beings.

Keywords: Activated sludge; *Daphnia magna*; Inhibition; Metal doping; Nanoparticles; Oxygen uptake rate; Sol-gel; Titanium dioxide

1. Introduction

In recent years, nanotechnology has been very common in environmental applications and other self-cleaning applications of textiles [1–5]. Face to the growing industrial use of nanomaterials, nanoparticles (NPs) are expected in aquatic and terrestrial environments [6]. Titanium dioxide (TiO_2) NPs are among the most frequently reported engineered materials used in nanotechnology-based consumer products that have been used for decades [7]. TiO_2 NPs are widely applied in catalysts [8], sunscreens [9], cosmetics [10], paints [11], plastics [12] and wastewater treatment processes [13]. Besides, several modifications for TiO_2 have been reported in

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Presented at the 4th International Conference on Recycling and Reuse 2018 (R&R2018), 24–26 October 2018, Istanbul, Turkey
1944-3994/1944-3986

the literature such as metal-ion doped TiO_2 (by using; Co, Cu, Fe, Ni, Cr, Au, Ag, Pt, etc.) [14,15].

The fate of NPs in wastewater treated by the activated sludge process has been frequently examined in literature [16–19]. Park et al. [20] have studied the removal characteristics of NPs by activated sludge and indicated that (Ag, TiO_2, and SiO_2) were removed by adsorption on the surface of the microorganisms and agglomeration of NPs within the extracellular polymeric substances (EPS) in the activated sludge.

Furthermore, toxicity studies for TiO_2 NPs on the number of aquatic organisms were performed [21]. Results indicate that no toxicity was noted for *Vibrio fischeri* and *Daphnia magna*. On the contrary, Zebrafish reproduction and growth of *Daphnia magna* and algae were inhibited by TiO_2 NPs [22–24]. TiO_2 NPs caused more damage to algae than bulk particles [25]. The other toxicity study of NPs conducted on human keratinocytes in vitro designated that the long term exposure induces more negative effects than short term exposure [26]. The occurrence of TiO_2 NPs in the environment has recently aroused public attention regarding their potential impact on the biota and human health [27].

Respirometry has been used very efficiently for identifying and understanding the biochemical mechanisms involved in the activated sludge process [28,29]. On-line oxygen uptake rate (OUR) measurements have been considered as more reliable methods compared to instant determination of the OUR response of the activated sludge systems for toxicity and inhibition studies [30–34].

This study aims to investigate inhibition effects imposed by sol-gel based TiO_2 NPs of the activated sludge system using OUR. Besides this, in this study, Degussa P25 which is a commercial product that contains TiO_2 NPs is used for toxicity tests on *Daphnia magna* to evaluate their effects on aquatic life in the case of wastewater discharge to the environment. In this context, this study will provide a better understanding of the inhibition mechanisms and the results of the study will be useful in the solution of operational problems of activated sludge systems.

2. Materials and methods

2.1. Chemicals

Peptone, meat extract, urea, NaCl, $CaCl_2$, $MgSO_4$, K_2HPO_4, $CuCl_2$, $AgSO_4$, H_2SO_4, and NaOH were all obtained from Merck and Co., (Germany). Titanium IV-isopropoxide ($Ti[OCH(CH_3)_2]_4$, 97%) was obtained from Aldrich (St Louis, United States). All chemicals used were of analytical grade. Distilled water was used throughout this study.

2.2. Sol-gel preparation

The standard solutions of TiO_2 NPs with an initial concentration of 1 or 2 g/L (Ti NPs) as well as metal-doped (3% Ag and 2% Cu) TiO_2 NPs soles were freshly prepared by dissolving 25 ml titanium isopropoxide in 500 ml distilled water. Acetic acid (5 ml) and nitric acid (3.5 ml) were added to the solution while stirring at 80°C for 30 min. Subsequently, the TiO_2 solution was stirred for 2 h at room temperature. Dialysis membranes were used to increase the pH of solutions to 2.7.

Degussa P25 TiO_2 NPs (5 g/L) were stirred and heated with a magnetic stirrer until reaching a boiling point. An appropriate amount of sol-gel derivative and Degussa P25 TiO_2 were added to the aqueous samples to prepare nanoscale TiO_2 suspensions (5 g/L). The suspension was ultrasonicated for 15 min and settled down for 1 h and 2 h periods. The supernatant was removed after the settling process, to be used in *Daphnia magna* immobilization tests.

2.3. Activated sludge inhibition tests

The experimental setup has been prepared by the acclimation of activated sludge obtained from a domestic wastewater treatment plant with synthetic sewage described in ISO 8192 [35], via fill and draw reactors (Fig. 1). The acclimated biomass was used in the inhibition tests defined by ISO 8192 [35].

Activated sludge inhibition tests of the NPs applied to the synthetic wastewater samples, were conducted following the test procedure described in ISO 8192 [35]. The experimental setup includes the acclimation of activated sludge of a municipal wastewater treatment plant in Denizli, Turkey. Activated sludge was acclimated in two lab-scale batch reactors, which had a working volume of 2 L. The hydraulic retention time of the reactors is 1 d and the reactors were fed with synthetic sewage representing the domestic wastewater at a fixed Food/Microorganism (F/M) ratio of 0.3 mg chemical oxygen demand (COD) per mg VSS per day. For preparing the synthetic sewage stock solution,16 g/L peptone, 11 g/L meat extract, were added into the standard synthetic sewage solution containing 0.012 M NaCl, 0.0031 M $CaCl_2$, 0.0009 M $MgSO_4$, 0.05 M urea ($CO(NH_2)_2$) and 0.016 M K_2HPO_4. Hach (Düsseldorf, Germany) nitrification inhibitor, Formula 2533™ was added to the reactors at a concentration of 0.16 g for 300 ml volume. The reactors were aerated so that the dissolved oxygen levels were kept above 5 mg/L, pH value of the solution was checked periodically, and it was always in the range of 6.5–7.5 during the experiments. Dissolved oxygen measurements were performed by a WTW Inolab OXI 740 oxygen meter (Weilheim, Germany) in 50 mL air-tight

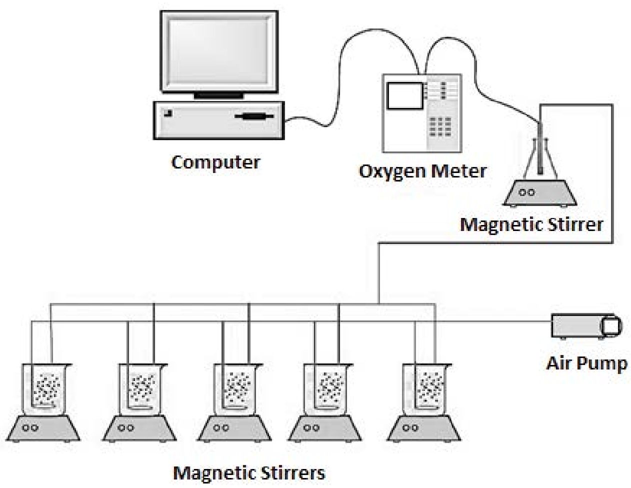

Fig. 1. Experimental set-up for measuring oxygen concentration in the aerated reactors.

vessels. Repeated trials were conducted by using TiO_2 NPs, prepared by the sol-gel method, for periods of 30–180 min.

The ISO 8192 [35] method describes the way to assess the inhibitory effects of a test substance on oxygen consumption of activated sludge by measuring the respiration rate under defined conditions in the presence of a defined biodegradable substrate and different concentration of the test substance [30]. The inhibitory effect of the test substance at a specific concentration can be calculated by using Eq. (1) compared with blank control. The blank control is prepared by using the same amounts of activated sludge seed and biodegradable substrate as the test dilutions. The physicochemical inspection is carried out to monitor the possible physicochemical oxygen consumption. This dilution includes the test substrate and substrate medium, without the addition of activated sludge. First, OUR measurement was obtained at 30 min. As indicated in the guidelines of ISO 8192 [35], if necessary, a second OUR measurement can be carried after 180 min.

$$\frac{R_B - \left(R_T - R_{\text{PC}}\right)}{R_B} = I_{\text{OUR}}\left(\%\right) \quad (1)$$

where R_T is the oxygen consumption rate by the test mixture, R_B is the oxygen consumption rate by the blank control, and R_{PC} is the oxygen consumption rate by the physicochemical inspection. Further details are described in ISO 8192 guidelines [35].

2.4. *Daphnia magna* inhibition test

Toxicity of TiO_2 NPs was tested by using *Daphnia magna* according to the standard ISO method given for *Daphnia magna* [36]. Five daphnids were used in each test beaker has 100 ml effective volume. Daphnids were cultivated in the laboratory by feeding with unicellular green algae *Selenastrum capricornutum* (300,000 cells/ml) and baker's yeast (*Schizo Saccharomyces cerevisiae*, 200,000 cells/ml) under 8 h dark and 16 h daylight (3,000 lux illumination) cycle. The constant room temperature was kept at 20°C ± 1°C, and a minimum of 6 mg/L of dissolved oxygen was supplied by air filter through activated carbon. Distilled water was used for preparing all solutions at pH 8.0, as described in Section 2.2. TiO_2 NPs suspensions (5 g/L) were prepared by using Degussa P25, after the 1 and 2 h settling processes, the supernatant which contains Ti NPs was fed to the daphnids.

After 24 and 48 h exposure times, the number of immobile organisms was divided by the total number of test organisms (20) and the percentage of immobilization was recorded. A higher immobilization percentage means greater toxicity of a sample.

2.5. Analytical procedure

TiO_2 was converted to ionic form due to its low solubility, by using HNO_3–H_2SO_4 digestion described in Standard Methods Section 3030 [37]. After the digestion process, TiO_2 concentrations that remained in the solutions were analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES) (PerkinElmer ICP-OES Optima 2100 DV, Massachusetts, United States). COD was determined using the closed reflux procedure defined by Standard Methods [37]. Vacuum filtration with 0.45 µm pore size Millipore membrane (Massachusetts, United States) filter was used for soluble COD analyses. The concentrations of total suspended solids and volatile suspended solids (VSS) were analyzed as defined in Standard Methods [37].

3. Results and discussion

3.1. Inhibition test

The EC_{50} level as defined by ISO 8192 [35] after 30 and 180 min of reaction time for TiO_2 NPs was calculated to be 1,654 and 1,670 mg/L for Ti, respectively (Figs. 2 and 3).

These results reflect that TiO_2 NPs alone have no adverse effect on the activated sludge system. Gartiser et al. [38] also stated that TiO_2 NPs did not affect the biodegradation and nitrification in the activated sludge. It is reported that methane production has not been affected by TiO_2 NPs during 105 d of sludge fermentation due to insoluble characteristics of TiO_2 NPs and EPS protection of sludge [39]. The two different periods were used to assess TiO_2 toxicity to monitor the effects of acute and long-time exposure. The results reflect that there were no significant differences between 30 and 180 min of exposure time. However, Chen et al. [39] indicated that 1, 10, and 100 mg/L TiO_2 NPs inhibited

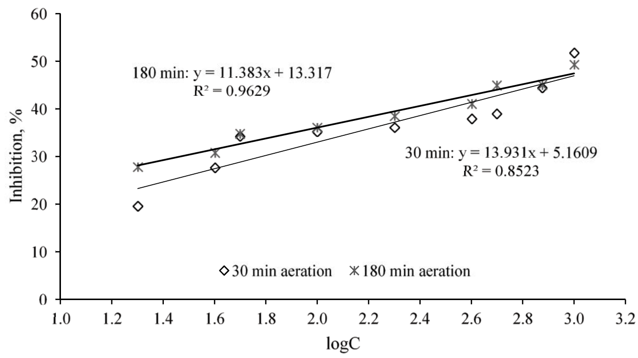

Fig. 2. Evolution of inhibition of TiO_2 NPs prepared by sol-gel method (aeration periods: 30 and 180 min, F/M ratio = 0.3 mg COD/mg VSS d, pH = 6.5–7.5).

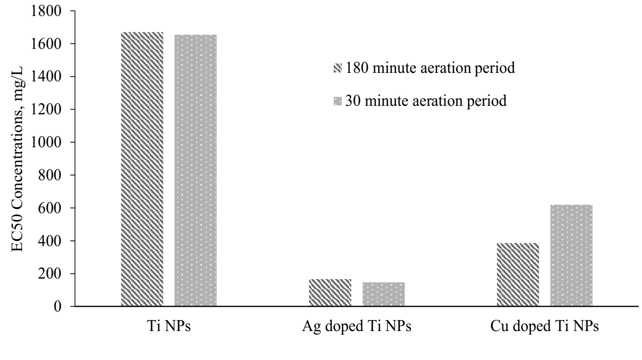

Fig. 3. Comparison of EC_{50} concentrations of TiO_2 with metal doped TiO_2 NPs (aeration periods: 30 and 180 min, F/M ratio = 0.3 mg COD/mg VSS d, pH = 6.5–7.5).

the O_2 fluxes of activated sludge when the exposure time extended to 4.5 h. The toxicity of doped-Ti NPs with metal ions which have the best antibacterial (3% Ag added to TiO_2 NPs sol-gel) and self-cleaning (2% Cu added to TiO_2 NPs sol-gel) properties was examined after 30 and 180 min of reaction time. As shown in Figs. 3 and 4, an inhibition effect was observed when copper was used as the additive.

Gonzalez-Estrella et al. [40] reported that 50% inhibition concentrations for Cu^0 NPs were 62 and 68 mg/L for acetoclastic and hydrogenotrophic methanogens, respectively. However, IC_{50} for Cu-doped TiO_2 NPs in our experiment was 386 mg/L on aerobic activated sludge. This indicates that aerobic activated sludge systems may be less affected by Cu NPs inhibition than different bacterial classes. At 30 and 180 min reaction times IC_{50}values were 147 and 166 mg/L for 3% Ag-doped NPs, respectively (Fig. 5).

Silver, which is known to possess anti-bacterial properties showed a higher inhibition effect on the microorganisms than Cu-doped solution, in lower concentrations. Çeçen et al. [41] have reported that Ag shows a high inhibition effect on nitrifying bacteria, even in low concentrations (0.33 mg/L). In another study Ag NPs (0.14 mg/L) caused 50% inhibition on nitrifying bacteria [42]. However, Cu NPs exhibited no inhibitory effect at the concentration of 10 mg/L [43]. Similarly, Ag-doped TiO_2 NPs showed inhibitory effects on activated sludge, two times higher than the Cu-doped TiO_2 NPs.

3.2. *Daphnia magna*

For the measurement of toxicity of TiO_2 NPs, analyses were carried out with *Daphnia magna*. Commercial TiO_2 (Degussa P25) and TiO_2 prepared with sol-gel were settled down for 1 and 2 h. After the settlement, *Daphnia magna* toxicity analysis was carried out, and the results are given in Table 1. After 1 h of settlement, Degussa P25 which Ti NPs' concentration was measured as 118 mg/L, has shown a 20% *Daphnia magna* toxicity and TiO_2 prepared with sol-gel (1,600 mg/L Ti NPs) shown 100% toxicity. On the other hand, the Ti NPs' concentration decreased to 90 mg/L with the 2 h settling period, and no acute toxicity was observed in 24 h. Ti NPs concentration did not change after the 2 h settling period in the sol-gel solution. However, it is reported that anatase and rutile TiO_2 (100 mg/L) did not present acute toxicity to

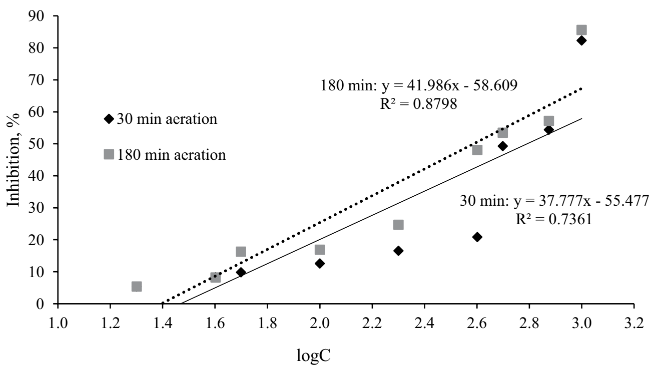

Fig. 4. Evolution of inhibition of Cu-doped (2%) TiO_2 NPs (aeration periods: 30 and 180 min, F/M ratio = 0.3 mg COD/mg VSS d, pH = 6.5–7.5).

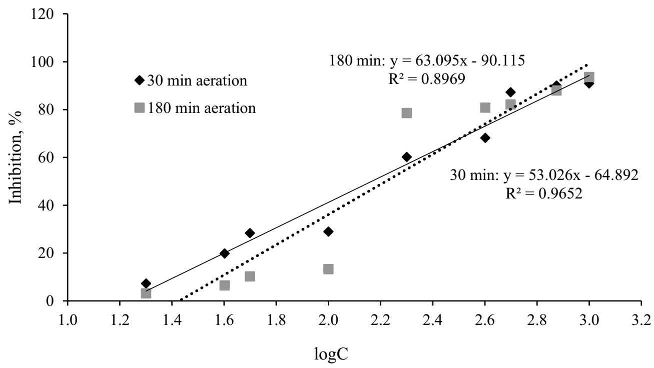

Fig. 5. Evolution of inhibition of Ag-doped (3%) TiO_2 NPs (aeration periods: 30 and 180 min, F/M ratio = 0.3 mg COD/mg VSS d, pH = 6.5–7.5).

Table 1
Toxicity of TiO_2 NPs to *Daphnia magna* for 24 and 48 h of exposure time (average results of quadruplicate experiments, <10% of standard deviation)

Settling time (h)	Immobilization (%)			
	1		2	
Exposure time (h)	24	48	24	48
Blank	0	0	0	0
TiO_2 Degussa P25	20	20	0	20
TiO_2 sol-gel	90	100	50	100

Daphnia similis [44]. Also, Zhu et al. [45] tested the exposure duration time that could affect the toxicity of nano-TiO_2 to *Daphnia magna*. After 48 h of the incubation period, 100 mg/L of nano-TiO_2 concentration caused 20% of immobilization, and no substantial mortality was seen. However, after 72 h 100% of immobilization and 13% mortality were observed. These results have similarities with our findings that sol-gel based Ti NPs exhibited 100% immobilization. On the other hand, Ti NPs (16–21 mg/L) inhibited the growth of *Chlorella* and *Scenedesmus* species [24].

Fan et al. [46] reported that the existence of NPs with Cu^{2+}increases concern over the enhanced biotoxicity of Cu^{2+} and nano-TiO_2 increased the accumulation of Cu^{2+} in *Daphnia magna* by 31%.

3.3. COD analysis

COD concentrations were also monitored while toxicity tests were conducted. The results are given in Fig. 6. The initial COD concentrations differ from each testing NPs due to chemical compositions of prepared TiO_2 solutions. The activated sludge used in the experimental procedure was acclimated to high COD concentrations. So high COD loading of metal-doped TiO_2 NPs did not affect the COD removal rates. It is clear that metal-doped Ti NPs significantly inhibit COD removal efficiency, whereas TiO_2 NPs did not affect the COD removal efficiency (85%). The experimental data on all figures indicate that an excellent logarithmic correlation between the inhibitor concentration and percent decrease

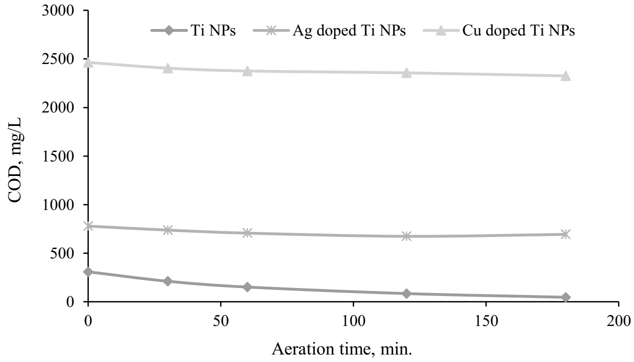

Fig. 6. Changes in COD concentrations in the aerated reactors exposed to TiO_2 NPs and metal-doped TiO_2 NPs (F/M ratio = 0.3 mg COD/mg VSS d, pH = 6.5–7.5).

in the corresponding OUR level for the range of TiO_2 NPs concentrations tested in the study.

4. Conclusions

The toxicity effects of TiO_2, Cu-, and Ag-doped TiO_2 NPs on the microorganisms in the activated sludge system were investigated to observe NPs' possible adverse effects. Cu-doped TiO_2 NPs showed less toxic effects than Ag-doped TiO_2 NPs. However, it was still toxic concerning TiO_2 NPs alone. It can be concluded that Ag, which has anti-bacterial properties, showed more inhibition effect on the activated sludge microorganisms than Cu in solution even in lower concentrations. Additionally, the COD removal efficiencies were also adversely affected by metal-doped TiO_2 NPs. The COD removal efficiencies dropped from 85% to 5% in the presence of Ag and Cu. This indicates that TiO_2 NPs alone did not affect the COD removal efficiency; inhibition of activated sludge is caused by the metal-doped TiO_2.On the contrary toxicity test results, conducted with *Daphnia magna* illustrate the TiO_2 NPs were utterly toxic. This phenomenon should be investigated in detail.

Acknowledgments

The authors would like to thank the Scientific and Technological Research Council of Turkey (TÜBİTAK), for the research grant 108M211.

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