RESPONSE TO SHOCK LOADS OF ENGINEERED NANOPARTICLES (TiO_2, SiO_2 OR ZrO_2) ON ACTIVATED SLUDGE MICROBIAL COMMUNITIES

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Abstract

The environmental impact of the use of engineered nanoparticles (NPs) is undeniable based on many studies. Considering that NPs end up in wastewater treatment systems, the potential impact of selected NPs (TiO_2, SiO_2 or ZrO_2) on the activated sludge, especially on *Microthrix parvicella* or *Nocardia* bacteria cells, was investigated using laboratory-scale batch reactors through short-term, 7-day exposure to 100, 200, 300 mg/L of NPs. Changes in the oxygen uptake rate (through respirometric measurements), viability of cell (fluorescence microscopy using a live/dead analysis), morphology of flocks or filaments (based on Gram staining and image analysis) and fluorescence in situ hybridization (FISH) of the activated sludge compared with the controls were investigated. The shock loads of NPs caused no lethal effects on activated sludge. The presence of NPs in the activated sludge (depending on the concentration) supported the presence of *Nocardia* cells in the case of TiO_2 (oxygen consumption was maintained); however, SiO_2 and ZrO_2 NPs caused a restriction in the activity of the sludge (oxygen consumption and *Microthrix parvicella* cell presence); viability was not significantly affected for any of the NPs or concentrations.

Keywords: Nanoparticle toxicity, activated sludge, microbial respiration, *Microthrix parvicella*, *Nocardia*

1. INTRODUCTION

The use of products containing nanoparticles (NPs) is significantly growing (antiperspirants, body washes, clothing cleaners, cosmetics, and others). The possible danger of releasing these NPs into the surroundings must be explored to recognize their harmful ecological hazard [1]. The first contact of NPs with microorganisms takes place in wastewater treatment plants [2]. Wastewater treatment plants (WWTPs) gather NPs from the inflow (production of domestic or industrial waste) [3]; and microorganisms in activated sludge are real sample cells not only model cells.

The risk of NPs is mainly due to their size, shape and reactivity [4]. A significant quantity of NPs can be reduced via aggregation [5], settling or biosorption [1]. Different forms of bacteria strains (in activated sludge) have differing responses to the NPs [6]. Floc-forming bacteria are more tolerant to NPs (e.g. Ag, TiO_2, SiO_2) than suspended bacteria in sludge, because of the extracellular polymeric substances (EPS) which have a protective function [7]. The presence of NPs in activated sludge influences the performance of the WWTPs as they cause changes in membrane permeability, they enter the bacterial cells and cause cellular enzyme deactivation, membrane permeability disruption, and accumulation of intracellular radicals, finally resulting in microbial growth inhibition, cell lysis and death [8].

SiO_2 NPs are employed in different areas as biomedical applications, in food packaging, and abrasives [9]. TiO_2 NPs have industrial and medical applications, i.e. solar cell technologies, self-cleaning surfaces of facades, paints, sunscreens, food additives and in environmental remediation [10]. ZrO_2 NPs show photocatalytic activity [11]. The concentration of NPs in wastewater is increasing because the use of these NPs is still growing.

In our experiment we used a mixed culture of microorganisms of activated sludge from a real wastewater treatment plant in the Czech Republic. The aim of these experiments was to examine the effects of selected NPs (TiO_2, SiO_2 and ZrO_2) on sewage microorganisms; especially focused on *Microthrix parvicella* and *Nocardia* species. Our results provide basic data on the influence of NPs on the wastewater sludge based on respirometric assessment, morphology of flocs through image analysis, viability of cells and fluorescence in situ hybridization.

2. MATERIALS AND METHODS

2.1. Nanoparticles (NPs)

NPs were provided by SkySpring Nanomaterials, Inc., Houston, USA. The characteristics of the NPs are as follows: Titanium dioxide NPs (TiO_2, anatase, 99.5 %, Product #: 7910DL), size 10 - 25 nm, white nanopowder, specific surface area 50 - 150 m^2/g, morphology: flat texture of the surface with smooth edges; Silicon dioxide NPs (SiO_2, 99.5 %, product #: 6807NM), size 15 - 20 nm, porous white nanopowder, specific surface area 640 m^2/g, morphology: porous and nearly spherical; Zirconium dioxide (ZrO_2, 99.9 %, Product #: 8512QI), size 20 - 30 nm, white nanopowder, specific surface area > 35 m^2/g, morphology: spherical.

2.2. Experiment set-up

A model water medium (BSM culture medium) was prepared according to [12] (with Tween 80 and Peptone of 4 g/L as a carbon source and other elements as K_2HPO_4, $(NH_4)_2SO_4$). The activated sludge (dry matter in concentration of 1 g/L) was obtained from a real municipal wastewater treatment plant (aeration tank) in the Czech Republic.

NPs (firstly 20 minutes sonicated in distilled water as a concentrated solution of 20 g/L) were dispersed into the model water medium containing sludge microorganisms to the final concentrations of 0.0, 0.1, 0.2 and 0.3 g/L. The experiment was run for 7 days; using 200 mL of working volume in 250 mL Erlenmeyer flasks, which were placed on a horizontal shaker to provide oxygen to the sludge and keep the NPs in suspension. A control sample was prepared in the same way but without the NPs. The temperature was within a range of 22 ± 2 °C.

2.3. Respirometry

A Micro-Oxymax respirometer (Columbus Instruments International, USA) was used according to EN ISO 9408, using inorganic NPs instead of the tested compounds. CO_2 release (produced by cells) and O_2 consumption were measured in hermetically sealed 500 mL flasks containing 200 mL of media, cultivated at 22 ± 2 °C under aerobic conditions and shaken continuously for seven days. Respiration provides first-hand information regarding the toxicity of a test substance to activated sludge respiration.

2.4. Microscopy and image analysis

Fluorescence microscopic assessment (LIVE/DEAD as well as FISH) was performed using a ZEISS Axio Imager.M2 microscope fitted with an AxioCamICc1 camera with a Colibri.2 fluorescent lamp. Image analysis was proceeding in Matlab (The MathWorks) as a ratio of green (live) and red (dead) object (bacteria cells) for LIVE/DEAD; and as a ratio of green (all cells) and red (*Microthrix parvicella* or *Nocardia* cells) for the FISH assessment.

2.5. LIVE/DEAD fluorescence analysis

The LIVE/DEAD BacLight™ kit was used for evaluating cell membrane integrity.

2.6. Fluorescence in situ Hybridization (FISH)

A total of 5 µl of the activated sludge sample was applied to microscope slides and dried for 10 min at 42 °C. Permeabilization of samples: the dried samples are overlaid with 100 µL of lysozyme at a concentration of 0.5 mg/mL and incubated for 20 min at room temperature, then they are washed with lysozyme and the slide is immediately immersed in ice-cold PBS (Phosphate Buffered Saline) for 15 seconds. Dehydration: The dried slide is gradually immersed in 50 %, 80 % and 96 % EtOH for 3 minutes each and then allowed to dry in a thermostat at 43°C for 5 minutes. In-situ hybridization: *Microthrix parvicella*: MPA645 (5´- CCG GAC TCT AGT CAG AGC - 3´), MPA60 (5´- GGA TGG CCG CGT TCG ACT - 3´), MPA223 (5´- GCC GCG AGA CCC TCC TAG - 3´). *Nocardia*: Gor596 (*Gordonia family*, 5´- TGC AGA ATT TCA CAG ACG C - 3´), G.am205 (*Gordonia amarae*, 5´- CAT CCC TGA CCG CAA AAG C - 3´), Spin1449 (*Skermania piniformis*, 5´- CCG CTC CCT CCC ACA AAG - 3´). Subsequently, 7 µL of citifluor was added to each sample for fixation due to its long-term stability.

2.7. Gram staining and microscopy

Gram staining (generally procedure) represents gram positive and gram negative microorganisms. ZEISS Axio Imager.M2 microscope monitors the structure and morphology of the microorganisms in the activated sludge. Image analysis (sum of flocks and porosity) was proceeding in Matlab (The MathWorks).

3. RESULTS

The same reduction of viability was observed in all of the samples in a similarly way. NPs did not significantly affect the viability of the activated sludge cells regardless of the type of NPs or their concentration (of up to 22 % in comparison to control sample), **Figure 1** and **Figure 2**.

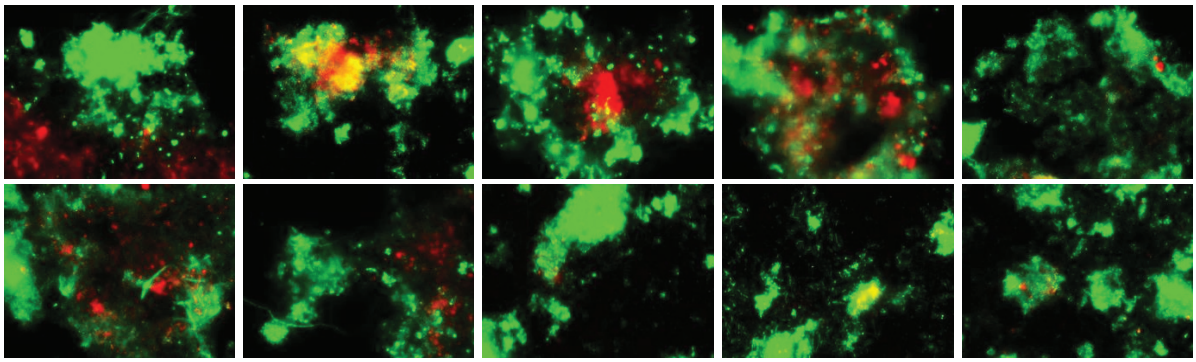

Figure 1 Documentation of cell viability of activated sludge with/without NPs (no NPs, TiO2 as 0.1, 0.2, 0.3 g/L, SiO2 as 0.1, 0.2, 0.3 g/L, ZrO2 as 0.1, 0.2, 0.3 g/L, respectively).

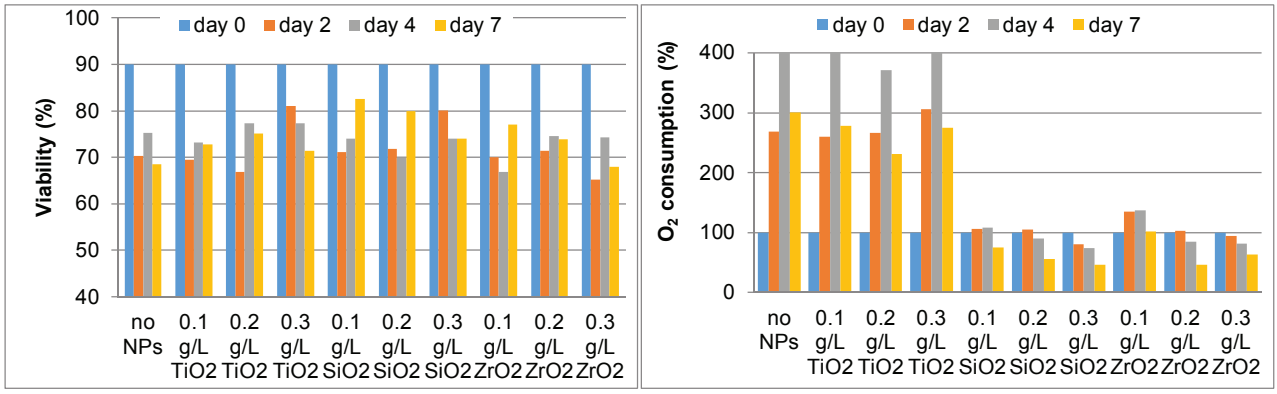

Figure 2 Result of the viability of cells and respirometric measurements of activated sludge with/without NPs

The highest increase in O_2 consumption (i.e. maximum respiration rate, **Figure 2**) was observed in the sample with no NPs (+200 % on day 7, compared to day 0). TiO_2 NPs showed a difference of +177, +131 and +175 % (0.1, 0.2 and 0.3 g/L respectively, on day 7), i.e. TiO_2 NPs showed no concentration effect, nor any significant effect compared to the control (sample with no NPs). SiO_2 NPs showed a difference of -25, -44 and -54 % (0.1, 0.2 and 0.3 g/L respectively, on day 7), i.e. SiO_2 NPs showed a slight influence of concentration and significant effect against the control sample (more than 100 %). ZrO_2 NPs showed a difference of +2, -54 and -37 % (0.1, 0.2 and 0.3 g/L, on day 7), i.e. ZrO_2 NPs showed no concentration effect, but had a significant effect compared to the control sample (over 100 %). Therefore, the sample with no NPs thrives on Tween 80 and peptone very well (respiration values were high). Samples affected by TiO_2 NPs have no effect on respiration; however, SiO_2 and ZrO_2 NPs affect the respiration rate significantly.

The presence of TiO_2 NPs in the activated sludge (depending on the concentration) supported the presence of *Nocardia* cells (**Figure 3, 4**) from 20 % to 30 %, *Microthrix parvicella* cells were mostly unaffected (the difference is within the range of 10 %). On the other hand, ZrO_2 NPs caused a mild restriction of *Microthrix parvicella* (from 5 % up to 20 % depending on the concentration) and also *Nocardia* cells (from 6 % up to 13 % depending on the concentration). SiO_2 NPs has differing effects depending on the concentration; 0.1 g/L of SiO_2 NPs supported *Nocardia* cells (40 %) as well as *Microthrix parvicella* cells (10 %); 0.2 g/L of SiO_2 NPs caused a mild restriction of *Microthrix parvicella* (7 %) and supported *Nocardia* cells (15 %); 0.3 g/L of SiO_2 NPs caused a restriction of *Microthrix parvicella* cells (23 %) and a mild restriction of *Nocardia* cells (9 %).

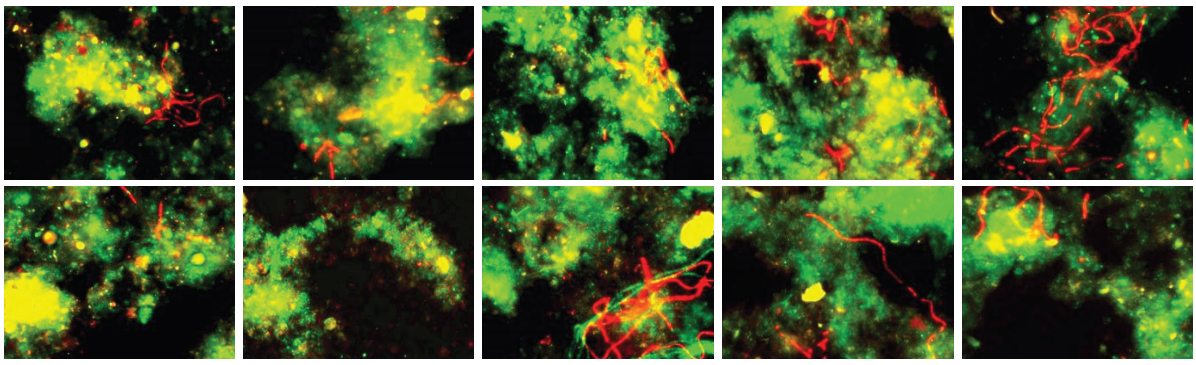

Figure 3 Documentation of FISH analysis of activated sludge with/without NPs (no NPs, TiO_2 as 0.1, 0.2, 0.3 g/L, SiO_2 as 0.1, 0.2, 0.3 g/L, ZrO_2 as 0.1, 0.2, 0.3 g/L, respectively)

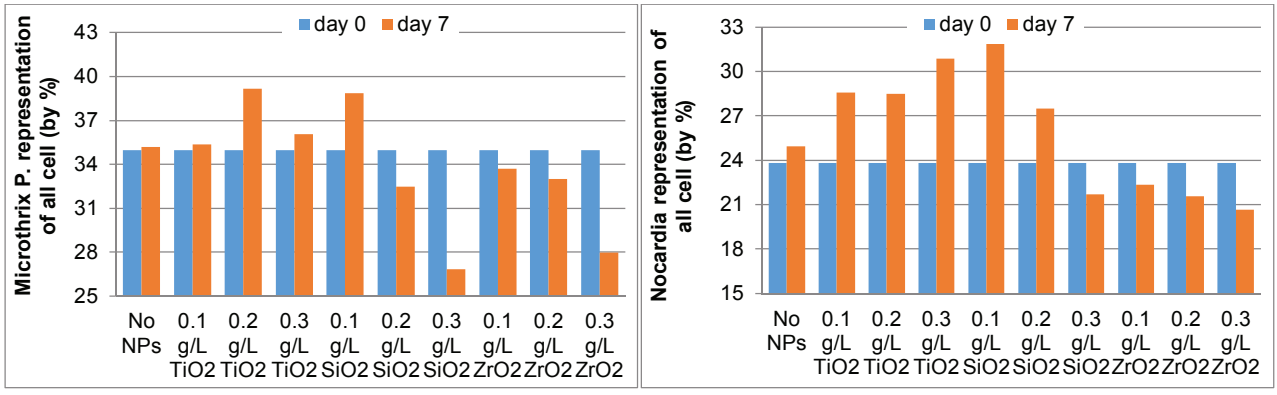

Figure 4 Results of the FISH analysis of activated sludge with/without NPs present

Flock porosity (**Figure 5**) is almost comparable to the control (with no NPs) for TiO_2 NPs within a range up to 20 % (day 7). The porosity of flocks affected by SiO_2 or ZrO_2 NPs was comparable at the beginning of the test (day 0 and day 2) but is lower in subsequent days (days 4 and 7); the flocks are less porous in the range of

40 % to 54 % for SiO_2 (compared to the control on day 7); flocks are less porous in the range of 38 % to 50 % for ZrO_2 (compared to the control on day 7).

The sum of flocks (**Figure 5**) for the TiO_2 NPs is almost comparable to the control sample (with no NPs), in the range of up to 11 % (on day 7). The sum of flocks with SiO_2 NPs is affected from the start of the test; the sum is higher for the increasing concentration of SiO_2 NPs. The sum of flocks is increased by 6 %, 92 % and 154 % (0.1, 0.2 and 0.3 g/L SiO_2 vs. the control on day 7). The sum of flocks with ZrO_2 NPs has been affected since the beginning of the test. The sum is higher than the control, however with a higher concentration is decreasing. The sum of flocks is higher by 88 %, 77 % and 53 % (0.1, 0.2 and 0.3 g/L ZrO_2 vs. the control on day 7).

The reduced respiratory activity (**Figure 2**) can be the result of low flock porosity (**Figure 5**) for SiO_2 and ZrO_2 NPs. Although the sum of flocks for ZrO_2 NPs is higher, it does not affect respiratory activity. None of the above facts affect the viability of the tested NPs i.e. the viability remains almost unchanged.

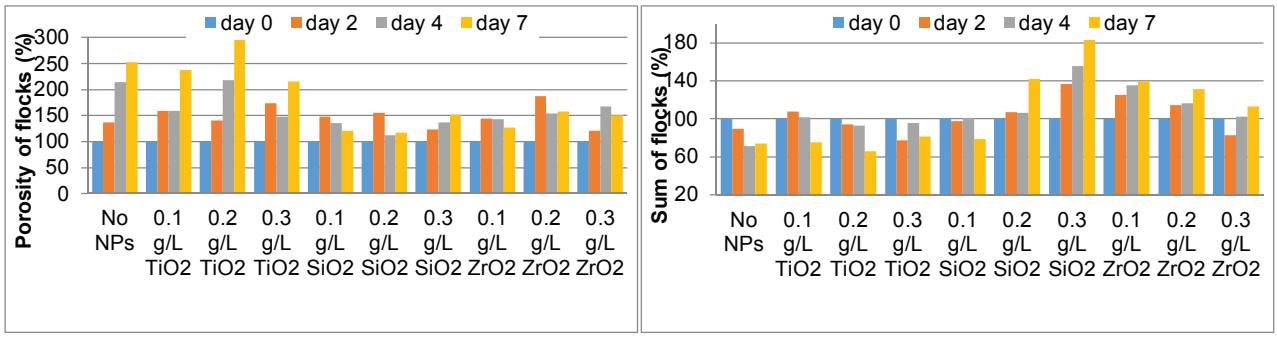

Figure 5 Result of the image analysis of the flock morphology (porosity and total sum) of activated sludge with/without NPs present

4. DISCUSSION

In [9] the inhibition of the oxygen uptake rate was 23 % (sizes of SiO_2 NPs of 12 nm); our results showed very strong inhibition of up to 97 %. In [6] the oxygen uptake rate of the activated sludge was reduced by 13 % (100 mg/L TiO_2 NPs); our results showed a slight reduction of 5 %. In [5] the smaller-sized NPs were more inhibitory than the large-sized NPs regardless of the concentrations; the degree of the inhibitory effect of TiO_2 NPs depended on the exposure time [5].

In [10] it was suggests that NPs may be adsorbed on and/or incorporated into the bacterial cell membrane without necessarily causing its disruption. However, in [13] the NPs destroyed the integrity of cytomembrane; this destruction could cause the variation of the morphology, which might alter the physiological functions of microorganisms in the activated sludge and affect the performance, as the slight difference caused by the NPs largely depended on exposure time rather than on NP type and NP concentration.

5. CONCLUSION

The shock loads of NPs caused no lethal effects on activated sludge (total viability was not significantly affected for any of the NPs or concentrations) but a very strong reduction of respiratory activity for SiO_2 and ZrO_2. The reduced respiratory activity can be also the result of low flock porosity for SiO_2 and ZrO_2 NPs. The presence of TiO_2 NPs in the activated sludge (depending on the concentration) supported the presence of *Nocardia* cells (up to 30 %) while oxygen consumption and *Microthrix parvicella* cells were maintained. ZrO_2 NPs caused a restriction in the activity of the sludge (oxygen consumption of up to 54 %); *Microthrix parvicella* cell presence (of up to 20 %) and *Nocardia* cell presence (of up to 13 %). SiO_2 NPs showed a significant restriction in oxygen consumption (more than 50 %) for all concentrations; while *Nocardia* cell presence is affected by the

concentration (0.1 g/L support of up to 40 %, 0.3 g/L restriction of up to 9 %); also the presence of *Microthrix parvicella* cells depends on the concentration (0.1 g/L support of up to 10 %, 0.3 g/L restriction of up to 23%).

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