EFFECTS OF NANOSIZED TITANIUM DIOXIDE (TiO₂) AND FULLERENE (C₆₀) ON WASTEWATER MICROORGANISMS ACTIVITY

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Abstract

This paper investigates the impact of nano-TiO₂ and fullerene (C₆₀) on the activity of micro-organisms present in wastewater treatment works (WWTWs). Results showed that low concentration levels of nano-TiO₂ (10 µg.L⁻¹) and fullerene (1 µg.L⁻¹) has little effect on both gas production and the microorganisms' morphology. However higher concentrations of nano-TiO₂ (100 and 1,000 µg.L⁻¹) and fullerene (10 and 100 µg.L⁻¹) reduced or stopped completely gas production. SEM micrographs showed that addition of nanoparticles reduced the microorganisms count up to 30 min of exposure, followed by an increase of selected micro-organisms after a 360 minutes exposure to TiO₂ and Fullerene. Overall, this study demonstrates that engineered nanoparticle concentration levels and

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exposure time play an important role in the toxicity of microbial communities present in WWTWs. It also shows that under stressed conditions, the micro-organisms appear to protect themselves via a spore forming mechanism that allows them to survive the environment.

1. Introduction

The use of Engineered Nanoparticles (ENPs) materials has risen considerably in the last decade. In 2006, 212 manufacturing companies were identified to be producing all types of nanoparticles. In 2009, this number exceeded 1,000, with silver being the most common, utilised primarily as an antimicrobial (1). Nanoparticles are utilised in several other products (2-4) and its increasing use has been investigated by several authors (5-13) to cause pollution in natural ecosystems. They claim those materials are released in aquatic systems and have influence on the microorganisms behaviour and fate.

The toxicity mechanisms of nanoparticles on microorganisms is subjected to size, shape, concentration and surface area, which the latter is the most critical parameter recognized to influence microbial activity (14). However the pathway of interaction between an ENP and a microorganism requires deeper investigation. (15) and (16) reported the presence of nano-TiO₂ inside microorganisms' cells on their studies. (15) investigated the interaction between nano-TiO₂ and planktonic bacteria under the influence of extracellular polymeric substances (EPS), which is a natural substance excreted by this type of bacteria. It provides a barrier for ENPs because it limits the interaction with the cell wall besides facilitating the attachment of nano-TiO₂. For instance, (17) removed loosely bound EPS from a sample of wastewater biofilm and observed significant increase in the toxicity of nano-Ag compared to regular samples, which did not showed major inhibitory effects when exposed to 200 mg.L⁻¹ of nano-Ag. (6) have described the effects of EPS in protecting intracellular protein and polysaccharide in conditions where EPS layer is thick and when is thin. Nevertheless EPS decreases the exposure of nano-TiO₂ to Ultra-Violet (UV), decreasing as well oxidation through photo-catalysis, which is the

main mechanism for this NP to inhibit bacterial activity. Besides photo-catalysis, nano-sized titanium oxide causes agglomeration inside mussel cell's digestive gland (16) resulting in metabolic inhibition not related to exposure to UV.

Fullerene has been reported to have antimicrobial activity. However, (18) reported in their study that fullerene stimulated the growth of a bacillus bacteria that is responsible for denitrification in wastewater treatments, which contrasts to other publications whose results were of decrease in biological activity and inhibition. Polymerase Chain Reaction (PCR) was used to quantify strains of the microorganisms present in the sample. *B.cereus* was identified as the major strain in the sample.

It is uncertain which nanoparticle is more harmful to the environment; moreover studies have shown all ENPs cause harm to ecosystems at certain degree. In this study the influence of TiO₂ and Fullerene C₆₀ on aerobic microorganisms from wastewater treatment samples taken after primary sedimentation were investigated. TiO₂ is one of the most used ENPs and its release is expected to increase in aquatic systems (19) while fullerenes were discovered in 1985 and have been linked to stimulation of growth and inactivation of bacteria. Hence this study aimed to assess the influence of nano-TiO₂ and Fullerene C₆₀ on the microbial community in wastewater samples taken from lamellas before aeration lanes from a wastewater treatment works in Reading, United Kingdom.

2. Materials and methods

Influence of nano-TiO₂ (25 nm) and Fullerene was observed and measured through two methods: Automatic Methane Production Testing System (AMPTS) and SEM. The first provided data of biological activity by gas production records and the latter allowed observation of the morphology and changes of the biota due to contact with contaminants. The methods were chosen to evaluate the influence of the two nanoparticles in aerobic wastewater samples under three concentrations. The concentrations were chosen according to the average levels found in wastewaters. Concentrations for nano-TiO₂ (25 nm, Sigma-Aldrich, United States) in solution were 10 µg.L⁻¹; 100 µg.L⁻¹; and 1,000

$\mu\text{g.L}^{-1}$. Concentrations for Fullerene (C_{60} , Sigma-Aldrich, United States) in solution were $1 \mu\text{g.L}^{-1}$; $10 \mu\text{g.L}^{-1}$; and $100 \mu\text{g.L}^{-1}$.

The aerobic wastewater samples were collected from a wastewater treatment works sited in Reading, United Kingdom. Samples were sampled randomly from six aeration lamellas following primary sedimentation. The wastewater physical and chemical parameters were measured at the wastewater works laboratory once the water was sampled from the lamellas. In order to compare the effects of the nanoparticles on the samples, blanks were sampled and analysed through SEM, and gas production. Blank samples consisted of wastewater sampled without nano- TiO_2 or fullerene C_{60} .

2.1 Gas production

Total gas production was measured using an Automatic Methane Production Testing System (AMPTS) equipment. The equipment was connected to a computer and a software recorded the gas produced during the experiment. The bottles were closed with the stir cap; when turned on, it stirred, thus homogenising the sample. Gas flowed in the tubes and was sent to cells. Each cell could hold 11 mL of gas. When 11 mL gas was collected, the cell lifted, released the gas contained in it and recorded a gas production of 11 mL. The analyses were performed in triplicates and in two batches of 10 bottles each. They were composed of samples of 180 mL wastewater and 20 mL stock solution, and a blank of 180 mL wastewater and 20 mL deionised water (DW). Gas production was expected to be presented in four stages, following the pattern of bacterial activity: lag (phase 1), log (phase 2), stationary (phase 3), and decay (phase 4) (20).

2.2 SEM Analysis

Scanning electron microscope analyses were carried out with an analytical field transmission SEM model JEOL JSM-7100F with a spatial resolution of 1.2 nm at 30 kV. Samples were placed in 5 mm x 7 mm silicon chips and fixed with 2.5% (v/v) glutaraldehyde in a PBS solution for 120 minutes, dried at ambient temperature, dehydrated with ethanol at three concentrations: 30%, 60% and 100%

v/v for 15 minutes then rinsed with deionised water. SEM micrographs were taken for samples at initial time, 30 and 360 minutes exposure.

3. Results and discussion

3.1 Effects of time and concentration of nano-TiO₂

Fig.1. Effect of nano-TiO₂ concentration on total gas production by microorganisms present in a wastewater sample

The comparison of gas production is presented in Fig.1. Results showed the total gas production of samples spiked with TiO₂ at concentrations of 10, 100, and 1,000 µg.L⁻¹ as a function of time compared with blanks (sample with DW only).

There were no records of gas production after 191 minutes. The Lag phase occurred for a short period of time, mostly less than 10 minutes. Then, microbial activity became enhanced in the log phase, followed by a stationary phase that lasted between 60 to 120 minutes; and finally biological activity stopped after 191 minutes exposure. Results indicated that the sample with 10 µg.L⁻¹ nano-TiO₂ was initially beneficial to gas production. The total volume of gas produced was 0.058 mL against 0.044 mL for the blank. The following concentration of 100 µg.L⁻¹ showed that the volume of gas produced

was 0.029 mL, which was slightly less than the blank. And finally, the highest concentration of nano-TiO₂ (1,000 µg.L⁻¹) was clearly shown to be toxic and inhibited entirely the biodegradation of the organic content since no gas production was measured.

The biodegradation process stopped entirely after 191 minutes which could be explained by the lack of oxygen in the bottles. Since the sampling bottles were closed in order to measure the gas produced, and no source of oxygen was introduced, the conditions could have become anoxic or anaerobic, as such it could have inhibited bacterial activity once aerobic microorganisms were predominant. SEM images of samples subjected to addition of nano-TiO₂ are presented in fig.2.

Fig.2. Effect of nano-TiO₂ concentration and time on microorganisms morphology using SEM.

Fig.2 illustrates the impact of different concentrations of nano-TiO₂ on the microbial characteristics present in the samples. It illustrates only a representative number of samples that have been studied by SEM. An EDX was run in order to detect the nanoparticles in the samples. However due to its small size of 25 nm they could not be detected by the equipment used.

In fig.2a (10 µg.L⁻¹/30 min) a *streptobacilli* is shown surrounded by organic matter. It showed little impact on the microbial community due to the addition of 10 µg.L⁻¹/30min. There were no indication of excessive EPS release, but it showed a number of small white spheres of approximately 100 nm. An energy dispersive x-ray (EDX) was run and showed it was made of carbon, oxygen and nitrogen, suggesting it was organic, which could indicate the release of bacterial spores. In Fig. 2b (10 µg.L⁻¹/360 min) microorganisms (mainly bacteria) of various shape were observed, including bacillus, rods and spiral-like after 360 minutes exposure. The micrograph showed a decreased level of organic and

inorganic matter in flocks, bacteria shapes and types were varied and there were higher counts of bacteria.

The effects of $100 \mu\text{g.L}^{-1}/30 \text{ min}$ in fig.2c were not significant, however there were minerals present in the samples, bacteria attached to organic and inorganic matter, varied-shaped bacteria including bacillus and coccus-like. The effects were also observed in fig. 2c as the arrow indicates an organic substance, which has been identified in other studies as EPS (15). The release of EPS could be explained by the behaviour of microorganisms to attempt to protect the outer part of the cell by limiting the interaction of the cell wall with a pollutant. This substance is also released once a microorganism attaches to other materials. In Fig. 2c a bacillus and rod-like bacteria attached to the substrate were observed, with the abundant presence of an organic substance which could be EPS. Images of $100 \mu\text{g.L}^{-1}/360 \text{ min}$ presented in fig. 2d did not show evidence of release of EPS, and illustrated the presence of mineral forms which EDX identified as Na and Cl, and few counts of calcium, carbon and oxygen. This suggests that NaCl and calcium carbonate were present in the sample. Bacteria were predominantly bacillus-like, showing that the bacillus species in this particular sample could potentially be resistant to nano-TiO₂.

The most drastic effects on microorganisms could be observed on samples at $1,000 \mu\text{g.L}^{-1}/30 \text{ min}$ (fig. 2e). Very few micro-organisms could be observed, which were mostly bacillus and spiral-like and appear to be attached to organic matter only. Organic matter was abundant, which indicated that it could not be bio-transformed. The few bacterial cells spotted appeared to have been disrupted. The observations on fig. 2e were in accordance to the findings of other studies presented in the literature review (21-22). The images were similar to observations of wastewater after various treatments aimed at to cause cell lysis. Thus, it could be implied that 1 ppm of nano-TiO₂ was toxic to aerobic heterotrophic microorganisms. Subsequently, samples after a six hour exposure (fig. 2f, $1,000 \mu\text{g.L}^{-1}/360 \text{ min}$) displayed the organic matter in the form of flocks, with little presence of the effects seen on fig. 2e. Bacillus-like bacteria were predominant and were mostly attached to organic matter. Images

of the sample exposed to 1ppm of nano-TiO₂ at a shorter time indicated that bacteria decreased in number and species when compared to the blank. However, at a longer time (360 minutes) the number of bacteria increased, in contrast to the effects observed when samples were exposed for thirty minutes to the nanoparticle. The decay of bacteria once nano-TiO₂ was added followed by recovery indicates that the addition of nano-TiO₂ was toxic and lead bacteria to release spores. Once conditions were more favourable, spores could have evolved into bacteria once again.

Finally, in order to better compare the impact of nano-TiO₂, blank samples with DW were analysed and results presented in fig. 2g and 2h. They displayed abundant amounts of inorganic material. An EDX analysis indicated the presence of sodium (Na) and chloride (Cl). There were organic matter dispersed in the sample and bacteria attached to it. Potential bacterial spores appeared as small white spheres in fig. 2g and 2h. Bacterial shape was varied – bacillus, rods, spiralled and coccus. Once the samples were placed in a warm bath, it allowed the bacteria to be active and biodegrade the organic material, utilizing the contents of the wastewater as a nutrient source. Hence the observation of flocks decreased with time as the bacterial count increased. The predominant bacteria in the samples were in the shape of potential aerobic bacteria (bacillus and spiral shape) due to the conditions the samples were held in. It was not possible to identify the types of bacteria through SEM observations only, as more sophisticated methods (e.g. PCR) would have been required for bacterial identification. However, it was clear that the bacteria present in the samples were heterotrophic aerobic (sample bottles were left open to allow aerobic degradation) and the organic matter content decreased as it was utilized as the main carbon source. The white spheres could be potential bacterial spores as a result of the primarily (chemical) treatment to the wastewater, which consists of the addition of coagulants salts in order to destabilize the zeta potential of wastewater and allow suspended particles to form flocks and precipitate. It could have been toxic to the biota and have caused bacteria to release spores in order to preserve the species (23).

3.2 Effects of time and concentration of fullerene (C_{60}) on CO_2 production

Figure 3. Effect of fullerene C_{60} concentration on total gas production by microorganisms present in a wastewater sample

Fig. 3 illustrates the total gas production from the samples at concentration of 1, 10, and 100 $\mu g.L^{-1}$ compared to the blank. Results showed that the lag phase lasted approximately 17 minutes for the sample exposed to 1 $\mu g.L^{-1}$ fullerene C_{60} . Then microbial activity commenced and produced 0.22 L of total gas, until it naturally stopped at approximately 83 minutes. The blank showed constant flow in the stationary phase for approximately one hour, producing 0.44 L of total gas between 0 and 83 minutes.

30 minutes exposure

360 minutes exposure

Fig. 4. Effect of fullerene C₆₀ concentration and time using SEM.

Fig.4 illustrates the effects of fullerene C₆₀ concentration and time on the morphology of the microbiological community using SEM. The effects of the lowest concentration of fullerene C₆₀ (1

$\mu\text{g.L}^{-1}$) were reported in fig. 4a and 4b. In Fig.4a there were bacteria attached to organic material. Their morphology appeared to be almost intact and have not suffered modifications after 30 minutes contact at $1 \mu\text{g.L}^{-1}$ fullerene C_{60} . In Fig.4b a sample that had been exposed for 360 minutes exhibited a streptobacillus-like bacteria in the centre. Bacteria of varied shapes, presented as sole cells or attached to organic and inorganic material were also observed. Comparison with the images obtained for the "blank", the addition of $1 \mu\text{g.L}^{-1}$ fullerene C_{60} did not cause disruption of cells or caused changes in the morphology of the sample.

Fig. 4c and 4d illustrate the effects of the addition of $10 \mu\text{g.L}^{-1}$ fullerene C_{60} when samples were exposed to 30 minutes and 360 minutes consecutively. A 30 minutes exposure (fig. 4c) showed irregular shapes of organic matter, and possible release of EPS. The effects were similar to those in fig.2e. It was observed that cells appeared to have suffered lysis, few bacteria could be counted, and the presence of sole bacteria could not be identified. Fig. 4d illustrates the results following a six-hour exposure to $10 \mu\text{g.L}^{-1}$ fullerene C_{60} . It could clearly be observed that bacillus and spiral-like bacteria were predominant in the sample. Few white spheres were identified to be organic, which could be bacterial spores. Organic and inorganic matter were identified in flocks using EDX. Vestiges of disrupted organic matter could also be observed in the samples after 30 minutes exposure.

Fig. 4e and 4f display the effects of $100 \mu\text{g.L}^{-1}$ fullerene C_{60} to wastewater microbiota. Fig. 4e displays dispersed organic and inorganic matter as being predominant in the sample following 30 minutes exposure. Few bacteria could be observed, which were mostly attached to a mineral compound identified by EDX analysis as being composed of sodium and chloride. EDX also pointed to the occurrence of organic material as white spheres. Fig. 4f displays similar effects to samples exposed for 360 minutes to $1,000 \mu\text{g.L}^{-1}$ nano- TiO_2 - the organic matter seemed to have decreased and isolated bacillus, rods and spiral-like cells were observed.

Exposure time was shown to have no negative effects on the microorganisms. Instead, longer exposure times assisted bacterial proliferation which could be explained by adaptation to the environment, and release of bacterial spores. The results showed that when the microbial sample was exposed to nanoparticles solution, the toxic effects were immediate but could be reversed once the microorganism adapted to the environment.

3.3 Implications of TiO_2 and C_{60} in WWTW

Several publications have been made linking ENPs with microorganism inhibition and/or decay. However this study showed that the implications of TiO_2 and C_{60} in WWTW were temporary since spores were formed and microorganisms thrived the toxic environment created by the addition of ENPs. If the stressed conditions to which the microorganisms present in wastewater are temporary then it can be implied, based on our results, that the biodegradation of organic matter in wastewater can still be achieved. On the other hand, if high concentrations of TiO_2 and C_{60} are kept constant then biodegradation might be affected due to the fact that mostly only spore-forming microorganisms survived the stressed environment.

4. Conclusions

This study is the first to show time related effects of two nanoparticles added to microorganisms from a WWTW using SEM observations and gas production as a measurement of biological activity. The addition of nano- TiO_2 and fullerene (100 and 1,000 $\mu\text{g.L}^{-1}$; 10 and 100 $\mu\text{g.L}^{-1}$ respectively) to wastewater microorganisms caused adverse effects at 30 minutes exposure time. Microorganisms recovered after a 360 minutes exposure, possibly due to the species were spore-forming. The lowest concentration of fullerene did not affect the microorganisms and 10 $\mu\text{g.L}^{-1}$ nano- TiO_2 addition favoured gas production. Overall, ENPs concentration levels and exposure time played an important role in the toxicity effects of microorganisms in WWTWs. Moreover, under stressed conditions the bacteria appeared to protect themselves via a spore forming mechanism that allows them to resist the

toxic environment. It is suggested to future authors to research the reasons for the reappearance of microorganisms since the nanoparticles remained in the solution, which lead us assumption that the microorganisms adapted in such a short time to the nanoparticle rich environment.

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6. References

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