SCIENTIFIC REPORTS

OPEN

Assessment of agglomeration, co-sedimentation and trophic transfer of titanium dioxide nanoparticles in a laboratory-scale predator-prey model system

Received: 30 December 2015

Accepted: 20 July 2016

Published: 17 August 2016

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Nano titanium dioxide (nTiO₂) is the most abundantly released engineered nanomaterial (ENM) in aquatic environments. Therefore, it is prudent to assess its fate and its effects on lower trophic-level organisms in the aquatic food chain. A predator-and-prey-based laboratory microcosm was established using *Paramecium caudatum* and *Escherichia coli* to evaluate the effects of nTiO₂. The surface interaction of nTiO₂ with *E. coli* significantly increased after the addition of *Paramecium* into the microcosm. This interaction favoured the hetero-agglomeration and co-sedimentation of nTiO₂. The extent of nTiO₂ agglomeration under experimental conditions was as follows: combined *E. coli* and *Paramecium* > *Paramecium* only > *E. coli* only > without *E. coli* or *Paramecium*. An increase in nTiO₂ internalisation in *Paramecium* cells was also observed in the presence or absence of *E. coli* cells. These interactions and nTiO₂ internalisation in *Paramecium* cells induced statistically significant ($p < 0.05$) effects on growth and the bacterial ingestion rate at 24 h. These findings provide new insights into the fate of nTiO₂ in the presence of bacterial-ciliate interactions in the aquatic environment.

Engineered nanomaterials (ENMs) are used in diverse applications, owing to their unique optical, chemical, mechanical, thermal, magnetic and catalytic properties¹. Currently, more than 1800 nano-based consumer products derived from 45 different ENMs are manufactured globally². ENMs can enter into the environment at various stages in their life cycle: production, manufacturing, transportation, consumer use and product disposal^{3–5}.

Nano titanium dioxide (nTiO₂) is one of the most abundant materials in products such as cosmetics, paints, food additives, pharmaceuticals, electronics, and textiles as well as in construction and wastewater treatment^{6–8}. Moreover, the unique photocatalytic and UV-reflecting properties of nTiO₂ have enhanced the exponential growth of low-cost and safer consumer products^{9–11}. Risk assessment studies have predicted nTiO₂ to be the most abundant ENM in the environment [21–10000 ng/L in surface water, 1–100 µg/L in waste water treatment plant (WWTP) effluent, 100–2000 mg/kg in WWTP sludge]¹².

Aquatic environments act as a sinks for chemicals as well as emerging metal pollutants such as ENMs¹³. Aquatic bodies contain a dominant and ubiquitous community of bacteria (~10⁶ cells/ml) as well as the bacterial predators ciliated protozoans (10²–10⁴ cells/ml)^{14,15}. ENMs affect the organisms within and across trophic levels in the aquatic food chain. Poor water solubility and long-term persistence of ENMs in aquatic systems^{16,17} facilitate their bioaccumulation and biomagnification in aquatic organisms such as bacteria, ciliated protozoans, rotifers, algae, crustaceans, zebrafish, and mussels^{18–24}. The accumulation of ENMs can also affect the growth, reproduction, ingestion and digestion behaviour of aquatic organisms^{18,20,21}. Factors such as surface interactions (adsorption or hetero-agglomeration), internalisation, oxidative stress, membrane damage and mitochondrial perturbations have been reported to be responsible for the acute toxicity of ENMs in microorganisms, cell lines

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Groups	1 h	24 h
nTiO ₂ -UZ	395 ± 8	299 ± 44
nTiO ₂ -MZ	411 ± 6	318 ± 41
nTiO ₂ -LZ	411 ± 9	402 ± 28
Ec + nTiO ₂ -UZ	699 ± 9**	425 ± 10
Ec + nTiO ₂ -MZ	521 ± 6**	530 ± 4*
Ec + nTiO ₂ -LZ	601 ± 6**	814 ± 15**
Pm + nTiO ₂ -UZ	742 ± 8**	734 ± 6**
Pm + nTiO ₂ -MZ	657 ± 11**	790 ± 19**
Pm + nTiO ₂ -LZ	781 ± 10**	1211 ± 47**
Ec + Pm + nTiO ₂ -UZ	1036 ± 27**	1138 ± 46**
Ec + Pm + nTiO ₂ -MZ	967 ± 6**	1400 ± 68**
Ec + Pm + nTiO ₂ -LZ	875 ± 17**	ND

Table 1. Hydrodynamic diameter (d-nm) of nTiO₂ in the microcosm, reflecting agglomeration. Pm, *Paramecium*, Ec, *E. coli*. ND, not detected. Values represented are the mean ± SE of three independent experiments. *p < 0.05 was considered significant compared with the control.

and eukaryotic organisms²⁵. The surface interactions of ENMs with microbial cells, the first step in ENM toxicity, are predominantly governed by charge interactions between ENMs and microbes^{26,27}. ENMs with positive surface charges have been found to have higher toxicities than ENMs with negative charges. This finding has been attributed to the negative charges of cell surfaces^{28,29}. In the natural environment, bacterial cells are ubiquitously present and have a high ratio of surface area to their volume; thus, the cells interact with and absorb high levels of ENMs^{15,30}. Additionally, the presence of exopolymeric substances (EPS) on the outer membranes of bacterial cells complements the adsorption of ENMs from the aquatic environment^{15,31,32}. Ciliated protozoans such as *Tetrahymena* secrete mucus from their mucous membranes under stress conditions, and this surface coating affects the fate of ENMs in the medium³³.

To understand the actual behaviours and toxicities of ENMs in aquatic systems, it is necessary to study the surface interactions, such as adsorption and hetero-agglomeration, of ENMs with microorganisms. For instance, the physical properties of *E. coli* cells are affected by exposure to hematite nanoparticles (NPs)³⁴. The adsorption of ENMs on the *E. coli* surface is dependent on size: large hematite NPs adsorb faster than smaller NPs do³². In another study conducted in *Paramecium multimicronucleatum*, nTiO₂ has been found not to cause toxicity, owing to weak surface interaction energy³⁵. Furthermore, different pH and ionic strength conditions play roles in the hetero-agglomeration and co-sedimentation of discharged oxide nanoparticles with chlorella cells³⁶. Studies examining the surface adsorption and hetero-agglomeration of ENMs with biotic factors have been limited to the single organism level.

No studies have examined the adsorption, hetero-agglomeration and co-sedimentation of ENMs in the presence of a predator-prey interaction model of a real-world environmental situation. Such an interaction model, involving two organisms in lower trophic levels of the food chain, can be represented by a bacterium and a ciliated protozoan. *E. coli* has been used extensively as a model bacterium in toxicity assessments of ENMs because it divides rapidly and is easily cultured^{32,37}. *Paramecium caudatum*, a ubiquitous single-celled ciliated protozoan that feeds on bacteria, is a significant ecological link between microbes and multicellular organisms^{33,35}.

Therefore, in the present study, an experimental aquatic microcosm involving *Escherichia coli* as prey and *Paramecium caudatum* as a predator was established to understand the hetero-agglomeration and co-sedimentation of nTiO₂ in the presence of predator-prey interactions. The microcosm was also used to determine the bioavailability, trophic transfer and effect of nTiO₂ on the food chain.

Results and Discussion

Characteristics of nTiO₂. The commercial nTiO₂ used in the present study was heterogeneously distributed, with a particle distribution ranging from 100 nm to 400 nm, as determined by dynamic light scattering (DLS) analysis (SI-Fig. S2a). Transmission electron microscopy (TEM) indicated that most particles ranged from 10 to 70 nm, with an average size of 40 nm (SI-Fig. S2c). The zeta potential of nTiO₂ in Dryl's buffer was −31 mV (SI-Fig. S2b). The size of nTiO₂ determined by DLS was higher because of the formation of the hydrodynamic layer on its surface.

Rationale for selection of sampling time points. In all the experiments, the initial time point was 1 h to allow optimum adsorption of nTiO₂ on the surface of test organisms. The final time point was selected as 24 h, coinciding with the life cycle of *Paramecium*³⁸. This duration was sufficient to allow recurrent ingestion and digestion of bacteria. It also ensured that nanoparticle agglomeration achieved a steady state³⁹.

Adsorption and hetero-agglomeration of nTiO₂ in the microcosm. The average hydrodynamic diameters of nTiO₂ agglomerates observed in different groups (nTiO₂, nTiO₂ + *E. coli*, nTiO₂ + *Paramecium* and nTiO₂ + *E. coli* + *Paramecium*) at 1 h and 24 h are presented in Table 1. A concomitant and significant (p < 0.001) increase in the size of nTiO₂ agglomerates was observed because of the presence of test organisms (*E. coli* and/or *Paramecium*) in the exposure medium. The agglomerate size of an individual nTiO₂ suspension at the initial time point (1 h) was uniform across all characterised zones (upper zone, UZ; middle zone, MZ and lower zone, LZ) of

Figure 1. Zeta potential of nTiO₂ in different zones of the experimental tube at 1 h and 24 h. (a–c) nTiO₂ incubated with *E. coli*, (d–f) nTiO₂ incubated with *Paramecium*, (g–i) nTiO₂ incubated with *Paramecium* and *E. coli*. (a,d,g) = upper zone; (b,e,h) = middle zone and (c,f,i) = lower zone. Ec, *E. coli*, Pm, *Paramecium*. Values represented are the mean $\pm$ SE of three independent experiments.

the simple microcosm. However, a statistically significant ($p < 0.001$) increase in agglomerate size was observed after the addition of *E. coli* and *Paramecium* cells, individually or in combination.

At the final time point (24 h), the size of nTiO₂ agglomerates in the LZ of the microcosm increased significantly ($p < 0.001$), with sedimentation of larger agglomerates in all the groups (nTiO₂ + *E. coli*, nTiO₂ + *Paramecium* and nTiO₂ + *E. coli* + *Paramecium*) (Table 1). Sedimentation occurred because of the increased adsorption of nTiO₂ on the surface of the *E. coli* and the release of mucus exudates by *Paramecium* cells³³. The agglomerate size observed in the lower zone was maximised at 24 h in the following order: nTiO₂ + *E. coli* + *Paramecium* > nTiO₂ + *Paramecium* > nTiO₂ + *E. coli*. The agglomerate size of nTiO₂ in the *Paramecium* co-incubation group was greater than that in the *E. coli* co-incubation group because of differences in binding interactions between nTiO₂ and organisms. The negatively charged surfaces of nTiO₂ (−32 mV) and *E. coli* (−39 mV) do not permit electrostatic interactions (SI-Figure S3 and Fig. 1). A weak electrostatic association may be possible between nTiO₂ (−32 mV) and *Paramecium* (−18 mV), given the extreme difference in the intensity of negative charges of both surfaces (Fig. 1d–f). Further, the combination of *Paramecium* with *E. coli* cells in the microcosm decreased the overall zeta-potential of *E. coli* in the absence of nTiO₂ but increased slightly after the addition of nTiO₂, possibly because of adsorption on the *E. coli* surface. This observation shows that poor electrostatic interactions between nTiO₂ and *E. coli* in the presence of *Paramecium* are possible (Fig. 1i). At 24 h, these interactions were negligible, owing to the equilibration of the overall charges between *E. coli* and nTiO₂ (Fig. 1c,i).

Previous studies have shown that *E. coli* cells produce exopolymeric substances that are involved in nTiO₂ adhesion^{40–42}. Jucker *et al.*⁴³ and Host *et al.*⁴¹ suggested that numerous bacterial exopolymers, such as lipopolysaccharides (LPS) and siderophores (in *Pseudomonas aeruginosa*), participate in the adsorption of nTiO₂ and other metals on the bacterial surface. Li and Logan⁴⁴ have observed that long-chain LPS in *E. coli* cells adheres more widely to nTiO₂ than short chain LPS. Hahn *et al.*⁴⁵ and Pernthaler¹⁴ have also shown that the predation of planktonic bacteria by a ciliated protozoan in the aquatic environment induces the biosynthesis of exopolymeric substances in bacteria, which protect against predation by facilitating biofilm formation. In the present study, a similar mechanism may be responsible, thus facilitating the adsorption of nTiO₂ onto the *E. coli* surface in the presence of *Paramecium*.

The adsorption and hetero-agglomeration of nTiO₂ on the surface of organisms in the microcosm was further assessed through dark field (SI-Fig. S4) and scanning electron microscopy coupled with EDS (SEM-EDS) (Fig. 2). Dark field microscopy revealed the hetero-agglomeration of nTiO₂ after 1 h of incubation with *E. coli* and *Paramecium* cells (SI-Fig. S4). SEM-EDS observations further confirmed the hetero-agglomeration of nTiO₂ in the presence or absence of *E. coli* and *Paramecium* at 1 h (SI Figs S5 and S6) and 24 h (Fig. 2). The images showed the adsorption and agglomeration of nTiO₂ on the surface of *E. coli* and *Paramecium* cells within 1 h. Moreover, at 24 h, the size of the nTiO₂ agglomerates increased in co-incubated and individual suspensions (Table 1). The increased size of individual nTiO₂ agglomerates in suspension was attributable to homo-agglomeration in the presence of Na⁺ and Ca⁺⁺ ions in Dryl's buffer, which was used as an exposure medium throughout the experiments^{36,46,47}.

Figure 2. Adsorption and homo- and hetero-agglomeration of nTiO₂ in an aquatic microcosm. SEM images with corresponding EDS spectra after 24 h of incubation with and without organisms. (a,a') nTiO₂ in Dryl's buffer, (b,b') nTiO₂ incubated with *E. coli* in Dryl's buffer, (c,c') nTiO₂ incubated with *E. coli* and *Paramecium* in Dryl's buffer. The white circle corresponds to the point where the EDS spectrum analysis was performed. The yellow arrow indicates nTiO₂ agglomerates. The inset image in (b) shows the surface interaction of nTiO₂ with *E. coli*.

Co-sedimentation of nTiO₂ in the microcosm. Figure 3 shows that the co-sedimentation of nTiO₂ occurred only with *E. coli* and not with *Paramecium* in the microcosm. This result may be attributed to the low motility of *E. coli* cells (25 μm/s) compared with the high motility of *Paramecium* cells in the water column (1.7 mm/s)^{48,49}. The adsorption of nTiO₂ on the *E. coli* surface led to co-sedimentation because the presence of titanium was detected by EDS scanning of bacterial cells recovered from the LZ of the microcosm (Fig. 2).

nTiO₂ and *E. coli*. The most significant co-sedimentation of nTiO₂ with *E. coli* was obtained at 5 μg/ml nTiO₂, a concentration at which hetero-agglomeration is the favourable option, because of the availability of an optimum ratio between the number of nanoparticles and the surface area of *E. coli* cells (SI-Table S1). However, at higher concentrations (50 and 100 μg/ml) homo-agglomeration was favoured, owing to dominant nanoparticles-nanoparticle interactions that reduced the co-sedimentation of NPs with *E. coli* cells (SI-Fig. S9b). These observations are consistent with results from previous studies examining the homo- and hetero-agglomeration of ENMs^{39,40,47,50}. Furthermore, the analysis of additive sedimentation of *E. coli* and nTiO₂ permitted the calculation of nanoparticle-cell interactions, thus supporting our findings regarding hetero-agglomeration (Fig. 3d)³⁶. The maximum interaction of *E. coli* with nTiO₂ was observed at the lowest concentration of nTiO₂ (5 μg/ml), as evident in the discrepancy (ΔOD) between nTiO₂-bacteria co-sedimentation and additive settling in the LZ (Table 2). ΔOD > 0 represents the occurrence of NP-cell hetero-agglomeration and co-sedimentation, whereas zero or negative ΔOD values indicate no or very weak agglomeration between NPs and cells³⁶.

nTiO₂ and *Paramecium*. Co-sedimentation of nTiO₂ was not observed with *Paramecium* in the absence of *E. coli* because the ΔOD value was highly negative (> -0.85; Table 2) for all concentrations of nTiO₂ (Fig. 3b). Slight sedimentation of nTiO₂ in LZ was observed at higher concentrations (50 and 100 μg/ml; Fig. 3b). This observation may be attributable to the continuous release of larger nTiO₂-loaded vesicles from the cytoproct region of the *Paramecium*, which is a normal physiological process to egest un-digested material (SI-Fig. S10c,d). These vesicles are covered with the mucous membrane, which may further influence agglomeration in exposure medium^{33,51}.

Figure 3. Co-sedimentation (a–c) and additive-sedimentation (d–f) plots of nTiO₂ in the presence of *E. coli* and *Paramecium* in an aquatic microcosm. The ratio of initial to final absorbance at 600 nm in the upper, middle and lower zones of the microcosm depicting the effects of *E. coli* and *Paramecium* individually and in combination on the sedimentation of nTiO₂. Ec, *E. coli*, Pm, *Paramecium*. Values represented are the mean $\pm$ SE of three independent experiments.

Concentration of nTiO ₂ (μg/ml)	<i>E. coli</i> + nTiO ₂			<i>Paramecium</i> + nTiO ₂			<i>Paramecium</i> + <i>E. coli</i> + nTiO ₂		
	Upper Zone	Middle Zone	Lower Zone	Upper Zone	Middle Zone	Lower Zone	Upper Zone	Middle Zone	Lower Zone
5	−0.90	−0.73	−0.18	−1.17	−0.87	−1.00	−0.76	−0.72	0.20
25	−0.91	−0.74	−0.71	−1.39	−0.98	−1.08	−0.85	−0.70	0.06
50	−0.71	−0.59	−0.56	−1.22	−0.85	−1.04	−0.72	−0.49	−0.16
100	−0.69	−0.61	−0.89	−1.27	−0.98	−0.99	−0.67	−0.49	−0.41

Table 2. Differences (Δ OD) in the A/A₀ values of co- and additive sedimentation of nTiO₂ in an aquatic microcosm. Values represented are the mean $\pm$ SE of three independent experiments. A/A₀ = Final absorbance/Initial absorbance.

nTiO₂ in combination with *Paramecium* and *E. coli*. A significant increase in the co-sedimentation of nTiO₂ with *E. coli* cells was observed in the presence of *Paramecium*, as evidenced by the high positive Δ OD (0.20) value at the lowest concentration of nTiO₂ (5 μg/ml), although the Δ OD value was close to zero (0.06) at 25 μg/ml (Table 2; Fig. 3c). This result indicated that the interaction of nTiO₂ with *E. coli* was stronger in the presence of *Paramecium* at lower concentrations. The enhanced interaction and hetero-agglomeration of nTiO₂ on the surface of *E. coli* in the presence of a predator species (*Paramecium*) indicated that the biotic interactions of nTiO₂ in aquatic systems influence the fate as well as toxicity of this NP in bacteria and other organisms. These observations are supported by an earlier report examining the effects of surface interactions between nanoparticles and bacteria on toxicity²⁷. In addition, co-sedimentation of nTiO₂ may adversely affect sediment dwelling organisms⁵².

The interaction of nTiO₂ with *Paramecium* in the presence of *E. coli* was also observed by using dark field and scanning electron microscopy. The dark field microscopy demonstrated that nTiO₂ was taken up by *Paramecium* in the form of vesicles (SI-Fig. S4c,d), which were later released from the cytoproct in the form of aggregates. Further, SEM-EDS revealed the presence of nTiO₂ in the oral groove regions of *Paramecium* cells, thus indicating its internalisation via phagocytosis (Fig. 2c,c' & SI-Fig. S8c). Phagocytosis is a well-defined mechanism for NP uptake in ciliates such as *Tetrahymena*³³.

Figure 4. Growth and bacterial ingestion rate of *Paramecium* cells after exposure to nTiO₂ at different concentrations. Values represented are the mean $\pm$ SE of the three independent experiments. * $p < 0.05$ was considered significant compared with control.

The behaviour of the test organisms in the presence or absence of nTiO₂ in the microcosm was also determined. The observations revealed that *Paramecium* cells remained alive throughout the experiment as there were no signs of mortality, such as membrane leakage or loss of motility, at the tested time points. Furthermore, measurements revealed compromised bacterial ingestion and growth of *Paramecium* at 24 h after exposure to a higher dose of nTiO₂ (100 µg/ml; Fig. 4). The *Paramecium* ingestion rate was ~1600 bacterial cells/ciliate/h. The morphological observation of *E. coli* cells by SEM revealed the shrinking size of *E. coli* cells recovered at 24 h from the LZ of the microcosm (SI-Figs S7a and S8a,b). This effect was also observed in the DLS analysis, which showed a decrease in the size of *E. coli* cells (SI- Table S2).

The shrinking and co-sedimentation of *E. coli* cells in the presence of nTiO₂ may be attributable to the adsorption of NPs on the *E. coli* surface, thus affecting their physico-mechanical properties and possibly play a critical role in maintaining the cellular morphology and motility of bacterial cells. In an earlier study, Zhang *et al.*³⁴ have shown that the adsorption of hematite nanoparticles on the *E. coli* surface exerts multiple effects on physiological and mechanical properties, such as the loss of adhesiveness, elasticity, hardness, electrical potential and deformities in appendages such as flagella. These effects have the potential to inflict numerous consequences on bacterial cells, such as the loss of cellular motility, adhesion to surfaces and stability. The compromised motility and stability of bacterial cells due to the adsorption of nanoparticles may underlie the co-sedimentation of *E. coli* cells with nTiO₂. Protistan grazing on bacterial cells in the environment has been shown to affect the morphology, physiological behaviour and activity of bacterial cells⁵³. In general, bacterivorous protists preferentially feed on medium-size bacterial cells rather than smaller or larger bacteria. In this context, del George *et al.*⁵⁴ have suggested that bacterial cells become inactive and thus adapt to powerful grazing pressure generated by the efficient grazing of bacteria by protists. Further, Gasol *et al.*⁵⁵ have shown that inactive bacterial cells are smaller than active cells. Therefore, the presence of *Paramecium* in the microcosm facilitates the shrinking and co-sedimentation of *E. coli* cells with nTiO₂.

The natural assemblage contains diverse communities that include motile, less-motile and sessile organisms in similar or diverse habitats. Therefore, under natural conditions, sessile organisms may also promote the co-sedimentation of NPs after interactions. To test this hypothesis, the adsorption of nTiO₂ on sessile embryos (4 h post-fertilisation) of an established zebrafish ecotoxicity model was evaluated. Preliminary observations using optical and scanning electron microscopy revealed the extensive binding of nTiO₂ on the chorion, which protects early stage embryos (SI-Figs S11 and 12). The presence of titanium on the chorion surface was further confirmed by EDS analysis (SI-Fig. S12). Therefore, it can be reasoned that interactions and co-sedimentation in water columns are mediated by members of the natural assemblage, including the highly motile *Paramecium* and less-motile organisms such as *E. coli* or sessile zebrafish embryos.

Previous studies have demonstrated that the fate and toxicity of ENMs may change because of the presence of natural colloids and organic matter in the environment⁵⁶. The present study elucidated the role of predator-prey interactions in the hetero-agglomeration and co-sedimentation of ENMs in an aquatic environment. The findings also suggest that the adsorption and internalisation of nTiO₂ on highly motile *Paramecium* cells may further increase bioavailability to higher trophic level organisms in the food chain.

Real-time monitoring of nTiO₂ intracellular interactions in *Paramecium*

Internalisation of nTiO₂ in *Paramecium* in the absence of *E. coli*. Flow cytometry is a well-established technique to measure the cellular density of cells and microbes. The side scattering intensity (SSC-intensity) parameter correlates with changes in cellular granularity due to the uptake of nanoparticles^{57,58}. The real-time monitoring of nTiO₂-exposed *Paramecium* cells in a flow cytometer revealed a dose-dependent and statistically significant ($p < 0.05$) increase in SSC-intensity, thus demonstrating the cellular internalisation of nTiO₂ (Fig. 5.1a). The

Figure 5. Real-time monitoring of nTiO₂ intracellular interactions in *Paramecium*. (5.1) Direct exposure of *Paramecium* cells to nTiO₂ up to 9 h. (a) A concentration-dependent and statistically significant ($p < 0.05$) increase in the internalisation of nTiO₂ in cells determined by flow cytometry. (b–f) Internalisation of nTiO₂ confirmed by bright field microscopy: (b,c) controls at 1 and 9 h, (d–f) 50 µg/ml-treated cells captured at 1, 7 and 9 h. (5.2) Exposure of *Paramecium* cells to nTiO₂ through Dryl's buffer and *E. coli* up to 9 h. (a) A concentration dependent and significant ($p < 0.05$) increase in the internalisation and retention of nTiO₂ in cells determined by flow cytometry. (b–f) Internalisation of nTiO₂ confirmed by bright field microscopy: (b,c) controls at 1 and 9 h, (d–f) 50 µg/ml-treated cells captured at 1, 7 and 9 h. (All bright field microscopy images were captured at 200x magnification). Values represented are the mean $\pm$ SE of three independent experiments. * $p < 0.05$ was considered significant compared to the control.

SSC-intensity was ~ 0.8 -fold higher at an exposure concentration of 100 µg/ml compared with control conditions at 1 h. Further, the intensity of SSC remained constant up to 5 h, possibly because of the saturated uptake of nTiO₂ within 1 h, as represented by the dark vesicles packed into the cytoplasm of nTiO₂-treated *Paramecium* (Fig. 5.1b–e). At 7 h, a decrease in intracellular levels of nTiO₂ was observed, evidenced by a reduction in the number of dark cytoplasmic vesicles and a consistent decrease in the SSC-intensity of *Paramecium* cells (Fig. 5.1a,f). The reduction in the intracellular levels of nTiO₂ in *Paramecium* cells was attributable to the egestion of NPs loaded with undigested food vacuoles from the cytoproct and was confirmed by images captured through dark (SI-Figure S4d) and bright field microscopy (SI-Fig. S10c). The uptake rate of nTiO₂ was also decreased after 7 h because of agglomeration and co-sedimentation, as evidenced by DLS measurements (Table 1) and observations under bright field microscopy (SI-Fig. S10d).

Internalisation of nTiO₂ in *Paramecium* through exposure medium and *E. coli*. Figure 5.2 shows a concentration-dependent (5, 10, 50 and 100 µg/ml), statistically significant ($p < 0.05$) increase in the SSC-intensity of *Paramecium* cells exposed to nTiO₂ in the presence of *E. coli* cells. The *Paramecium* cellular density increased because of the internalisation of nanoparticles. The SSC-intensity of the *Paramecium* cells exposed to 100 µg/ml nTiO₂ was 1.04-fold higher than that of the control. This result demonstrated that the density of nanoparticles within *Paramecium* cells was slightly higher in the presence of *E. coli* than direct exposure (*Paramecium* + nTiO₂). Thus, additional exposure through feeding was involved in the uptake of nanoparticles into *Paramecium* cells. The internalisation of nTiO₂ was maximal at 1 h and remained constant up to 9 h in contrast to 7 h after direct exposure in the absence of *E. coli* (Fig. 5.2a and 5.1a). Under co-exposure (*Paramecium* + *E. coli* + nTiO₂) conditions, the retention of nTiO₂ in *Paramecium* cells was extended because of the presence of nTiO₂ and *E. coli* cells (Fig. 5.2b–f).

In addition, nTiO₂ bioavailability through *E. coli* was observed after the exposure of *Paramecium* cells to nTiO₂-pre-exposed *E. coli*. Figure 6 shows an increase in the SSC-intensity of *Paramecium* within 1 h of exposure to nTiO₂-pre-exposed *E. coli* and was significantly ($p < 0.05$) constant for more than 9 h. An increase in the cellular density of *Paramecium* cells was attributable to the transfer of nTiO₂ from *E. coli* cells. *E. coli* cells adsorb a mass of nTiO₂ within 1 h of exposure that is further transferred to their predator protozoan via phagocytosis³³. Further, the digestion of ingested bacteria in *Paramecium* cells is complete within 1 h, and thus nTiO₂ is released from *E. coli*³⁹. There is increased biocompatibility between nanoparticles and *Paramecium* cells, owing to the presence of bacterial components on the surface that prevent immediate release from the cytoproct of *Paramecium* cells. Here, the SSC-intensity of *Paramecium* cells was normalised to control levels at 24 h compared to 7 and 9 h under direct and co-exposure conditions. The cytoplasmic vacuoles of treated *Paramecium* cells also returned to control levels at 24 h (Fig. 5b–f). Overall, the presence of a prey species (*E. coli*) plays significant role in the bioavailability and persistence of nTiO₂ in a predator (*Paramecium*). These observations also provide evidence, supporting the trophic transfer of nTiO₂ during prey-predator interactions.

Figure 6. Trophic transfer of nTiO₂ in *Paramecium* relative to *E. coli*. (a) A statistically significant ($p < 0.05$) increase in the cellular granularity of *Paramecium* cells fed with nTiO₂-contaminated *E. coli* as determined by flow cytometry. (b–f) Trophic transfer of nTiO₂ further confirmed by bright field microscopy: (b,c) controls at 1 and 24 h, (d–f) 50 µg/ml-treated cells captured at 1, 7 and 24 h. (All bright field microscopy images were captured at 200x magnification). Values represented are the mean $\pm$ SE of the three independent experiments. * $p < 0.05$ was considered significant compared with control.

Transmission electron microscopy further confirmed the presence of nTiO₂, *E. coli* and nTiO₂-containing *E. coli* in the food vacuoles of *Paramecium* (Fig. 7a–f). TEM images clearly revealed the presence of nTiO₂ inside and outside the *E. coli* cells as well as those lodged in the food vacuoles of *Paramecium* cells, thus indicating that NPs were internalised into the cells either directly from Dryl's buffer or through *E. coli* (Fig. 7e,f). The role of *E. coli* as a vehicle for the transfer of nTiO₂ to *Paramecium* cells establishes the trophic transfer of nTiO₂ from the lower to the upper trophic levels in the microbial food chain. The trophic transfer of nTiO₂ was further supported by the presence of packaged bacteria and nTiO₂ in the egested *Paramecium* food vacuoles (SI-Fig. S10a).

Conclusion

Bacterial abundance and their high surface-area-to-volume ratio increases the probability of interactions with ENMs in the environment. The magnitude of interaction between ENMs and the bacterial surface can significantly alter the environmental fate and consequent toxicity of ENMs. Bacteria are the simplest organisms in the lower trophic levels linked to ciliated protozoans in the upper trophic levels of the food chain. The present study demonstrates that the presence of a predator, *Paramecium*, significantly influences the surface interactions between nTiO₂ and its prey, *E. coli*. The increased surface interactions between nTiO₂ and *E. coli* further enhanced the hetero-agglomeration and co-sedimentation of nTiO₂ in the modelled predator-prey-based microcosm. Although co-sedimentation reduces the bioavailability of nTiO₂ to organisms present in surface water columns, sediment-dwelling organisms may be at a higher risk of exposure to nTiO₂. The strong adsorption and internalisation of nTiO₂ in *Paramecium* cells increases the bioavailability of nTiO₂ via *Paramecium* to organisms such as rotifers and fish larvae, which are present at higher trophic levels in the food chain. The present study addresses a one-consumer (protozoan), one-resource (bacterium) system to assess the fate of nTiO₂. However, in a real-world situation, the effects of predation and interactions with prey species may be much greater than those observed in composite natural communities.

Methods

Chemicals and biological materials. Titanium dioxide nanopowder (anatase; 99.7%; CAS No. 1317-70-0) was purchased from Sigma Aldrich (St. Louis, USA). The *E. coli* K12 substrain DH10B was purchased from the Microbial Type Culture Collection Centre (MTCC, Chandigarh, India). *Paramecium caudatum* and protozoan pellets were purchased from Carolina Biological Supply Co. (Burlington, USA). Details regarding the use of *E. coli* and *Paramecium* as model organisms are provided in the supplementary information (SI-method section 1). All other chemicals were of analytical reagent grade and purchased from HiMedia Pvt. Ltd. (Mumbai, India).

Figure 7. The interaction of *Paramecium* with *E. coli* and nTiO₂ after 1 h of incubation. Transmission electron microscopy images, (a,b) control, and (c–f) treated cells (25 µg/ml) showing the presence of *E. coli* and nTiO₂ in food vacuoles (c,d). nTiO₂ is present inside (e) and outside *E. coli* cells (f). Red arrowhead represents nTiO₂, yellow arrow represents food vacuoles and white arrow represents bacteria.

***E. coli* and *Paramecium* culture.** *E. coli* cells were cultured in Luria Bertani (LB) broth (HiMedia Pvt. Ltd., Mumbai, India) at 37 °C in an environmental shaker incubator. *Paramecium caudatum* was cultured in a protozoan pellet medium at 22 °C in a BOD incubator (Model LBI-500M, Daihan Labtech, India). A detailed protocol for medium preparation and *E. coli* and *Paramecium* culture is given in the supplementary information (SI-method section 2).

Nanoparticle preparation and characterisation. A stock suspension of 200 µg/ml nTiO₂ was prepared by adding 8 mg of nTiO₂ powder (anatase) to 40 ml of filtered Dryl's buffer (0.22-µm membrane filter). The suspension was then sonicated for 10 min using a probe sonicator (Sonics Vibra Cell, Sonics & Material Inc., New Town, USA) at 30% amplitude and 30 watts at a pulse rate of 50 sec on and 10 sec off. Before use, the suspension was maintained in a BOD incubator for 10 min to optimise the temperature to 22 °C. Subsequently, it was diluted to four different working concentrations of 5, 25, 50 and 100 µg/ml. A detailed description of the selection of concentrations is given in the supplementary information (SI-results section 2.0).

Hydrodynamic size and zeta potential were measured by dynamic light scattering (DLS) and phase analysis light scattering (PALS) using a Zeta-sizer Nano-ZS equipped with a 4.0-mW, 633-nm laser (Model ZEN3600; Malvern Instruments, UK). The distribution of different sizes of nTiO₂ was also observed by transmission electron microscopy (TEM). The samples for TEM analysis were prepared as described by Shukla *et al.*⁵⁸. Briefly, a drop of nTiO₂ suspension (25 µg/ml) was placed on a formvar-coated copper grid. The film on the TEM grid was allowed to dry in the dark at room temperature. Images were captured using a Tecnai™ G2 Spirit electron microscopy (FEI, The Netherlands) at an accelerating voltage of 80 kV.

Microcosm setup. A simple aquatic microcosm was designed in a 50-ml polystyrene tube to assess the effects of biotic factors on the fate and stability of nTiO₂. The experiments were conducted in Dryl's buffer in seven different groups at 22 °C in a BOD incubator (LabTeK, India). Each group contained a 40-ml suspension of nTiO₂ or Dryl's buffer as well as predator-prey organisms and was marked into three zones, an upper zone

(UZ), a middle zone (MZ) and a lower zone (LZ), as shown in the supplementary information (SI Fig. S1). One millilitre of sample was withdrawn carefully from each zone at 1 h and 24 h for analysis. Dryl's buffer was used because of its unique chemical composition [sodium citrate (2 mM), $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (1 mM), Na_2HPO_4 (1 mM), CaCl_2 (1.5 mM), pH 7–7.2], which helps organisms to survive without stress for long durations, and the presence of citrate ions maintains the environmental relevance of the study because citrate is present in abundance in the natural environment³⁹. Detailed information on the microcosm setup is given in the supplementary information (SI-method section 3).

- Group 1: nTiO_2
- Group 2: *E. coli*
- Group 3: *E. coli* + nTiO_2
- Group 4: *Paramecium*
- Group 5: *Paramecium* + nTiO_2
- Group 6: *Paramecium* + *E. coli*
- Group 7: *Paramecium* + *E. coli* + nTiO_2

Measurement and analysis of nTiO_2 agglomeration and adsorption onto organisms in the microcosm. The agglomeration of nTiO_2 in the microcosm was measured by dynamic light scattering, which was further validated by dark field microscopy and scanning electron microscopy (SEM). The SEM was equipped with an energy-dispersive X-ray spectroscopy (EDS), which complemented the qualitative images with compositional analysis of the adsorption of nTiO_2 .

Sample preparation and analysis of hydrodynamic diameter and electro-kinetic measurements. Nanoparticle suspensions at different working concentrations were incubated with organism/s according to the experimental setup described for groups 2, 5 and 7 to measure the hetero-agglomeration.

Samples for analysis were withdrawn from each group at the initial (1 h) and final time points (24 h) from three different zones (upper, middle and lower) in the microcosm. The samples were analysed immediately using a Zeta-sizer Nano-ZS equipped with a 4.0-mW, 633-nm laser (Model ZEN3600, Malvern instruments Ltd., Malvern, UK). The zeta-sizer was equipped with a He-Ne laser (wavelength 633 nm) with a scattering angle of 173° and a constant temperature of $25 \pm 1^\circ\text{C}$. Water was selected as the experimental medium, with a refractive index of 1.330, and the refractive index of nTiO_2 was 0.200. A minimum of ten runs per analysis was carried out in automatic mode, and the data were analysed using the Zetasizer Software version 7.01 (Malvern, UK).

Measurement of hydrodynamic diameter. Measurements of the z-average intensity-based hydrodynamic size and size distribution were carried out with the DLS on the basis of the Stokes-Einstein equation. The modality of nTiO_2 size measurement by DLS was also verified by running *E. coli* and *Paramecium*. The multi-modal peaks of the individual organisms before and after incubation with nTiO_2 did not interfere with the measurement of nTiO_2 agglomeration, as shown in the supplementary information (SI Fig. S13). Given that the detection limit of the DLS equipment was $\sim 8\ \mu\text{m}$, *Paramecium* cells could not be detected because of their large size (length $\sim 110\ \mu\text{m}$ and width $\sim 40\ \mu\text{m}$) (SI-Fig. S7b and Table S2). *E. coli* was represented by a small peak in the size range of $2\text{--}6\ \mu\text{m}$ by DLS (SI-Fig. S13 and Table S2) and $3\text{--}5\ \mu\text{m}$ in length and $\sim 0.7\ \mu\text{m}$ in width by SEM (SI-Fig. S7a). The addition of *E. coli* did not interfere with the measurement of nTiO_2 , owing to differences in the refractive index of *E. coli*. In an earlier study examining hematite nanoparticles, similar measurements were performed by DLS for bacterial cells³⁴. The data are presented as the mean $\pm$ standard error of three independent experiments.

Zeta potential measurements. Zeta potential measures the total surface charge on particles and is independent of nanoparticle size and shape.⁴² Zeta potential changes if the chemistry of the exposure medium is altered. The zeta potential of nTiO_2 and *E. coli* was measured in Dryl's buffer. Briefly, 1 ml of sample was injected into a capillary zeta cell (Folded Capillary Cell, DTS 1060, Malvern, UK). During injection of the sample, caution was taken so that bubbles did not form in the capillary cells and interfere with the analysis. Data were generated in the form of electrophoretic mobility, which was further converted to zeta potential by application of the Smoluchowski equation⁴¹.

Microscopic observation and sample preparation. Samples from different zones of the microcosm were taken at 1 h and 24 h and analysed for homo- and hetero-agglomerates using dark field microscopy as well as scanning electron microscopy linked with energy dispersive X-ray spectroscopy (SEM-EDS).

Detailed protocols for sample preparation for dark field microscopy and SEM are given in the supplementary information (SI method section 4.1 and 4.2).

Measurement and analysis of nTiO_2 co-sedimentation and hetero-agglomeration in the microcosm. The experiments were carried out in the established microcosm as described above, with seven distinct groups. Four different concentrations of nTiO_2 (5, 25, 50 and $100\ \mu\text{g/ml}$) were used to determine the effects of nTiO_2 dilution on co-sedimentation.

Samples from each group and all zones were withdrawn at the initial (1 h) and final time points (24 h), immediately transferred to a quartz cuvette with a 1-cm path-length, and the absorbance was recorded at 600 nm using a UV-Vis spectrophotometer (Synergy HT, BioTek, Winooski, USA).

Co-sedimentation. Individual sedimentation and co-sedimentation patterns of nTiO₂ were measured and plotted using the method described by Ma *et al.*³⁶ with minor modifications. Briefly, the ordinate was calculated as the ratio of absorbance at a given time point (A) to the initial absorbance (A₀), and the graph was plotted against the concentrations of the nTiO₂. Independently, an additive sedimentation curve was also plotted as the sum of the individual absorbance of two different groups.

Hetero-agglomeration. The hetero-agglomeration of nTiO₂ with *E. coli* in the microcosm was measured as described by Ma *et al.* in algae³⁶. The difference (OD_{mix}-OD_{sum}) between co-sedimentation [co-(OD_{mix})] and additive sedimentation [additive-(OD_{sum})] was calculated to determine the intensities of NP-cell interactions.

Measurement of bacterial ingestion rate of *Paramecium* cells. The assay was performed by counting the numbers of bacterial and *Paramecium* cells/ml in the control (*Paramecium* + *E. coli*) and treated (*Paramecium* + *E. coli* + nTiO₂ at 5, 25, 50 and 100 µg/ml) groups at 0, 1, 2, 3, 4, 5, 24 and 48 h after incubation. At each time point, 2 ml of culture was withdrawn from each group and divided into two separate 1-ml Eppendorf tubes. Separate tubes were used to count *Paramecium* and bacterial cells. The detailed protocol for bacterial and *Paramecium* cell counting is given in the supplementary information (SI-Method 2.1). To show the growth of *Paramecium* cells and the reduction in *E. coli* cells over time, a graph was plotted with the number of *E. coli* and *Paramecium* cells/ml on a double y-axis and time (h) on the x-axis.

The ingestion rate of *Paramecium* was determined by applying the formula suggested by Ali & Saleh⁶⁰.

$$\text{Ingestion rate (bacteria/ciliate/h) I.R.} = \frac{N_t - N_0}{t(h)} \times \frac{1}{N_p}$$

N₀ = number of prey (bacteria) at 0 h; N_t = number of prey (bacteria) at time (t);

N_p = number of predators (bacterivorous ciliate).

Uptake of nTiO₂ in *Paramecium* cells via direct exposure and co-exposure. An experiment was designed to determine the intracellular fate of nTiO₂ in *Paramecium* cells under three different exposure conditions: (1) direct exposure of *Paramecium* cells to nTiO₂ in Dryl's buffer, (2) co-exposure of *Paramecium* cells to nTiO₂ in the presence of *E. coli*, and (3) feeding of *Paramecium* cells with nTiO₂-preloaded *E. coli*.

The experimental setup included: *Paramecium* alone, *Paramecium* + *E. coli* and nTiO₂ without cells (Control; Group 1), *Paramecium* + nTiO₂ and *Paramecium* + *E. coli* + nTiO₂ (Treatment; Group 2) and *Paramecium* + nTiO₂-preloaded *E. coli* (Treatment; Group 3).

The experiments were performed in 6-well plates, and each well contained 3 ml of Dryl's buffer with *Paramecium* (600–800 cells/ml) and/or *E. coli* (OD₆₀₀ 0.4). *Paramecium* in the treatment group was exposed to different concentrations (5, 25, 50 and 100 µg/ml) of nTiO₂. The cellular internalisation of nTiO₂ was assessed using flow cytometry and validated by both bright field and transmission electron microscopy. Each sample was analysed after 1 h of exposure and subsequently every 2 h until experimental completion (9 h). Additionally, samples from Group 3 were also analysed at 24 h to verify the dilution of NPs in the cells because the dilution was not evaluated at 9 h.

The details regarding sample preparation and flow cytometry data analysis are given in the supplementary information (SI method section 5).

***Paramecium* sample preparation for dark field, bright field and transmission electron microscopy.** Detailed protocols for the sample preparation of *Paramecium* for dark field, bright field and transmission electron microscopy are given in the supplementary information (SI method section 4.1 and 4.3).

Statistical analysis. The three independent experiments were performed for all assays, and the results were expressed as the mean ± standard error (SE). Statistical analysis was carried out using Microsoft Excel 2007 and GraphPad Prism version 3.02. The significance of the data were analysed using one-way analysis of variance (ANOVA) with Dunnett's post hoc test as well as t-tests (non-parametric). In all cases, p < 0.05 was considered significant.

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Acknowledgements

The authors acknowledge funding from CSIR Network Projects NWP35 and BSC0112 (NanoSHE), India, and the EU-FP7/2007-2013/Grant Agreement 263147 (NanoValid-Development of reference methods for hazard identification, risk assessment and LCA of engineered nanomaterials), Europe. Funding received from the GICT, India, under the CENTRA project Grant Agreement (ILS/GICT/2013/003) is also acknowledged. GSG acknowledges UGC, India, for a Junior Research Fellowship. The CSIR-IITR and DBLS-Ahmedabad University manuscript communication numbers are 3345 and DBLS 65, respectively.

Author Contributions

R.S. conceptualised the experimental design. A.D. discussed the experimental design and critically evaluated the manuscript. G.S.G. executed the experiments. A.K. helped with flow cytometry analysis. All authors contributed to the writing of the manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Gupta, G. S. *et al.* Assessment of agglomeration, co-sedimentation and trophic transfer of titanium dioxide nanoparticles in a laboratory-scale predator-prey model system. *Sci. Rep.* **6**, 31422; doi: 10.1038/srep31422 (2016).

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