WHAT IS THE FEEDBACK OF SEWAGE MICROORGANISMS TO NANOSTRUCTURES?

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Abstract

The growth in the application of nanomaterials (of any structure in any use) leads to increasing concentrations in the environment. These nanomaterials may also enter into wastewater treatment plants and interact with activated sludge, so at least partial knowledge of the behaviour of nanoparticles in the activated sludge is essential. This paper deals with the feedback of sewage microorganisms to nanostructures (nanoparticles or nanofibers). The main task was to observe the impact of nanostructures on the microorganisms based on microscopy access and image analysis procedures. The examination included changes in bacterial morphological properties (size and roughness), and the viability of the microorganisms. The experiments demonstrated an increase in the adhesion of the microorganisms to the nanofibers during the first few hours, a significant influence of the microorganism's viability in the presence of nanoparticles (reduced by up to 99 %); and a changeover of the bacterial morphological properties (dispersed into floc-forming clusters or vice versa) of the nanofibers and nanoparticles.

Keywords: Nanoparticles; nanofibers; sewage microorganism; wastewater treatment plant; image analysis

1. INTRODUCTION

Sources of nanomaterials (NMs) principally originate from every day products, including personal care (antiperspirants, body wash), clothing (cleaners), cosmetics, and many others. In addition, the use of these products has significantly increased. The potential hazard of releasing these NMs into the environment endangers ecosystems and also human health. The fate of NMs should be investigated carefully to minimize their negative environmental risk. [1, 2]

Wastewater treatment plants (WWTPs) are important barriers to prevent NMs from entering to the natural environment [3, 4]. A considerable amount of NMs can be removed via aggregation, settling, biosorption, or other biomass mediated processes in WWTPs, as biomass has a strong affinity to NMs and plays an important role in their removal [2]. Different forms of activated sludge (bacteria strains) may respond differently to the NMs. Biofilm (or floc-forming bacteria) was reported to be more tolerant to NMs (e.g. Ag, TiO_2, SiO_2) than planktonic/suspending sludge due to the protective function of extracellular polymeric substances (EPS) and the interactions within the microbial community [3, 5]. On the other hand, due to the properties of NMs (composition, size, shape, surface charge and capping molecules), the interaction of activated sludge with NMs may also influence the performance of WWTPs i.e. toxicity; NMs may attach to cell membranes and cause changes in membrane permeability; NMs may enter the bacterial cell and cause cellular enzyme deactivation, membrane permeability disruption, and accumulation of intracellular radicals, finally resulting in microbial growth inhibition, cell lysis and death [3, 6-9]. Unfortunately, there are still many unanswered questions regarding the fate of NMs and their impact on wastewater facility and treatment operations [1].

The aim of our study was to examine the basic effect of engineered NMs (i.e. nanoparticles [NPs] or nanofibers [NFs]) on sewage microorganisms. Two type of NMs, NPs (Ag, TiO_2) and NFs (polyurethane, 260 nm in

diameter), were selected for the experiments because they are among the most common NMs in use [8, 10-14]. Still not enough is known to predict the fate of NMs in the wastewater environment and further experiments are needed. Our results provide basic data to assess the fate of NMs in the wastewater environment and potential risks to the wastewater treatment system.

2. MATERIAL AND METHODS

2.1. Nanoparticles and nanofibers

Pure TiO_2 was supplied by VWR Chemicals, USA (EC number 215-280-1); the particles were crushed using a CryoMill "nano-grinder" (Retsch, Germany, **Fig. 1a**). Reduction of silver nanoparticles from silver nitrate was carried out using UV radiation (**Fig. 1b**). Nanoparticles were dispersed directly into the natural water medium containing sewage microorganisms in concentrations of 0.0, 0.2, 0.5 and 0.9 g/L. The experiment took place over six days, using 100 mL of working volume. The solution was intimately mixed to provide oxygen to the reactor and to keep the nanoparticles in suspension.

Polyurethane (electrospinning method, diameter approx. 260 nm, **Fig. 1c**) was tested as the NFs. The nanofiber carrier is composed of three parts: a basic (supporting) fibre composed of polypropylene and a coating composed of polyurethane nanofibers, which are both twice-wrapped in a protective polyethylene fibre for fixation. The experiment took place over 90 days, using 10 L of working volume containing 234 meters of nanofiber carrier. The NFs in the reactor were raised in the water using an air pump (fine-bubble aeration).

The final size of the particles was determined using scanning electron microscopy (Carl Zeiss Ultra Plus, Germany). Samples were deposited with a thin layer of gold.

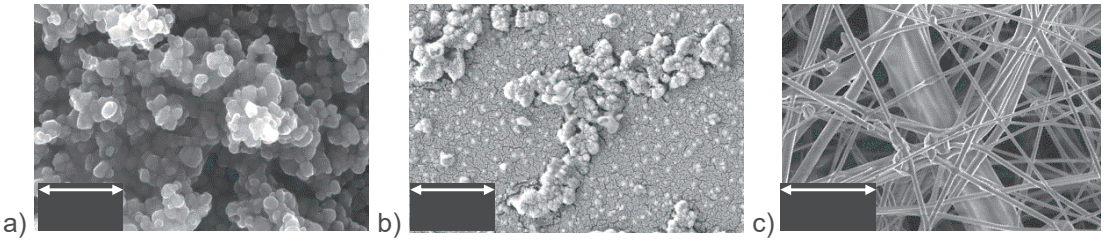


Fig. 1 SEM images of a) TiO_2NPs, b) Ag NPs, and c) polyurethane NFs

2.2. Sewage microorganisms

The activated sludge sample was collected from the aeration tank of a municipal wastewater treatment plant in the Czech Republic. The temperature was maintained at 22 °C ± 2 °C and pH was controlled at 7.0-8.0.

2.3. Determination of live/dead cells and degree of adhesion to nanofibers

The distribution of live/dead cells (evaluation of cell membrane integrity in the sludge or on the NFs before or after exposure of the NPs or NFs) was investigated through staining with fluorescence dyes using a LIVE/DEAD BacLight™ kit (Thermo Fisher Scientific Inc., Life Technologies) and observed using a ZEISS Axio Imager.M2 fluorescence microscope. Sample preparation was carried out according to the protocol of this kit. Images of at least 20 fields of view were taken along the samples and were analyzed (individual cells or aggregates, size, viability and roughness (Roughness is a parameter characterizing the smoothness of an object; if the perimeter changes according to the area, the roughness of the object is low (cell colonies contain no (or less) protuberances), if the roughness of the object is high it contains numerous protuberances (i.e. hairy objects).). Samples of the nanofibers with biofilm were deposited onto a microscope slide with the same staining kit. A set of approximately 10 images was taken on the NFs carrier. The biofilm characteristics (number of objects = individual biofilm colonies or cell clusters, viability, cell cluster size and roughness) were calculated.

3. RESULTS AND DISCUSSION

We calculated the basic parameters of abundance (number of objects/cells), viability, size and roughness of sewage microorganisms in contact with the NFs and NPs based on the image analysis procedures within the Matlab software (The Mathworks, Inc.).

3.1. Effects of nanofibers on the abundance, viability, size and roughness of sewage microorganisms

Cell fluorescence microscopy (**Fig. 2**) indicated that microorganisms response to the environment with NFs. The experiment demonstrated an increase in the adhesion of microorganisms to nanofibers during the first few hours, as indicated by the number of cell clusters on the NFs. During the experiment, the number of objects decreases as the biofilm fills the surface of the carrier (small colonies join into larger units; also see the parameter size of the cell cluster on the NFs). The colonies on the NFs have a low degree of roughness (the objects are smooth) because of shear forces (aeration) in the reactor during the entire experiment. The viability of cell clusters on the NFs changed marginally. The number of cells in suspension decreases, the suspension cells almost disappears at the end of the experiment. The size of cells in suspension firstly grows (small cell clusters adhere to the NFs, only large cell clusters remain in suspension), followed by a reduction in size (floc-forming bacteria disappear; only small single cells remain in suspension). The roughness of cell clusters in suspension follows the same course as the size (larger cell units have greater roughness; small cell units, single cells, are smoother). The viability of single cells was higher than the floc-forming cell clusters (inside of the larger flakes were dead cells possibly due to the formation of anaerobic zones, as showed in **Fig. 2b**).

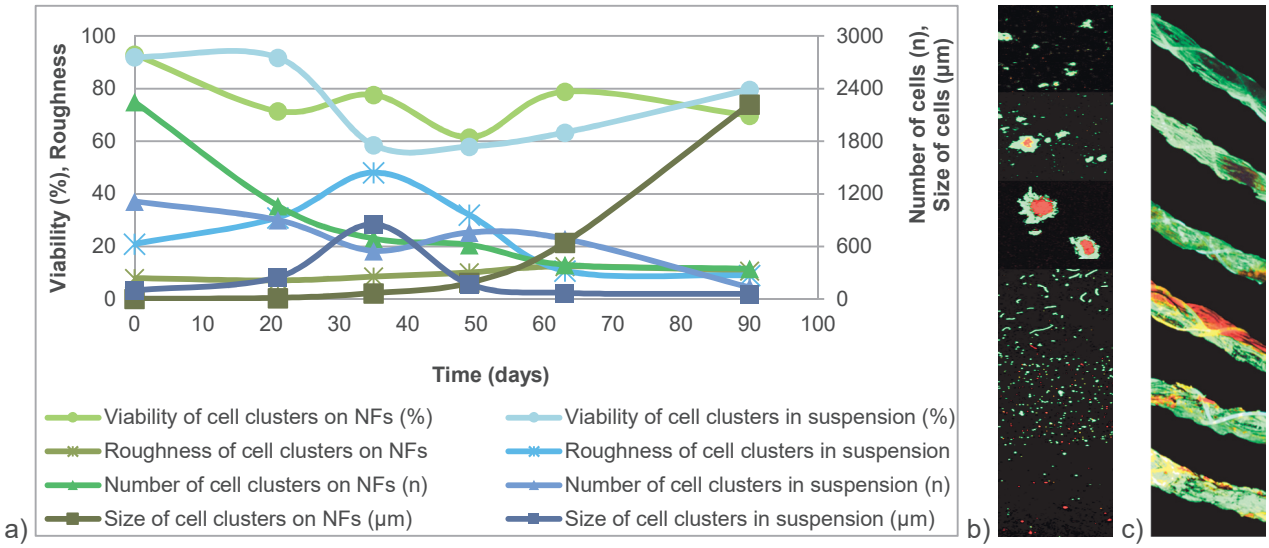


Fig. 2 a) Results of the image analysis of cell fluorescence microscopy; b) Live/dead staining of suspension cell clusters; c) Live/dead staining of cell clusters on NFs

3.2. Effects of nanoparticles of Ag and TiO_2 on the abundance, viability, size and roughness of sewage microorganisms

Cell fluorescence microscopy (**Fig. 3** and **Fig. 4** and **Table 1**) indicated that the response of microorganisms to the environment with NPs is dissimilar. The experiment demonstrated the decrease in cell abundance (number of live cell clusters) with an increase in NP concentrations for both types of NPs; however with different degrees. Ag NPs had a significant impact on live cells during the first six hours (acute toxicity) and the effect of NPs was also significant after six days of exposure; the viability corresponds to the abundance (conformity of approx. 75 %) and was reduced by up to 99 %. TiO_2 NPs has less of an influence on the live cells during the first six hours (only a concentration of 0.9 g/L has a significant power); after six days of exposure the effect

of the NPs was significant only for concentrations higher than 0.5 g/L; the viability corresponds to the abundance (conformity of approx. 85 %) and was reduced by up to 95 %.

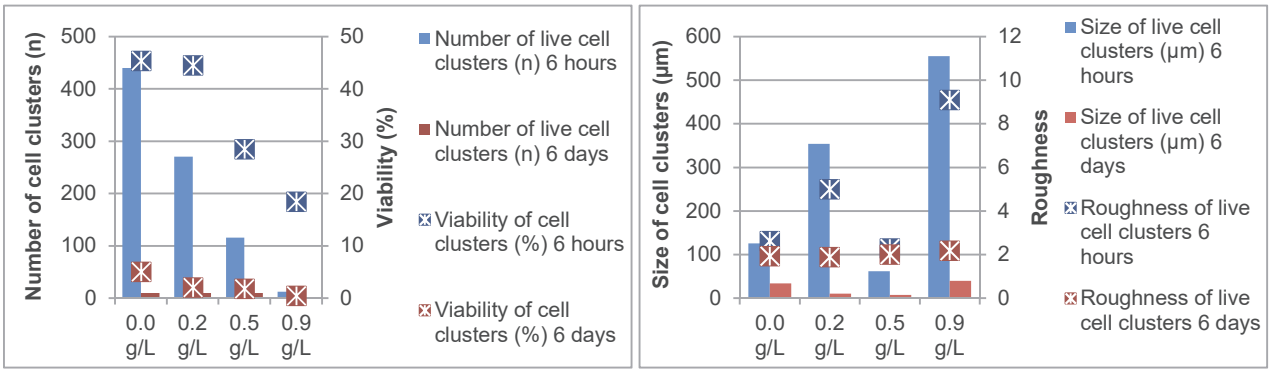


Fig. 3 Results of the image analysis of cell fluorescence microscopy for cells in the presence of Ag NPs

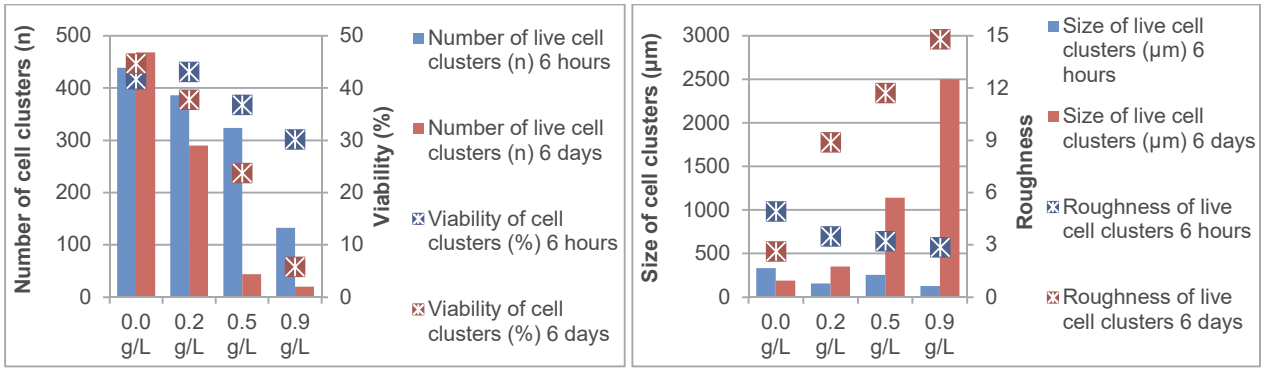


Fig. 4 Results of the image analysis of cell fluorescence microscopy for cells in the presence of TiO_2 NPs

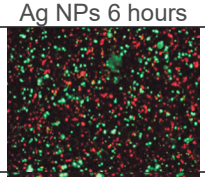
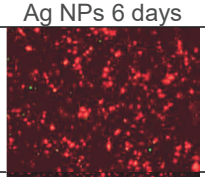
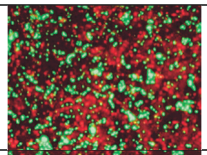
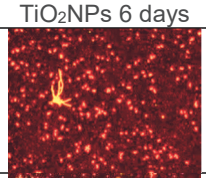
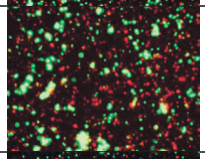
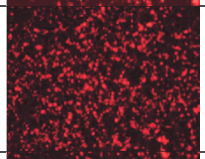
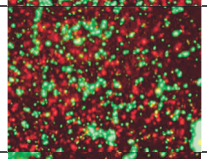
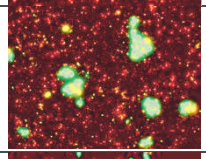
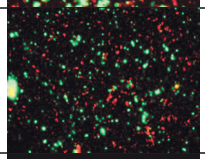
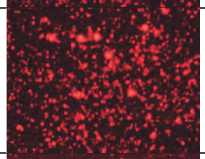
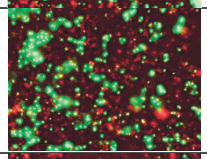
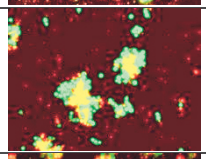
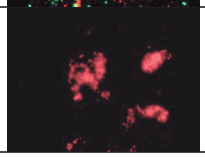
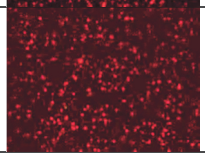
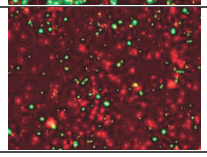
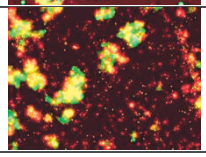
The decrease in cell abundance corresponds to the increase in cell size for Ag NPs during the first six hours, which confirms the significant influence of Ag NPs in the short-term (acute toxicity). The Ag NPs also influence the cell viability after six days; however, the size of the cells is more or less constant. The roughness corresponds to the cell size (conformity of approx. 70 %), whereby the larger cell clusters are rougher. The decrease in cell abundance corresponds to the increase in cell size for TiO_2 NPs after six days, which confirms the significant influence of TiO_2 NPs after long-term use (chronic toxicity) i.e. the influence of cell size was less during the first six hours. The TiO_2 NPs also influence the cell viability after six days and significantly for concentrations of NPs higher than 0.5 g/L, whereby the size of the cells greatly increases. The roughness corresponds to the cell size (conformity of approx. 70 %), whereby the larger cell clusters are rougher.

Moreover, the dead cells were mainly located in the outer layer of the floc-forming cells for concentrations of 0.0 and 0.2 g/L of TiO_2 and 0.0 g/L of Ag. More dead cells were found in the interior of the floc-forming cells for concentrations of 0.5 and 0.9 g/L of TiO_2 and 0.2, 0.5 and 0.9 g/L of Ag NPs. This observation suggests that Ag or TiO_2NPs can penetrate into the inner part of the cell clusters and can cause the toxicity to the interior cells. These findings agree with the conclusions of Gu et al. [3].

The analysis indicates that an increase in the concentration of TiO_2 NPs influences the abundance of bacteria in the sludge system; however, concentrations up to 200 mg/L have only a marginal influence (in accordance with [15], which also demonstrated that the presence of TiO_2 NPs shows marginal influences on the activities of key enzymes related to methane production and the abundances of bacteria and methanogenicarchaea in sludge fermentation reactors after long-term exposure for 150 mg/(g total suspended solids) of TiO_2 NPs). However, in some cases TiO_2 NP is nontoxic to certain aquatic microorganisms even at

20 g/L [16]. The results indicate that different cell strains (common in activated sludge) may show dissimilar responses to TiO_2 NPs. A reduction in biological activity by toxic nanoparticles could decrease the effectiveness of contaminant removal, and in the worst case a total failure of the biological process could be experienced.

Table 1 Live/dead staining of a suspension of cell clusters with Ag or TiO_2 NPs for different concentrations

	Ag NPs 6 hours	Ag NPs 6 days	TiO_2 NPs 6 hours	TiO_2NPs 6 days
0.0 g / L				
0.2 g / L				
0.5 g / L				
0.9 g / L				

Extracellular polymeric substances (EPS) in sludge flocs are usually considered to protect the inner microorganisms against demanding external environmental conditions by impeding the access of these pollutants to bacterial cells. The EPS protection may be the reason for the lower toxicity of TiO_2 NPs for microorganisms in sludge [15]. It still needs to be determined whether the bacteria in the activated sludge is protected significantly enough by the EPS and whether or not the toxicity is in the form of respiration when exposed to NPs. Moreover, SEM images can show whether the NPs may adsorb onto the activated sludge; these measurements will be the aim of our further work.

4. CONCLUSION

Nanoparticles have been found to inhibit (TiO_2) or even prevent (Ag) biological activity of cells in terms of the properties, concentrations and exposure time (up to 99 %) of NPs. Image analysis revealed that the decrease in cell abundance corresponds to an increase in cell clusters size; moreover the roughness corresponds to cell size (larger cell clusters are rougher); both probably due to the toxic effects of NPs (it may be a defence mechanism of the cells to protect the inner microorganisms). On the other hand, nanofibers can promote cell growth (cells adhere to nanofibers due to a suitable surface structure) and the biological activity and also the contaminant removal can be more effective.

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