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Understanding Time-dependent Toxicity of Titanium Dioxide Nanoparticles to Activated Biomass

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Abstract

This study characterized toxic effect of titanium dioxide (TiO2) nanoparticles (NPs) on aerobic microorganisms in batch kinetic studies for different concentration levels. Initially no difference in lag periods or exponential growth periods was found due to presence. of NPs. Also, a considerable amount of decrement in the absorbance values of the bacterial growth phase was observed with the highest toxicity effect (i.e., reduction of almost 52% in presence of 2 mg/L TiO2 NPs and 72% in presence of 20 mg/L TiO2 NPs) obtained during the stationary phase (i.e., after 72 hours of exposure). Further, counts of culturable bacteria was. also reduced to almost 151 and 154 colony forming units (CFU) because of exposures to 2 mg/L and 20 mg/L TiO2 NPs for 12 hours. Scanning electron morphological analysis revealed retention of nanoparticles on bacterial cell surface, explaining toxicity of NPs to biomass. Findings of this study provided proper estimation of time-dependent toxicity. of TiO2 NPs to aerobic biomass, useful in determining hydraulic retention time for aerobic biological reactors. However further observations of these effects at pilot-scale reactors are required to properly understand interaction of nanoparticles with activated biomass.

Keywords: Aerobic biomass; Titanium dioxide nanoparticles; Toxicity; Wastewater

Introduction

Recent studies have reported occurrence of nanoparticles (NPs) in wastewater [1] and documented their adverse effects on different organisms as well as on humans [2]. In biological systems, nanoparticles have been observed to be. accumulated in biomass which gives toxicity to mix consortia of bacteria present in biological reactors. For example, monitoring of the titanium NPs in wastewater treatment. plants (WWTPs) [1] showed the accumulation of TiO_2 nanomaterials in biosolids ranging from 1-6 μg/mg (for raw sewage concentration: 100 to 3000 μg/ L), indicating possible threat to existence of mix consortia of sludge bacteria. These NPs either adhere to microorganisms or are taken up by microorganisms, resulting in creation of a potential gradients of NPs in and outside the microbial cells. Further, NPs are effective. bactericidal agents against both gram positive and negative bacteria. This necessitates the need to examine the effects on microbes in natural communities of bacteria in natural waters [3]. Among NPs, Ag and TiO_2 are two of the most widely used NPs in consumer products. (Gottschalk et al., 2009) and thus, chances of their coming in wastewater are more. Thus, it is important to understand their interaction with microorganisms in biological processes. A literature review indicates that some studies have focused on understanding effects of interaction of NPs with biomass (both aerobic and anaerobic) (effect on oxygen uptake rate, nitrification-denitrification rate, methane generation rate). Interaction of aerobic biomass with different NPs were studied (for Ag NPs: [4-12] for CeO_2 NPs: [11,13] for TiO_2: [10,11] for C60: 10). Similarly, studies have also been done to understand interaction of NPs with anaerobic biomass (for example, C60: [14], and for ZnO: [15]). However, most of these studies do not provide information on effect of contact time of NPs to biomass and how biomass behaves with increasing contact times of NPs exposure. This information is important to decide about contact time of NPs to biomass with focus on toxicity to biomass and its effect on their biological activities. The objective of this study was to understand the effect of toxicity of TiO_2 NPs on growth kinetics of the mix consortia of bacteria present in the sewage. TiO_2 NPs was selected due to its increased usage and high possibility of it

coming in wastewater [1]. Findings of this study are expected to provide an understanding about toxicity of the nanoparticles on mixed culture of bacteria present in the wastewater.

Materials and Methods

Materials

TiO_2 NPs (particle size < 25 nm, purity 99.7%; anatase crystal phase) was purchased from Sigma-Aldrich Chemicals Pvt. Ltd. (India). These NPs were properly sonicated in an ultrasonic cleaner (model: TOSHCON, Toshniwal Instrument Manufacturing Pvt. Ltd., India) for 30 minutes for properly dispersing in media. All chemicals and biological media were obtained from HiMedia Laboratories (India) and Merck Ltd. (India), unless mentioned otherwise. Domestic sewage was collected from the internal drainage system of Indian Institute of Technology (IIT) New Delhi, India for conducting growth kinetic studies. Further, biomass samples were also collected from the aeration tank of a sewage treatment plant (STP) in New Delhi (India) for understanding toxic effect of NPs on viability of aerobic biomass. All biomass samples were characterized for dissolved oxygen, pH, and total dissolved solids parameters using the APHA methodologies (Table 1).

Table 1: Characterization of biomass (number of samples analyzed =3) (Values are expressed as average ± one standard deviation).

Parameters	III Delhi Domestic sewage	WWTP New Delhi
pH	8.6±0.06	7.95±0.06
Conductivity	118±0.02 mS	120±0.02 mS
Total dissolved solids	62±0.02 mg/L	80±0.02 mg/L
Dissolved oxygen	4.4±0.05 mg/L	4.85±0.05 mg/L
Mixed liquor suspended solids	Not applicable as this biomass was used to start biological reactors in laboratory	3850±128.5 mg/L

Generation of Mixed Culture Biomass from Domestic Sewage

Biomass acclimatization reactor of one liter volume was set up for generation of appropriate amounts of biomass containing 0.5% of glucose, 0.5% of potassium di-hydrogen phosphate and 0.5% of peptone and 100 ml of domestic sewage, freshly collected and aerated for 48 hours. The generation of mixed culture of microbial population was visible from the appearances of turbidity inside the reactor. The mixed liquor was centrifuged and then supernatant was discarded to finally collect cell pellets. This cell pellets (105-107) were re-suspended in nutrient media to study their growth.

Effect of TiO_2 NPs on Growth Kinetics of Aerobic Biomass

Three different semi-batch type bioreactors (R0, R1, and R2) were set up containing 3.75 g of glucose, 1.14 g of ammonium chloride and 7.5 ml of 0.2 m of phosphate buffer per 1000 ml reactor volume. In each of these reactors, 2000 mg/L of the biomass generated from the acclimatization reactor was added and aerated properly by automatic aerators (rate= 0.5 to 1 L/min). Two of three reactors were exposed to 2 mg/L and 20 mg/L of TiO_2 NPs (in R1 and R2, reactors, respectively) and the last reactor (R0) was not exposed to NPs (i.e., control reactor). The concentration of 2 mg/L TiO_2 NPs was chosen as an average experimental concentration using occurrence information reported in literature for TiO_2 NPs in wastewater. Also, it was overestimated up to 20 mg/L to represent TiO_2 pollution in wastewater for the worst-case scenario. The solution pH of 7-7.4 and a temperature of 36-37°C were maintained inside the reactor for optimum growth of the microbes. Bacterial growth at different time intervals for 7 days was studied by measuring absorbance at 600 nm [16-20] using UV-Vis spectrophotometer (PG Instruments, India). Further, two more separate reactors containing nutrient media and only NPs (two concentrations: 2 mg/L and 20 mg/L NPs) were also studied (i.e., nanoparticles-based control reactors: Rc1 and Rc2, respectively). These reactors were also sampled as per the sampling scheme used for exposed reactors (i.e., R0-R3) and analyzed for absorbance values. These reactors were set up to study interaction of NPs with nutrient media and its effect on absorbance of samples. Absorbance values of these two reactors (Rc1 and Rc2) were subtracted from the absorbance values of the R1 and R2 reactors, respectively to observe the actual growth of the bacteria during exposure to NPs for different time periods.

Effect of TiO_2 NPs on Viability of Activated Biomass

To study toxicity of TiO_2 NPs on viability of biomass (expressed in terms of heterotrophic plate count, HPC), biomass samples

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were collected from the aeration tank from the sewage treatment plant (STP) in New Delhi (India). These samples were diluted to 103 times and were mixed with 2 mg/L and 20 mg/L nanoparticle concentrations in sterilized test tubes. Exposure times of bacteria to NPs were varied for 2, 6, and 12 hours to study effect of exposure time on toxicity to bacteria. After the desired exposure, 100 μl of mixed sample was spread on petri dishes containing nutrient agar and kept for incubation at 37°C for 24 hours. At every time interval, duplicate samples were kept. After the stipulated time of incubation, the number of bacterial colonies was counted as colony forming unit (CFU) [21]. A nutrient agar plate containing only bacteria was studied as a control. All CFU values were multiplied by dilution factors to get the original population of the bacteria per ml of the sample. Further, reduction in the CFU values was calculated with respect to the control (i.e., devoid of NPs) and the subtracted CFU values were plotted against exposure times to understand effect of exposure time on viability of sludge bacteria.

Surface Morphology Analysis of Bacteria Exposed to TiO_2 NPs

Freshly collected activated biomass was exposed to 20 mg/L TiO_2 NPs for overnight. and was centrifuged at around 10000 rpm to collect the cell pellets. The cell pellets were fixed in 0.1 M phosphate buffer (pH =7.3) containing 2.5% glutaraldehyde for 2 h at 4°C. After fixation, sample was rinsed three times in 0.1 M phosphate buffer (pH=7.3 and 10 min/wash). Then the sample was dehydrated gradually after successive washing in ethanol solutions of increasing concentration (50, 70, 80, 90 and 100%) and each step was completed for 10 minutes. Drying of the sample was completed by incubating the samples for 2 hours at 60°C and was stored in the desiccator at room temperature. The sample was gold-coated before imaging to reduce charging of the sample [22]. The samples were adhered to the holder by double-carbon tape and were viewed under scanning electron microscope EVO 50 at IIT Delhi (India).

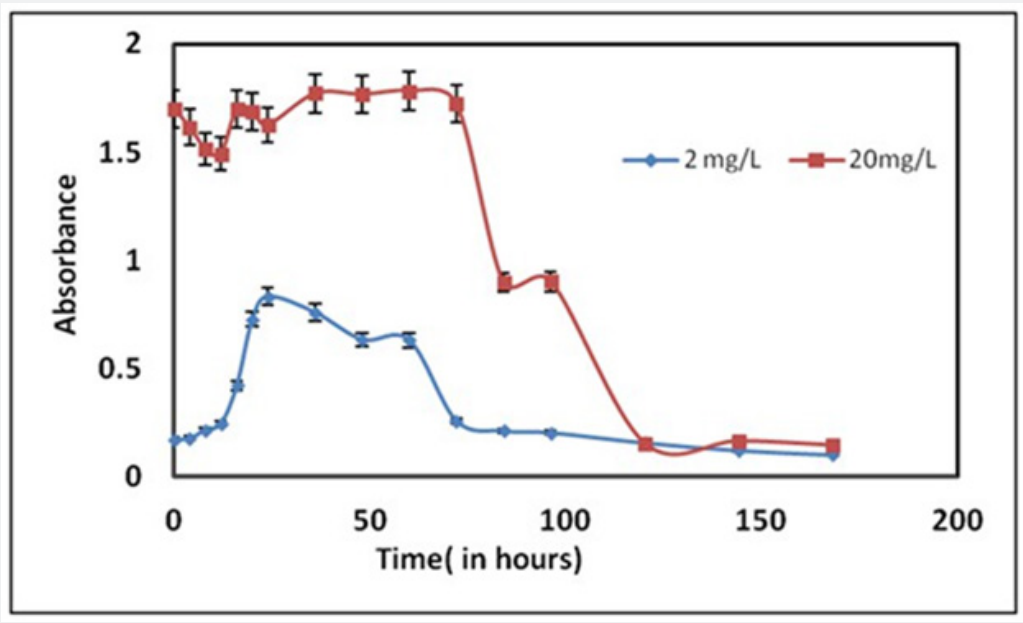

Figure 1: Variation of absorbance values of nanoparticles only-control reactor with time (average of duplicate observations are shown here).

Results and Discussion

Effect of TiO_2 NPs on Bacterial Growth

Figure 1 shows absorbance values of nanoparticles only based control reactor (Rc1and Rc2). In Figure 1, the 2mg/L concentration of TiO_2 NPs (Rc1) have a very low absorbance values in the first few hours, while the value suddenly rises on the 20th hour because of the probable agglomeration of the metal oxide nanoparticles. It remains almost static up to 60th hour and ultimately due to possible formation of large flocs and subsequent settling, much lower absorbance value was observed at the end of the experiment. While in reactor Rc2, the absorbance value is very high from the beginning of the experiment, and it remains almost static at the end of 72nd hour. It suddenly drops after that because of possible settling of NPs. This drop in absorbance values continued till the end of observation period. These values from Rc1 and Rc2 were used as background absorbance values due to NPs presence in solution and were accounted for during calculation of absorbance

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due to bacteria only for R1 and R2 reactors. Figure 2 shows toxicity data during TiO_2 NPs exposures to bacteria in batch studies. The control reactor (R0) had distinguished phases of bacterial growth (i.e., lag, log, stationery and decay phases). However, the presence of titanium dioxide NPs produced toxic effects on different phases of bacterial growth. Absorbance values of the bacterial population in presence of 2mg/L of NPs (R1) were found to be smaller for different time periods than that in control reactor, indicating toxic effects of TiO_2 on bacterial population. The most toxic effects on the microbial growth were observed during bacterial exposure to 20 mg/L TiO_2 NPs (R2). High NP concentration was found to impart more toxicity to bacteria than lower NP concentrations for all observation periods (Figures 2 & 3), with highest difference obtained at 48 hours since start of exposure (Figure 3). These results indicate that TiO_2 NPs pose threat to consortium of mixed bacteria present in wastewater and concentration also influences extent of toxicity to bacteria at different growth phases. However, this aspect needs to be investigated further for different NPs concentration and wastewater quality parameters, such as pH and conductivity values.

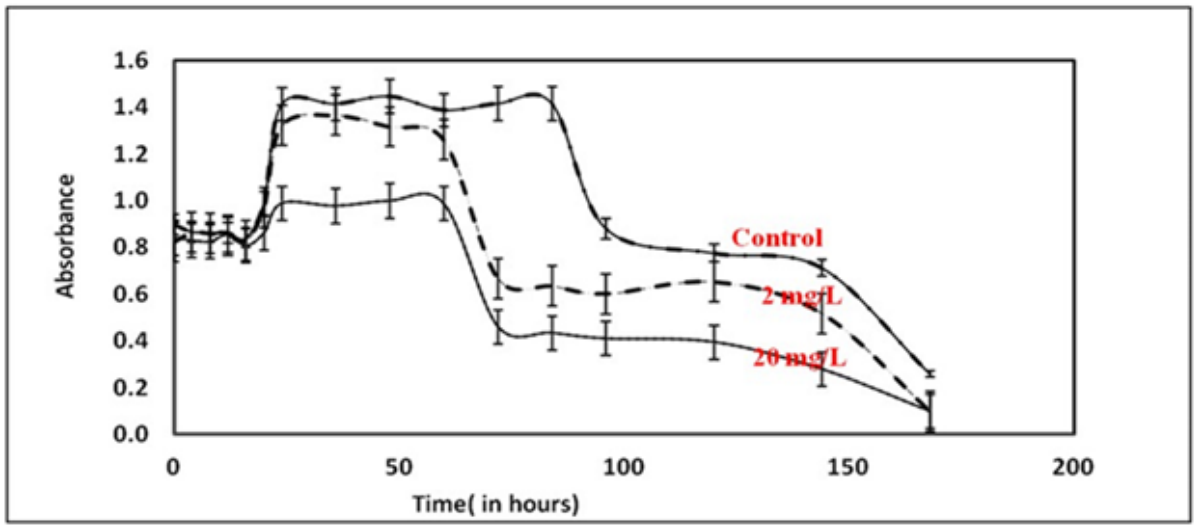

Figure 2: Microbial growth kinetics with and without titanium dioxide NPs (Error bars indicate one standard deviation value of duplicate samples) (here control reactor indicates reactor without any nanoparticles).

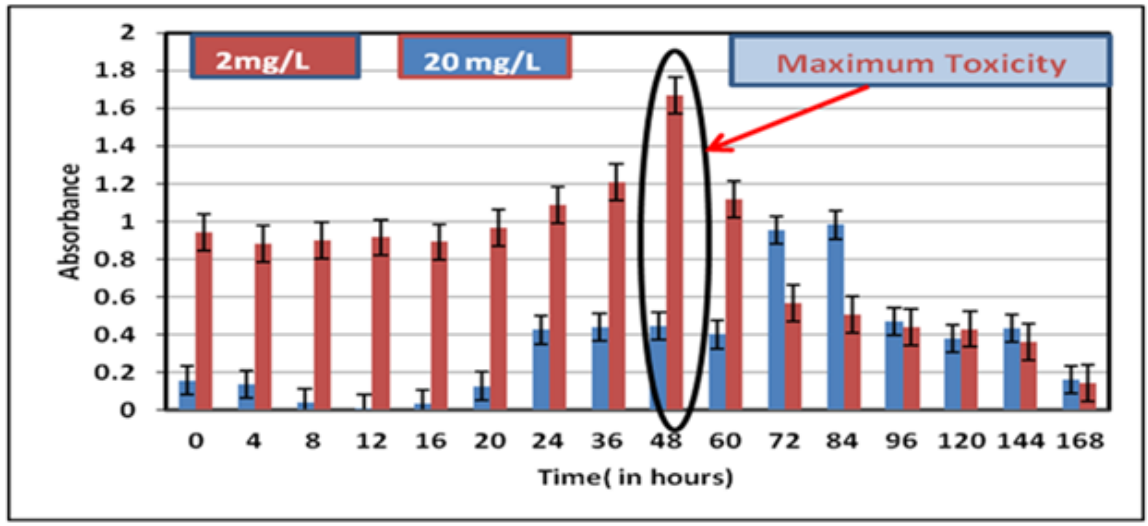

Figure 3: TiO2 NP-associated bacterial toxicity (in terms of reduction of absorbance compared to that of control) at different time intervals (Error bars indicate one standard deviation value of duplicate samples).

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Effect of TiO_2 NPs on Viability of Activated Biomass

The toxicity of the TiO_2 NPs to activate biomass was further observed during exposure of the bacterial culture to NPs on solid agar media with varying exposure times. Figure 4 shows a clear reduction in the number of microbial colonies (i.e., CFU values) on the nutrient agar plates when exposed to NPs. The toxic effect to bacteria was observed to be more for 20 mg/L TiO_2 NPs than 2 mg/L NPs. Figure 5 shows variation of CFU values with different exposure periods. The reduction in CFU values was noticed for exposure periods of 2 hours and 6 hours. Average CFU reductions were noted to be 20% and 92%, respectively for 2 hour and 6-hour exposure times (for the case of exposure of 2 mg/L TiO_2 NPs) and CFU reductions of 61 and 132, respectively for 2 hour and 6-hour exposure times (for the case of exposure of 20 mg/L TiO_2 NPs). However, the reduction in CFU values was observed to be almost similar for exposure times of 12 hours (i.e., 151 and 154), irrespective of exposure of 2 mg/L or 20 mg/L TiO_2 NPs.

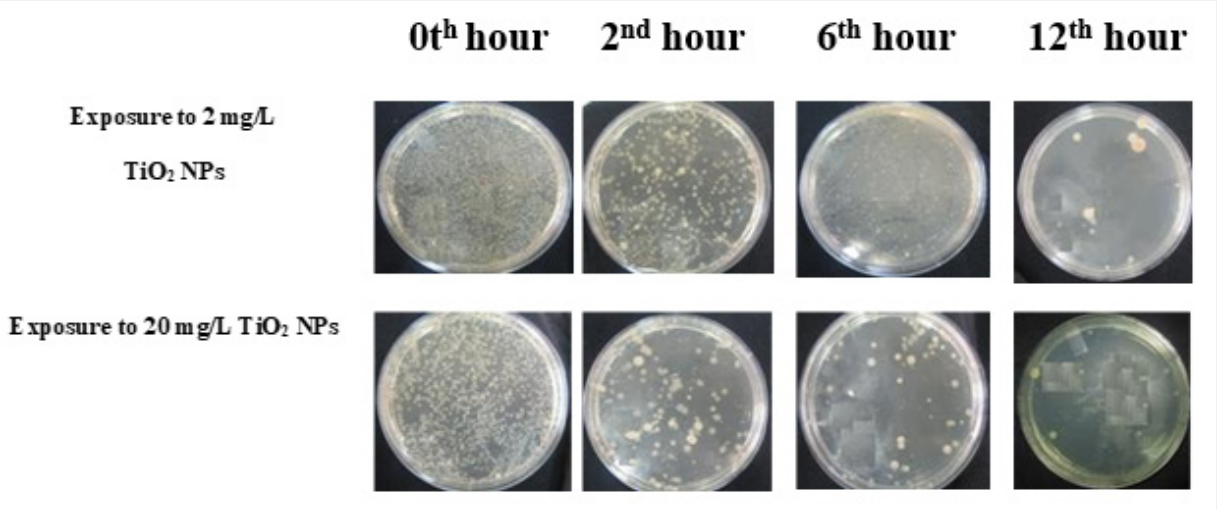

Figure 4: A Comparison of Growth (in terms of Colony Forming Units) of Bacteria Exposed to 2 and 20 mg/L TiO2 NPs for different exposure time (0, 2, 6 and 12 hour).

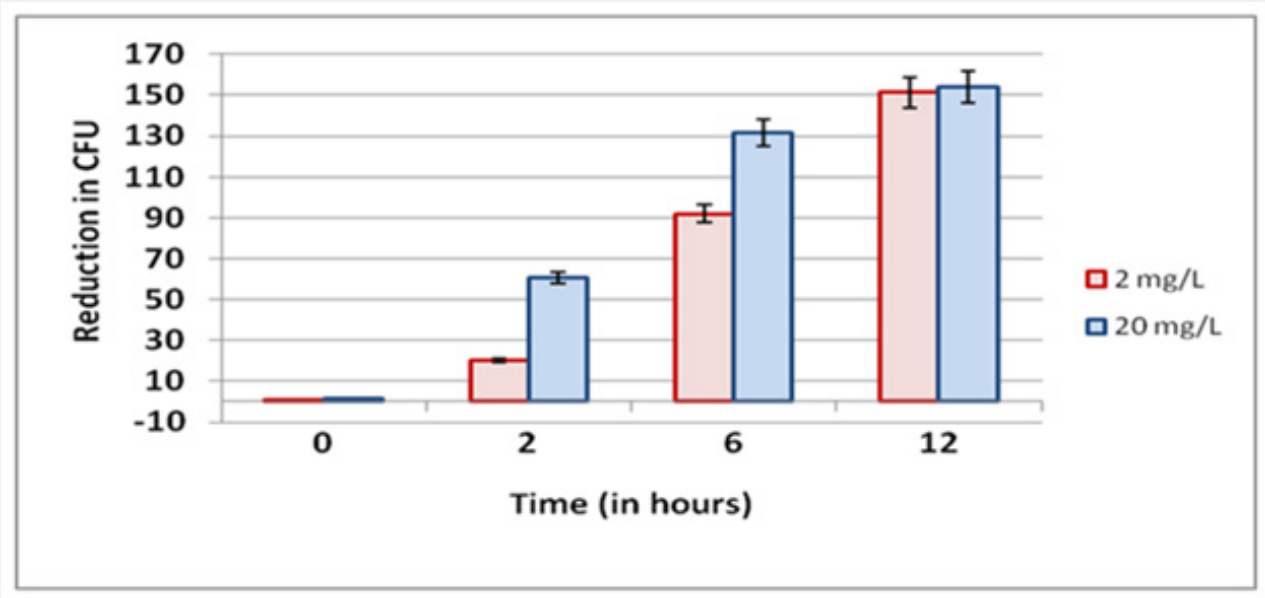

Figure 5: Variation of Reduction in Colony Forming Units of Bacteria Exposed to 2 and 20 mg/L TiO2 NPs for different exposure time (0, 2, 6 and 12 hour) (Error bars indicate one standard deviation value of duplicate samples).

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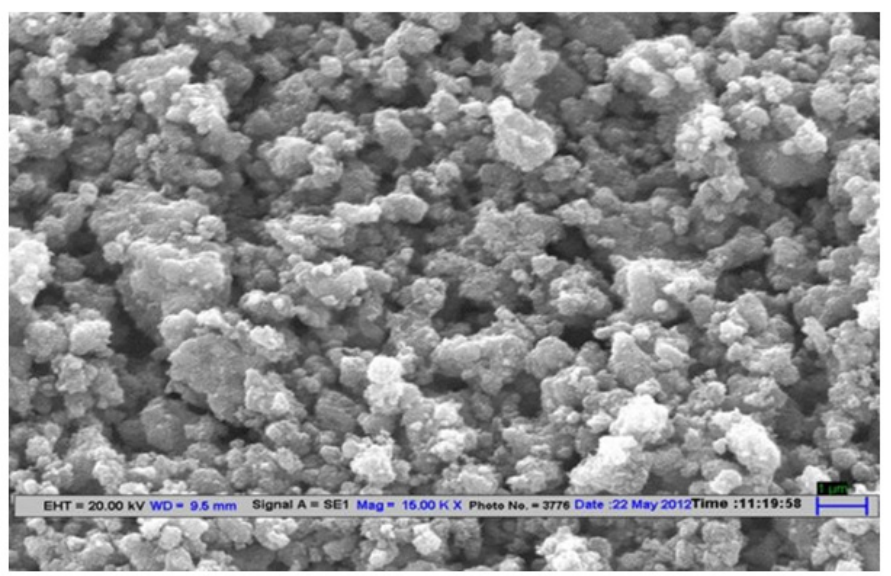

Figure 6: Surface Morphology of TiO2 NPs Dispersed in Nutrient Media.

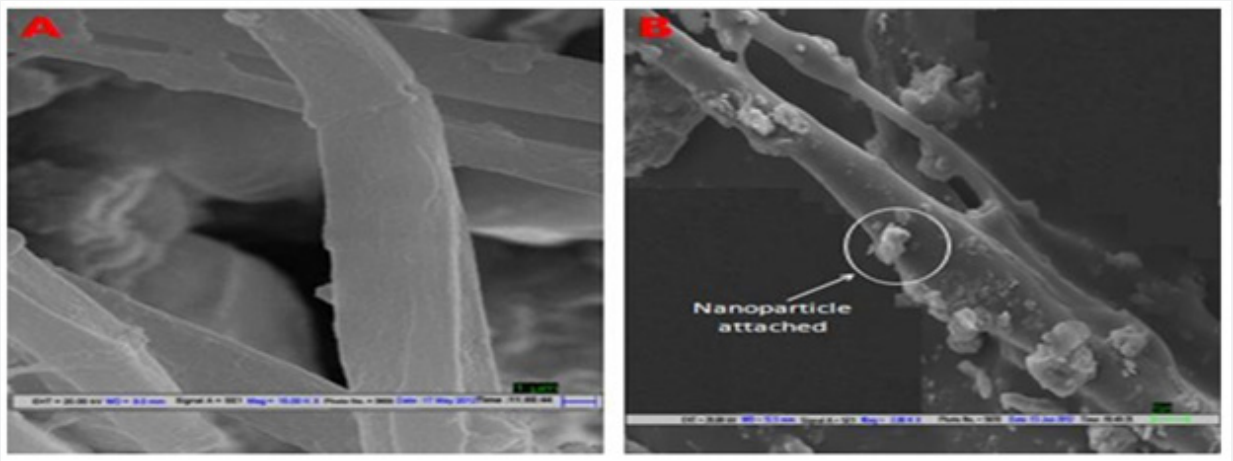

Figure 7: Surface Morphology: A) Bacterial Cells without any exposure to NPs (i.e., control), (B): Bacterial Cells after Exposure to NPs (here attachment of NPs on bacterial cell walls can be seen).

Figure 6 shows scanning electron morphology of TiO_2 NPs. Figure 7a shows surface morphology of activated biomass without any exposure to NPs, where different rod shaped, and filamentous bacteria can be seen. Figure 7b shows surface morphology of biomass exposed to NPs where aggregated NPs can be seen on surface of bacterial cells, indicating surface deposition of titanium dioxide NPs on bacterial cell wall. This deposition of NPs on bacterial cells in turn might increase exposure time of NPs to wastewater biomass, resulting in probable chronic toxicity to microbial cells in wastewater biomass (Luongo and Zhang,2010).

Conclusion

a) There was a decrement in the absorbance values of the bacterial growth in presence of NPs. During exposures of 2 mg/L TiO_2 NPs, absorbance was found to decrease by 6% after exposures of bacteria to NPs for 24 hours and 8% after exposures of bacteria to NPs for 48 hours. This decrease in absorbance values was observed to be 23% after 12 hours, 37% after 24 hours and 30% after 48 hours in presence of 20 mg/L of TiO_2 NPs, indicating different extents of toxicities to bacteria.

b) TiO_2 NPs posed maximum threat to bacteria during their stationary phase of growth which might affect the flocculation process of sludge bacteria during biological wastewater treatment. However, this aspect needs to be explored using detailed pilot-scale studies.

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c) The heterotrophic plate count method also revealed considerable decrease in the growth of microbial colonies due to the presence of NPs. Decrease in CFU values was found to be similar for two NP concentration values studied after 12 hours of exposure (i.e., reduction of 151 CFU value for the case of exposures of 2 mg/L TiO_2 NPs and 154 CFU value for the case of exposures of 20 mg/L TiO_2 NPs). However, effect of NP concentration on toxicity to bacteria need to be studied in detail.

d) Surface morphology analyses of NP exposed biomass indicated surface deposition of NPs on bacterial cell walls. This might increase exposure duration of bacteria to NPs, leading to increased toxicity of NPs to bacteria. These findings indicate that initially TiO_2 imparts high toxicity to biomass, which could be used in deciding hydraulic retention times for activated sludge processes. Future detailed studies are required to study TiO_2-bacteria interaction in activated sludge operations to understand long-term impact of NP exposures to bacteria.

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