Impact of TiO_2 nanoparticles on freshwater bacteria from three Swedish lakes

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Abstract

Due to the rapidly rising production and usage of nano-enabled products, aquatic environments are increasingly exposed to engineered nanoparticles, causing concerns about their potential negative effects. In this study we assessed the effects of uncoated titanium dioxide nanoparticles (TiO_2NP) on growth and activity of bacterial communities of three Swedish lakes featuring different chemical characteristics such as dissolved organic carbon (DOC) concentration, pH and elemental composition. TiO_2NP exposure concentrations were 15, 100, and 1000 μg L^{-1}, and experiments were performed *in situ* under three light regimes: darkness, photosynthetically active radiation (PAR), and ambient sunlight including UV radiation (UVR). The nanoparticles were most stable in lake water with high DOC and low chemical element concentrations. At the highest exposure concentration (1000 μg L^{-1} TiO_2NP) the bacterial abundance was significantly reduced in all lake waters. In the medium and high DOC lake waters, exposure concentrations of 100 μg L^{-1} TiO_2NP caused significant reductions in bacterial abundance. The cell-specific bacterial activity was significantly enhanced at high TiO_2NP exposure concentrations, indicating the loss of nanoparticle-sensitive bacteria and a subsequent increased activity by tolerant ones. No UV-induced phototoxic effect of TiO_2NP was found in this study. We conclude that in freshwater lakes with high DOC and low chemical element concentrations, uncoated TiO_2NP show an enhanced stability and can significantly reduce bacterial abundance at relatively low exposure concentrations.

Keywords:

Titanium dioxide nanoparticles, Natural lake water, Sweden, bacteria, DOC, UV radiation

1 Introduction

Nanotechnology is a rapidly growing industry and the steadily extending application of nano-enabled products reach from the technical-, medical, and research sectors, to a wide range of consumer products. The production of engineered nanoparticles (ENP) and nanomaterials is estimated to reach 58,000 tons within the next years (Maynard 2006). In 2005, the Woodrow Wilson International Center for Scholars listed 54 nano-enabled consumer products, while in 2013 this number had increased to over 1,600 (2014). Nano-sized titanium dioxide (TiO_2) is one of the most produced and used nano-materials, with typical use in solar cell technology, self-cleaning surfaces of facades, paints, sunscreen, food additive and environmental remediation (Weir et al. 2012). Recent studies have documented the release of nanoparticles from consumer products such as fabrics, paint and washing machines (Benn and Westerhoff 2008, Farkas et al. 2011, Kaegi et al. 2010, Kaegi et al. 2008). Kiser and coworkers reported incomplete removal of TiO_2NP in wastewater treatment plants, with concentrations of Ti in the effluents reaching from 10 – 100 μg L^{-1} (Kiser et al. 2009). Once released into the aquatic environment, TiO_2NP are expected to accumulate, with predicted environmental concentrations (PEC) ranging between 0.53 and 24 μg L^{-1} (Mueller and Nowack 2008, Tiede et al. 2009, Sun et al. 2014). Adverse effects of TiO_2NP have previously been reported on aquatic organisms such as fish, benthic organisms, zooplankton, and algae, with the toxic effects suspected to be triggered or enhanced by the presence of ultraviolet radiation (UVR) (Federici et al. 2007, Hund-Rinke and Simon 2006, Li et al. 2014a, Li et al. 2014b, Li et al. 2014c).

The stability of ENPs in the aquatic environment is dependent on environmental factors and nanoparticle properties. In aqueous ecosystems, dissolved organic carbon (DOC), pH and ionic strength influence particle stability (Christian et al. 2008, Keller et al. 2010, Ottofuelling et al.

2011). For example, DOC has been found to stabilize nanomaterials such as zinc sulfide nanoparticles, iron nanoparticles, fullerenes and carbon nanotubes (Baalousha et al. 2008, Chen and Elimelech 2007, Deonarine et al. 2011, Giasuddin et al. 2007, Keller et al. 2010). However, in high ionic strength environments bridging processes were observed in the presence of DOC, enhancing nanoparticle aggregation (Buffle et al. 1998, Liu et al. 2011). Such processes will affect bioavailability and thereby the toxicity of nanoparticles. However, the findings on the influence of DOC on nanoparticle toxicity differ between studies. Both, enhanced toxicity through nanoparticle stabilization, as well as a mitigated toxicity through reduced bioavailability of DOC bound nanoparticles or released ions were reported (Blinova et al. 2010, Fabrega et al. 2009, Hall et al. 2009, Yang et al. 2013).

Heterotrophic bacteria play a key role in freshwater ecosystems. Bacteria degrade and take up carbon from the DOC pool and their biomass forms the basis of the aquatic food web. However, bacterial communities are sensitive to disturbances, and alterations in community abundance and productivity may severely affect freshwater ecosystem functioning (Shade et al. 2012).

Previous studies reported adverse effects of TiO_2NP towards bacteria stream biofilms and soil bacterial communities (Battin et al. 2009, Ge et al. 2011). Toxic effects of TiO_2NP have also been reported for *Bacillus subtilis* and *Escherichia coli*, and they were enhanced in the presence of light (Adams et al. 2006).

The aim of the present study was to examine the effects of TiO_2NP on natural bacterial communities under different environmental conditions. Therefore, growth and activity of bacterial communities of three Swedish lakes, which feature different concentrations of DOC, pH conditions and elemental composition were assessed. In addition, the influence of light (UV radiation (UVR) and photosynthetically active radiation (PAR)) on the TiO_2NP effects was

studied. The *in situ* exposure further included the influences of other environmental factors such as light changes according to the diurnal cycle and water movement through wave action. Thus, the present study can provide valuable information on ecotoxicological effects of TiO_2NP in a realistic and environmentally relevant scenario. To our knowledge, the present study is the first ecotoxicological study assessing the effect of TiO_2NP on bacterial communities *in situ*, taking DOC concentrations and light regimes into consideration.

2 Materials and Methods

2.1 Experimental setup

We used a regrowth experimental setup to assess natural bacterioplankton community growth under different $TiPO_2$NP exposure concentrations under *in situ* conditions. For this, lake water from three lakes with different DOC concentration, pH and elemental composition was collected. A subsample of lake water was sterilized, amended with different concentrations of TiO_2NP and inoculated with the respective bacterial community. This allows testing the effects of TiO_2NP toxicity on bacterial growth without interference of nutrient release due to decaying bacteria or confusion of dead and viable microbes. The microcosm setups were transferred to sterile, UV-transparent bags and sealed. The microcosms were then placed in Lake Erken. To explore potential phototoxic effects of TiO_2NP, the light spectrum in the microcosms was manipulated to exclude all light (dark), to include photosynthetically active radiation (PAR) and to include PAR and UV radiation (UVR) as described in detail below (section 2.4). An overview over the experimental exposure groups is given in Figure 1.

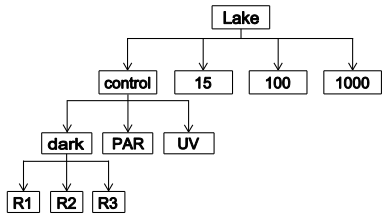

Figure 1. Experimental setup. Bacterial communities from three lakes, Lake Björklinge, Lake Erken and Lake Siggefora were isolated, and exposed to 0 (control), 15, 100, and 1000 µg L^{-1} TiO_2NP in their original lake water. The light regimes for the bacterial microcosms were manipulated to dark, photoactive radiation (PAR) and UV radiation (UVR). Each condition was present in triplicates (R1, R2, R3), resulting in *n*=108 microcosms.

2.2 Sample collection and preparation

Water samples were collected in early July 2011 from three Swedish lakes, Lake Björklinge (Björklinge Långsjön; 60°03'00"N 17°34'00"E), Lake Erken (59°51'00"N 18°34'00"E) and Lake Siggefora (Siggeforasjön; 59°58'00"N 17°08'00"E). The three lakes are located in central Sweden and their catchments are dominated by forest and rural areas. The lakes are circumneutral, mesotrophic, and dimictic. To isolate, cultivate and expose the bacterial communities, water samples from each lake were collected in acid-rinsed 50 L containers. Parallel to the water sampling, measurements of PAR attenuation in the respective lakes were taken at 0.5 m depth intervals with an IL 1400A radiometer (International Light, USA) connected a sensor for photosynthetically available radiation (PAR, 400-750 nm). Photosynthetically active radiation (PAR) was determined above the water surface, directly beneath the water surface, and subsequently every 1 m to the bottom in each of the three lakes. The DOC concentrations in the water samples were determined using a Sievers 900 TOC analyzer (GE Healthcare, Boulder, CO, USA) as non purgeable organic carbon. The pH of the

lake waters was analyzed with a pH meter Metrohm 744 (Metrohm Ag, Herisau, Switzerland). For determining the elemental composition, water was filtered through a 25 nm filter (Millipore Corporation, MA, USA) preserved with 0.1M HNO_3 and analyzed by high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS). Analyses were performed using a Thermo Finnigan model Element 2 instrument (Bremen, Germany). The radio frequency power was set to 1400 W. The sample introduction system (ESI, Elemental Scientific, Inc. Omaha, NE) consisted of a prepFAST sample/standard autodilution system equipped with S400V syringe pump and SC-2 DX autosampler. Samples were diluted on-line in ratio 4:1 v/v with internal standard consisting of 1 μg L^{-1} rhenium (Re) resulting in final flow 200 μL min^{-1} into the nebulizer. The instrument was equipped with a PFA-ST nebulizer, spray chamber (PFA Barrel 35 mm), demountable torch, quarts standard injector as well as Al sample skimmer and X-skimmer cones. The nebulizer argon gas flow rate was adjusted to give a stable signal with maximum intensity for the nuclides lithium (^{7}Li), indium (^{115}In) and uranium (^{238}U). Methane gas was used in the analysis to minimize interferences from carbon and to provide enhanced sensitivity. The instrument was calibrated using 0.1 HNO_3 solutions of matrix-matched multielement standards. The method was verified through the analysis of fresh water inter-comparison samples, SLP 13-22 (Blakseth 2014), the results of which are in good agreement with the reported average true values.

2.3 Nanoparticles

Stock dispersions of uncoated TiO_2NP in water at a concentration of 970 mg L^{-1} were purchased from Particular GmbH (Hannover, Germany). Measures of hydrodynamic diameter and zeta potential of the nanoparticles in the stock dispersion were provided by the manufacturer.

In order to determine the TiO_2NP crystal structure, X-ray diffraction analyses (XRD) were performed on dried samples. XRD measurements were conducted with a θ–θ Bruker D8-Advance DaVinci diffractometer (Bruker, Massachusetts, USA) utilizing Cu Kα radiation (wavelength of 1.54 Å) and equipped with a Lynxeye XE Superspeed position sensitive detector with 3 degrees opening. The scan was performed for the 2θ-range 15 to 95 degrees, in steps of 0.013 degrees and 1.15 sec/step.

Exposure concentrations of 15, 100, and 1000 μg L^{-1} were achieved by diluting the nanoparticle stock dispersions in the respective lake waters. The aggregation behavior and the interaction between nanoparticles and DOC in the three lake waters were investigated using energy filter transmission electron microscopy (EFTEM).Therefore, the TiO_2NP stock dispersion was diluted in MilliQ water and in samples from the three lakes to a final concentration of 1000 μg L^{-1} and then left for 5 days (equivalent to the exposure period). Subsequently, the dispersions were shaken and 100 μL of each sample was applied on carbon- coated copper grids (200 nm mesh). The samples were allowed to dry for several minutes to enable the attachment of the TiO_2NP and the remaining liquid was carefully removed. The nanoparticles were examined with a Zeiss Libra 120 EF TEM (Carl Zeiss AG, Germany) and the particle material identified by electron energy loss spectroscopy (EELS). The size of the particles was determined with the image processing and analysis software ImageJ (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/).

The hydrodynamic diameter of the particles in MilliQ water and the lake water samples were analyzed with dynamic light scattering (DLS, N5 submicron Particle Size Analyzer, Beckman Coulter Inc, CA, USA). The samples were filtered through a 200 nm filter prior to analysis.

Analyses were performed approximately 10 minutes after the addition of the particles to the respective water samples at a particle concentration of 100 mg L^{-1}.

The role of a potential ion release on the effects exerted by the particles on the bacterial communities was evaluated by adding TiO_2NP to the three respective lake waters at a concentration of 1000 µg L^{-1}, which represented the highest exposure concentration in this study. The samples were shaken at 50 rpm for 5 days (similar to the exposure duration), filtered through a 25 nm filter (Millipore Corporation, MA, USA). The filtrate was preserved with 0.1M HNO_3 and quantitatively analyzed for Ti by HR-ICP-MS (Element 2, Thermo Finnigan, Bremen, Germany) as described above.

2.4 Preparation of bacterial microcosms

The lake water samples were filtered through 0.2 µm polycarbonate filters (Supor, Pall, Sweden) and autoclaved for sterilization. In order to remove eukaryote predators from the inoculum, the lake water was screened through GF/F filters (Whatman, US). The microcosms were prepared by filling the sterile lake waters (300 mL) into UV-transparent polyethylene Bitran liquid-tight bags (Com-Pac) along with TiO_2NP to reach TiO_2NP exposure concentrations of 0 (control), 15, 100, and 1000 µg L^{-1}, respectively. Then, the bags were inoculated with 1 mL of the GF/F filtered bacterial inoculum, resulting in a starting abundance of approximately 3000 cells mL^{-1}. The bags were sealed and mounted horizontally on transparent buoyant racks. The racks were horizontally exposed beneath the lake surface, to allow for a high exposure to sun light. The solar radiation was manipulated to three levels; these were ambient light including PAR and UVR (UV radiation; the bags were uncovered), photosynthetically active radiation (PAR; the bags were covered with two layers of a UV cut-off foil (Ultraphan UV Opak, Digefra, Germany), and

darkness (dark; where bags covered with two layers of thick light-blocking black plastic). The microcosms were incubated for 5 days under sunny conditions in Lake Erken at 19°C water temperature. For each condition three replicates were incubated.

2.5 Bacterial abundance

Bacterial growth was determined as abundance in the water samples after the termination of the exposure period, which was analyzed by flow cytometry. Therefore the samples were preserved with 4% final concentration formaldehyde and stored at 4°C until they were analyzed using a nucleic acid stain (Syto13, Invitrogen) and flow cytometry (Cyflow Space, Partec, Germany) according to the protocol described previously (del Giorgio 1996). Gains for fluorescence (335) and side scatter signals (225) were adjusted to identify bacterial populations. To show relative changes in abundance, values were recalculated to the percentage (%) of the respective control (dark, PAR, UVR).

2.6 Bacterial activity

Heterotrophic bacterial activity was estimated by the incorporation of radioactive labeled L-[4,5-^{3}H] leucine into bacterial protein (Kirchman et al. 1985). This was achieved by incubating 1.7 mL of sample water with 20 nM leucine for 1 h under darkness. Leucine was prepared by a 1:10 dilution of radioactive leucine (1 mCi/mL, specific activity: 160 Ci mmole^{-1}, TRK 510, Amersham, Buckinghamshire, UK) with unlabeled leucine (Sigma Aldrich, St. Louis, USA). Blanks were treated with 90 µL 100% trichloroacetic acid (TCA). The incubations were terminated by addition of 90 µL 100% TCA and the samples were kept at 4°C. In the lab, the samples were cleaned with 5% TCA twice before 0.5 mL of the scintillation cocktail (OptiPhase

HiSafe 2, Perkin Elmer) was added. Radioactivity was measured as disintegrations per minute (DPM) with a liquid scintillation analyzer (Tri-Carb 2100TR, Packard, Perkin Elmer, Boston, USA). The DPM counts were converted to nmol leucine incorporated into bacterial biomass per hour assuming an intracellular isotope dilution of 2 (Simon and Azam 1989). The cell-specific bacterial activity was calculated as nmol leucine incorporation L^{-1} h^{-1} and normalized to the cell number. Relative changes in activity were calculated to % of the respective control (dark, PAR, UVR).

2.7 Statistics

The data were analyzed for differences in bacterial abundance and activity between exposed groups. Due to the non-normal distribution of the data, Kruskal-Wallis ANOVA by ranks and a 2 tailed p-test were applied to compare treatment groups. Data were analyzed using the software program Statistica 10.0 (StatSoft, Tulsa, OK, USA).

3 Results

3.1 Lake water characteristics

Concentrations of DOC were 6.7 mg L^{-1} in Lake Björklinge (low DOC), 11.4 mg L^{-1} in Lake Erken (medium DOC) and 16.7 mg L^{-1} in Lake Siggefora (high DOC) (Table 1). Correspondingly, PAR attenuation was highest in Lake Siggefora with a 1% penetration depth at 2.5 m depth, whereas in Lake Erken and Lake Björklinge 1% light penetration reached 8.3 m and 7.3 m, respectively (Supporting Fig S1). During the incubation period, sunrise was at 03:45 hrs and sunset at 22:00 hrs, resulting in up to 18 h of sunlight exposure per day. A stable clear

weather phase was observed during the experimental period, as also shown by a spatially-resolved model for solar radiation in the region (STRÅNG, SMHI) (Supporting Fig S2).

The lakes featured pH values between 7.19 (Lake Siggefora) and 8.23 (Lake Erken) (Table 1). The elemental composition between the lakes varied strongly. Concentrations of dissolved uranium (U), sodium (Na), magnesium (Mg), sulfur (S), chloride (Cl), and calcium (Ca) were highest in Lake Björklinge, followed by Lake Erken and were lowest in Lake Siggefora (Table 1).

Table 1. Lake water characteristics for Lake Björklinge, Lake Erken and Lake Siggefora. DOC is given as mean±SD (mg L^{-1}). Chemical element concentrations are given in µg L^{-1} and are rounded to three significant digits. Loq, below limit of quantification.

	Björklinge	**Erken**	**Siggefora**
pH	8.03	8.23	7.19
DOC	6.67±0.05	11.3±0.06	16.7±0.17
Cd	0.0903	loq	0.0271
Sn	0.0408	0.0252	0.099
Pb	0.116	0.009	0.184
U	20.9	4.26	0.477
Na	13400	7700	2450
Mg	9100	3540	1170
Al	3.73	0.657	81.7
Si	234	49.5	2420
P	1.01	3.64	1.06
S	24500	11400	150
Cl	21100	9320	2660
K	4030	2120	670
Ca	60600	41300	5100
Cr	0.0270	0.0314	0.253
Fe	0.124	0.447	102
Co	0.0290	0.0283	0.0185
Ni	0.790	1.35	0.684
Cu	2.26	1.88	8.89
Zn	4.25	0.555	8.11
Sr	128	66.0	17.5

3.2 Nanoparticle characteristics

The TiO_2NP were well dispersed in the stock dispersion. The hydrodynamic diameter of the particles in the purchased TiO_2NP dispersion was given by the producer as 70 nm (determined by DLS) with a zeta potential of -61.4 mV. Crystalline structure analysis (XRD) revealed that both, anatase and rutile particles were present (supporting information, Fig S4). Transmission electron microscopic analysis of TiO_2NP diluted in MilliQ water at a concentration of 1000 µg L^{-1} showed an average particle size of 59±36 nm (*n*=449). The size distribution of the particles determined with TEM is shown in Fig 2. The TiO_2NP were regularly spherical (Fig 3 a). The particle material was confirmed as Ti using electron energy loss spectroscopy (EELS; supporting information, Fig S3). In lake waters from Erken and Björklinge, the TiO_2NP were found to be mostly aggregated and complexed with either DOC or organic debris, and to mostly occur as aggregates (Fig 3, b, c). In contrast, the TiO_2NP in Lake Siggefora were mainly present as single particles or in groups of few particles (Fig 3, d). Dynamic light scattering measurements showed an average hydrodynamic diameter of 145 nm for the TiO_2NP in MilliQ water. In the lake waters, the hydrodynamic diameter was determined to be 318 nm, 522 nm, and 163 nm in Lake Björklinge, Erken, and Siggefora, respectively. However, despite filtering the samples prior to analysis, the background signal in the lake waters was high, and therefore there might be some impreciseness in the absolute numbers.

The concentrations of soluble Ti released from the TiO_2NP, here determined as the Ti < 25 nm following 5 days in the respective lake waters, were 2.8 µg L^{-1}, 0.280 µg L^{-1}, and 1.7 µg L^{-1} in Björklinge, Erken, and Siggefora, respectively. These concentrations accounted for 0.28%, 0.028% and 0.17% of the nominally added TiO_2NP concentrations, respectively. Electron

microscopic images revealed the presence of nanoparticles smaller than 25 nm, thus indicating a potential overestimation of ionic release.

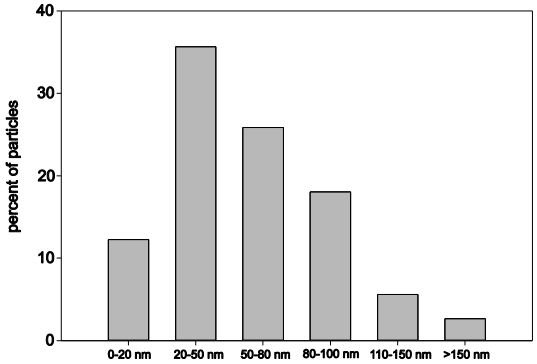

Figure 2. Size distribution (%) of TiO_2NP in MilliQ water determined by TEM, *n*=449.

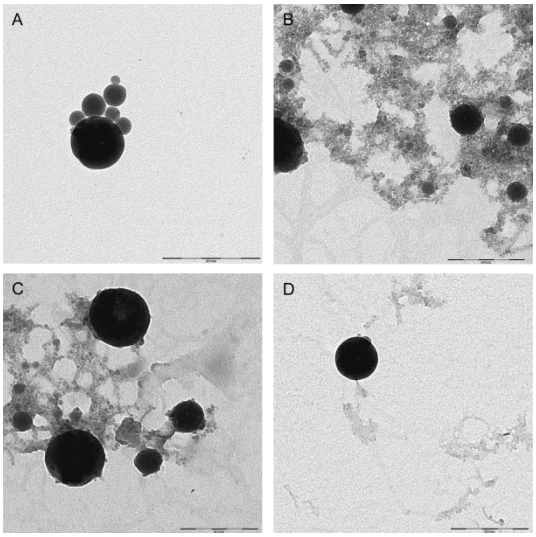

Figure 3. Typical TEM images of TiO_2NP in A) MilliQ water, B) water from Lake Björklinge, C) Lake Erken and D) Lake Siggefora. Scale bar: 200 nm.

3.3 Bacterial abundance after TiO_2NP exposure

Bacterial abundance in the control groups differed significantly among the lakes (ANOVA, $p<0.01$). The bacterial abundance in the control group from Lake Björklinge was 5.4 x 10^6 cells mL^{-1} , which was significantly lower ($p<0.001$ and $p=0.0054$) than in controls from Lake Erken and Lake Siggefora, which ranged between 9 and 10 x 10^6 cells mL^{-1}. There was no difference between controls from Lake Erken and Lake Siggefora ($p=0.98$).

The bacterial communities of all three lakes were affected by the exposure to TiO_2NP in a dose dependent manner. In Lake Björklinge, the bacterial abundance was the least affected, and was only significantly reduced at a concentration of 1000 µg L^{-1} TiO_2NP (ANOVA, $p=0.019$), while in Lake Erken and Lake Siggefora significant decreases in bacterial abundance were also identified at concentrations of 100 µg L^{-1} TiO_2NP (ANOVA, $p=0.013$ and $p=0.02$, respectively). In Lake Björklinge, the relative bacterial abundance compared to the control group increased in the 15 and 100 µg L^{-1} treatments to on average 105% and 111%, respectively, while abundance decreased on average by 36% in the highest exposure group (Fig. 4). In Lake Erken, all exposures led to a decrease in bacterial abundance, which was on average 0.6%, 27%, and 39% for 15, 100, and 1000 µg L^{-1}, respectively (Fig 4). In Lake Siggefora, the relative decrease in abundance was on average 7.6%, 28%, and 52% for the TiO_2NP exposures compared to the control (Fig 4).

3.3.1 Influence of light

In Lake Björklinge, the bacterial abundance in the control groups was significantly reduced ($p=0.022$) in the presence of UV light (5.4 x 10^6 to 3.6 x 10^6 cells mL^{-1}). The presence of PAR or UV did not enhance TiO_2NP toxicity, however, in Lake Björklinge a slightly reduced TiO_2NP toxicity was observed in the UV-exposed groups at 1000 µg L^{-1}.

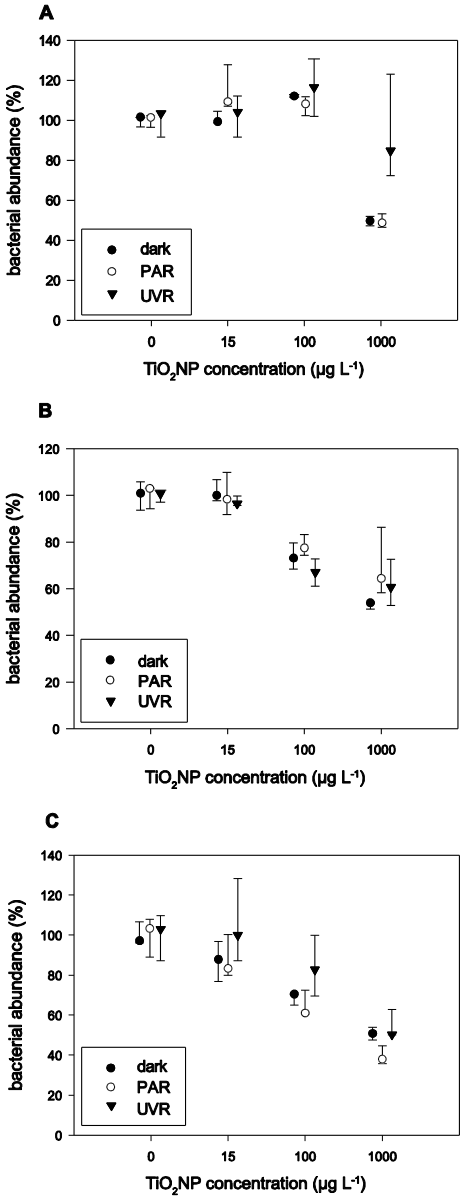

Figure 4. Bacterial abundance after 5 day exposure to control (0), 15, 100 and 1000 µg L^{-1} TiO_2NP in A) Lake Björklinge, B) Lake Erken and C) Lake Siggefora water, respectively. Different light regimes are shown as • dark, o PAR and ▾ UV. Data are normalized against controls of their respective light regime. Median±75^{th} and 25^{th} percentile; *n*=3.

3.4 Bacterial activity after TiO_2NP exposure

The bacterial activity in the control groups differed significantly among lakes (ANOVA, $p<0.01$). The highest activity was found in Lake Siggefora (0.11 nmol leucine L^{-1} h^{-1}), followed by Lake Erken (0.06 nmol leucine L^{-1} h^{-1}), and Lake Björklinge (0.02 nmol leucine L^{-1} h^{-1}). The total bacterial activity was affected by TiO_2NP only in Lake Siggefora, where a significant increase in activity was observed in the 1000 µg L^{-1} treatment group compared to control and 100 µg L^{-1} (ANOVA, $p<0.05$).

The cell-specific bacterial activity in the control group was highest in Lake Siggefora, followed by Lake Erken, with Lake Björklinge showing the lowest activity per cell. A comparison including all lakes and light regimes showed that at an exposure concentration of 1000 µg L^{-1} TiO_2NP, the cell-specific bacterial activity was significantly enhanced compared to the control groups (ANOVA, $p=0.008$) and to the 15 µg L^{-1} TiO_2NP exposure groups (ANOVA, $p=0.01$).

In Lake Björklinge, the cell specific activity was significantly higher in the 1000 µg L^{-1} TiO_2NP exposure group compared to the control group (ANOVA, $p=0.025$), 15 µg L^{-1} TiO_2NP (ANOVA, $p=0.0017$), and 100 µg L^{-1} TiO_2NP ($p=0.0014$) exposure groups. In Lake Siggefora, the cell specific activity was also significantly higher in the 1000 µg L^{-1} TiO_2NP exposure group compared to the controls (ANOVA, $p<0.001$) and the 15 µg L^{-1} exposure group ($p<0.001$), but not the 100 µg L^{-1} group. For Lake Erken, no differences were found between the exposure groups. Effects of nanoparticle exposure on bacterial activity in the lakes are shown in Fig. 5.

3.4.1 Influence of light on bacterial activity

The light regime did have an influence mostly in the low DOC lake, Lake Björklinge, where bacteria incubated in darkness had a significantly lower total activity compared to the UV-exposed group for the control, 15 µg L^{-1}, and 100 µg L^{-1} (ANOVA, $p=0.034$) exposures, but not

for 1000 µg L^{-1}. In Lake Erken, the light regime caused a significant difference between dark and PAR (ANOVA, p=0.034) at the highest TiO_2NP exposure concentration (Fig 5).

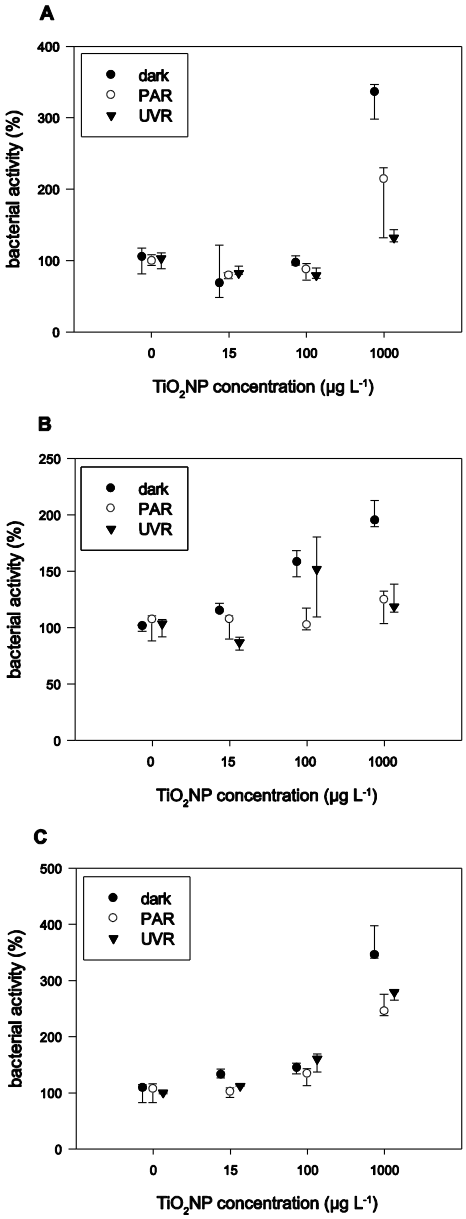

Figure 5. Cell specific bacterial activity after exposure to control (0), 15, 100 and 1000 µg L^{-1} TiO_2NP for 5 days in A) Lake Björklinge, B) Lake Erken and C) Lake Siggefora water,

respectively. Different light regimes are shown as • dark, o PAR and ▾ UV. Data are normalized against controls of their respective light regime. Median±75th and 25th percentile; *n*=3.

4 Discussion

The results of this study show that TiO_2NP can affect natural lake water bacterial communities in terms of bacterial abundance and bacterial activity. The impact of TiO_2NP varied among the three lakes, which featured different water characteristics such as DOC concentration, pH and elemental composition. Significant reductions in bacterial abundances were found at exposure concentrations of 100 µg L^{-1} Lake Siggefora and Lake Erken, and at 1000 µg L^{-1} in Lake Björklinge.

The effective concentrations in this study were low compared to in previous studies. For example, Heinlaan et al. (2008) found no TiO_2NP toxicity towards *Vibrio fischeri* below 20 g L^{-1} (Heinlaan et al. 2008). Furthermore, Adams et al. (2006) reported toxic effects of TiO_2NP on *Escherichia coli* and *Bacillus subtilis* at exposure concentrations of 500 and 1000 mg L^{-1}, respectively (Adams et al. 2006). The comparably low effect-concentrations determined in the present study as compared to some previous studies may derive from differences between nanoparticles, however, could also indicate that natural bacterial communities are more sensitive towards TiO_2NP than single strains due to differences in resistance. In complex bacterial communities, the sum of effects on different strains will determine the overall effect on bacterial abundance and productivity. Factors that are toxic to a subset of the community might negatively affect overall community functioning by altering interactions among strains. Furthermore, mortality of one strain may favor blossoming of others. Battin and co-workers (2009) reported enhanced cell damage in free-living planktonic cells and biofilms after a 24 h exposure to two

types of TiO_2NP at 5.3 mg L^{-1} (Battin et al. 2009). Further, TiO_2NP reduced soil microbial biomass and caused changes in community structure at an exposure concentration of 0.5 mg g^{-1} soil after 60 days (Ge et al. 2011).

In our study, the toxicity of TiO_2NP differed between the bacterial communities from the three lakes. The most pronounced toxicity in terms of reduction of bacterial abundance was observed for the bacterial community of Lake Siggefora: Although not significant, a slight reduction was observed at an exposure concentration of 15 µg L^{-1} of TiO_2NP. This is around the range of predicted environmental concentrations of TiO_2NP for the aquatic environment (Mueller and Nowack 2008, Tiede et al. 2009, Sun et al. 2014). It should also be noted that Kiser et al. (2009) found Ti concentrations of 10 to 100 µg L^{-1} in wastewater treatment plant effluents, which corresponds to concentrations at which we observed significant impacts on two of the three lake water bacteria. Thus, the results from the present study strongly indicate that TiO_2NP can affect bacterial communities (or abundances) at concentrations that are environmentally relevant.

The Lake Björklinge bacteria community appeared to be most resistant to TiO_2NP exposure. At the highest exposure concentrations the effects in terms of abundance reduction were similar for all lakes. The differences in effects at the lower exposure concentrations were strongly linked to the distinct water chemical parameters, especially DOC concentrations and elemental composition in the lakes with the effects being greatest in Lake Siggefora, featuring high DOC and low chemical element concentrations.

This is likely linked to the particle stability, as the TiO_2NP were most stable in Lake Siggefora. Organic matter has previously been reported to provide steric stabilization to engineered nanoparticles, thereby reducing aggregation and settling out of nanoparticles in natural

freshwater systems (Petosa et al. 2010). In a previous study NP stability was modelled for 6 water classes featuring different parameters, concluding that NPs have a higher stability in waters with high DOC and a low ion concentration (Lake Siggefora characteristics comparable to class II lakes), compared to a lower NP stability in waters with low DOC and a high ion concentration (Lake Björklinge comparable with class V lakes) (Hammes et al. 2013).
However, we also observed that nanoparticles appeared to be least attached to DOC in the highest DOC water. This may be attributed to differences in DOC composition among the lakes. In Lake Siggefora DOC is dominated by allochthonous sources, such as soil or peat. This DOC typically contains high concentrations of humic substances, which cause the brown color of such lakes. In contrast, DOC in Lake Erken and Lake Björklinge is dominated by internal production. However, to resolve the mechanisms of TiO_2NP interaction with DOC further investigations are required.

Analyses of lake water characteristics further revealed that elemental concentrations of dissolved Na, Mg, Cl, and Ca were lowest in Lake Siggefora, medium in Lake Erken and highest in Lake Björklinge (Table 1). The presence of monovalent and especially divalent ions was previously reported to enhance nanoparticle aggregation and agglomeration processes (Huynh and Chen 2011, Keller et al. 2010, Ottofuelling et al. 2011, Sillanpää et al. 2011). A major influence of pH on nanoparticle stability was not found in our study. Furthermore, the isoelectric point of TiO_2NP was previously reported to be between pH 4.8 and 6.25 (Guzman et al. 2006, Suttiponparnit et al. 2011), therefore the nanoparticles should carry a negative surface charge in all lake waters in this study.

In the present study, we found that both, the TiO_2NP stability and the TiO_2NP toxicity were highest in the high DOC lake water. The influence of DOC on the toxicity of nanoparticles varies between studies. An increased toxicity of Cu nanoparticles the presence of DOC in a bacterial-enzyme toxicity test was previously reported (Gao et al. 2009). Yang and co-authors found that the presence of humic acid increased the TiO_2NP toxicity towards developing zebrafish (*Danio rerio*) (2013). However, it should be noted, that, despite observing a stabilizing effect of Suwannee River Humic Acid (SRHA) on silver nanoparticles (AgNP), Fabrega and co-workers reported a reduced toxicity towards *Pseudomonas fluorescens* in the presence of SRHA (Fabrega et al. 2009). This may indicate differences among various types of NPs in respect to the interaction and effects of DOC on their toxicity. Especially the DOC-complexation of released ions, which is relevant for AgNP toxicity, could account for such differences. In contrast, ionic release from TiO_2NPs was seen to be low in the present study. In toxicity tests with aquatic invertebrates such as *Daphnia magna* and *Ceriodaphnia dubia*, reduced NP toxicity in the presence of organic matter was reported (Blinova et al. 2010, Gao et al. 2009, Kennedy et al. 2012). However, larger aggregates are probably taken up more efficiently by invertebrate organisms than single particles. In planktonic bacteria, uptake or interaction with single, non-aggregated particles may thus be responsible for the enhanced effect identified in high DOC water as compared to low DOC water.

The influence of the light regime on the bacteria abundance and activity varied among lakes. In the low DOC lake, Lake Björklinge, the control groups were affected by the presence of UVR, resulting in reduced bacterial abundance. This is in agreement with the study by Lindell and coworkers, who reported increased effects of solar radiation on bacterioplankton production in

clear lakes compared to humic lakes in Southern Sweden (Lindell et al., 1996). In the present study, the light regime did not have a major influence on the toxicity of the TiO_2NP, and therefore a phototoxic effect of the TiO_2NP used in this study could be ruled out. In contrast, Zuang and co-workers reported an UV induced photokilling in the presence of TiO_2NP of 5 different bacteria suspensions (Tsuang et al. 2008). Further, Miller and co-workers reported enhanced toxic effects of TiO_2NP and the formation of radicals in the presence of UVR in a study exposing marine phytoplankton (Miller et al. 2012). The different findings between studies can be due to differences in the TiO_2NP crystalline structure, as TiO_2 in anatase form is reported to exhibit strong photocatalytic activity, which is not found in its rutile form (Augustynski 1993, Xu et al. 2011). Analysis showed that TiO_2NP used in this study were of a mixed crystalline structure, with both, anatase and rutile particles present. This might at least partly explain the lack of phototoxic effects. Furthermore, in contrast to most other studies, the bacteria in the present study were exposed to natural sunlight under *in situ* conditions. Thus, the light regime followed a natural day-night cycle and the microcosms experienced reduced light exposure due to changes in the solar zenith angle (Madronich 1997), which may allow for repair of damaged cell components to a certain extent.

The light regime influenced the bacterial activity in control groups in the low DOC lake. The higher overall productivity in the UV exposed microcosms in the low DOC lake can be potentially explained by photodegradation of DOC and a resulting enhanced availability under carbon limitation. In the presence of UV radiation, larger, less metabolically available carbon molecules might be broken up which may allow for enhanced productivity of bacteria as compared to dark conditions.

Despite the reduction of bacterial abundance following nanoparticle exposure, the overall bacterial activity did, in most cases, not change significantly, which was due to a strongly enhanced activity per cell in the high TiO_2NP exposure groups. This indicates the presence of bacterial groups which are more resistant to TiO_2NP toxicity, or are even stimulated in the presence of TiO_2NP. This relative stimulation by TiO_2NP could be based on the removal of competitors from the community; however studies investigating the effects of TiO_2NP exposure on bacterial community composition are necessary to understand these mechanisms. Changes in bacterial community structure when exposed to AgNP have been previously reported (Das et al. 2012, Doiron et al. 2012). A reduction in abundance and number of Operational Taxonomic Units in a marine bacterial community following AgNP exposure was described by Doiron et al. (2012). Das and co-workers observed AgNP intolerant, - recovering, - tolerant and - stimulated bacterial groups in their experiment (Das et al. 2012). However, they reported a reduction in cell-specific bacterial activity after a 5-day exposure, which is in contrast to our study, showing enhanced activity rates under *in situ* conditions. Changes in community structure were also observed for soil bacteria following exposures to TiO_2, ZnO, Ag and Cu nanoparticles (Ge et al. 2011, Kumar et al. 2011).

5 Conclusions

Our work shows that TiO_2NP significantly affected natural lake water bacteria, with the effects varying among communities from lakes featuring different water chemical parameters. According to our study, TiO_2NP effects are strongest in lakes with DOC concentrations exceeding 16 mg L^{-1} and with low chemical element concentrations, leading to high particle

stability. Thus, we conclude that environmental characteristics should be considered in toxicity studies investigating effects of TiO_2NP for accurate risk assessment.

Acknowledgements

The authors would like to thank Kristin Høydalsvik for conducting XRD analysis and Syverin Lierhagen for HR-ICP-MS analysis.

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