Interactions of TiO₂ nanoparticles and the freshwater nematode *Plectus aquatilis*: particle properties, kinetic parameters and bioconcentration factors

C.W. Isaacsona,#, L. Sigg¹b, A.A. Ammann¹, J. Stadnicka-Michalak¹c and K. Schirmer¹b,c,*

¹Eawag (Swiss Federal Institute of Aquatic Science and Technology, Duebendorf, Switzerland), ²ETH Zürich, Institute of Biogeochemistry and Pollutant Dynamics, Zürich, Switzerland, ³EPFL, School of Architecture, Civil and Environmental Engineering, Lausanne, Switzerland

#Current address: Bemidji State University, Department of Environmental Science, Bemidji, MN, USA

*Corresponding author: Kristin.Schirmer@eawag.ch

Abstract

Principles for determining uptake kinetics and bioconcentration factors for nanoparticles and test organisms have only been cursorily explored. Here we report the derivation of bioconcentration factors (BCFs) and the role of surface functionalization on the interactions between the nematode *Plectus aquatilis* and titanium dioxide nanoparticles (TiO₂ NP) dispersed in freshwater. Because of the high background concentration of titanium in natural waters, TiO₂ NP irradiated to produce ⁴⁸V labeled TiO₂ NPs, or doped with 1% niobium, were used to determine BCFs taking either an equilibrium partitioning or a kinetic modelling approach. The BCFs based on equilibrium partitioning increase from 71±17 L kg⁻¹ at the highest exposure concentrations to 5.1 (±3.2) x 10³ L kg⁻¹ at the lowest exposure concentration, indicating that this approach is not valid. A kinetic modeling approach, based on uptake rate and elimination rates for a two-phase elimination, best reflected the experimental data and was then used to determine BCFs. To further rationalize the kinetic interactions between the TiO₂ NPs and the nematode, organisms were exposed to surface-functionalized TiO₂ NPs with positively charged, negatively charged, steric stabilizing and environmental relevant coatings. Correlations between the extent of TiO₂ NP associated (i.e. attached and internalized) with the nematode, and TiO₂ NP properties, were examined. For all parameters considered, association of the surface functionalized TiO₂ NP with the nematode best correlated with TiO₂ NP sedimentation rate. These results indicate that concepts developed for hydrophobic contaminants are not applicable to nanoparticles and concepts specific to nanoparticles will be of greater utility.

Introduction
Nanoparticles are an emerging group of environmental contaminants for which parameters describing their environmental behavior are insufficiently established.\textsuperscript{1-3} In the case of organic chemicals, general environmental behavior descriptors, such as partition or fugacity coefficients, have shown great utility in describing their behavior in the environment\textsuperscript{4} and there is increasing awareness that development of similar descriptors for nanoparticles is needed.\textsuperscript{1-3} The most commonly used approach to determine these descriptors is to assume equilibrium partitioning, which was originally developed for hydrophobic organic contaminants, where it is widely applied to, for example, determine bioconcentration factors (BCFs). While many doubt the utility of applying thermodynamics to determine BCFs for nanoparticles, this approach has been applied to carbon based, metal and metal oxide nanoparticles in both aquatic\textsuperscript{5-12} and sediment based systems;\textsuperscript{13-18} however, experimental data to prove or disprove this concept are lacking. When equilibrium is assumed, the number of nanoparticles moving back and forth between two phases is expected to be equal and a partition coefficient can be calculated based on the nanoparticle concentration in one phase (organism) over the nanoparticle concentration in the second phase (water or sediment), as in the case of BCFs. However, important differences between organic chemicals and nanoparticles have to be considered.\textsuperscript{3} For example, organic chemicals partition between phases based on the chemical potential of the compound in the different phases, whereas, interactions between nanoparticles and organisms are expected to be kinetically controlled by attachment of nanoparticles to organism surfaces based on their collision rate constant and their attachment efficiency.\textsuperscript{3} Moreover, nanoparticle internalization may be influenced by nanoparticle ingestion and endocytosis by cells at organism-environment barriers.\textsuperscript{5} Additionally, there are different methods for determining nanoparticles in organisms, ranging from dissection and identification of the nanoparticles in certain tissues,\textsuperscript{6,7} to use of whole organism uptake and depuration experiments to determine biologically-driven retention of test materials,\textsuperscript{8,9} in contrast to nanoparticles simply attached to the organism as determined by analysis of thoroughly washed and killed controls. We here use the latter approach to derive BCFs both based on equilibrium partitioning as well as on kinetic models (see below). We would like to note, however, that, given the difficulties of currently available approaches to
unequivocally and quantitatively prove full internalization of nanoparticles, there is debate over the
correct terminology when it comes to interactions between nanoparticles and test organisms and some
argue that a more general term, such as biosorption, maybe used instead.

An alternative approach to the equilibrium partitioning approach to determine BCFs is to use
kinetic models, which do not rely on assumptions of equilibrium between the test organism and the
water or sediment and are more accurate at determining accumulation for multiple routes of exposure
and non-steady state exposures. To develop kinetic models, uptake and elimination of the material are
measured, and these data are used to determine rates of uptake and elimination. The BCFs can then be
calculated by dividing the uptake rate by the elimination rate. With the exception of one report, uptake
and elimination rates have been measured for a metal and for metal oxide nanoparticles in both
aquatic and sediment based systems but the kinetic rates were not used to calculate BCFs. In the one
report in which kinetic models were used to determine BCFs, no comparison was made between BCFs
determined through kinetic models and BCFs determined through equilibrium partitioning.6
Additionally, thus far, there is at most limited discussion as to the implications for the uptake and
elimination rates on bioconcentration.

The aim of this study is to compare and contrast equilibrium partitioning and kinetic modeling
approaches for the determination of bioconcentration of TiO2 NP by the abundant freshwater nematode
Plectus aquatilis in its natural freshwater environment. Because of the high background concentration of
titanium in natural waters, TiO2 NPs, irradiated to produce 48V labeled TiO2 NPs or doped with 1%
niobium (Nb), were used to determine uptake and elimination kinetics and BCFs of exposed
nematodes. In the first exposure scenario, uptake and elimination experiments were conducted to
develop models from which BCFs can be determined, assuming equilibrium partitioning or by applying
kinetic models. In the second exposure scenario, nematodes were exposed to varying concentrations of
TiO2 NPs, then depurated on TiO2 NP-free media to determine if BCFs were constant for different
aqueous exposure concentrations as would be expected from the equilibrium partitioning approach.
Finally, to examine the effect of surface coating on nanoparticle binding and uptake by nematodes,
nematodes were exposed to TiO$_2$ NP functionalized with eight different coatings, including positively
charged, negatively charged, steric stabilizing and environmental relevant coatings (SI Table 1). As
labeled TiO$_2$ NPs are not available for these particles, surface functionalized particle exposures were
conducted at higher concentrations than in the first two exposure scenarios and titanium was quantified
by isotope $^{50}$Ti by ICP-MS.

Methods

Titanium Dioxide Nanoparticles

Commercially available P-25 TiO$_2$ NP (Degussa, Evonik Industries, Essen, Germany) were labeled
with $^{48}$V in cooperation with the Joint Research Center in Ispra Italy. Research grade flame synthesized
TiO$_2$ NP doped with 1 atomic % niobium was received as a gift (EMPA, Duebendorf, Switzerland). Solution synthesized TiO$_2$ NPs were produced by drop addition of titanium tetrachloride into ethanol
followed by condensation in benzyl alcohol at 80° C. After condensation in benzyl alcohol, particles
were diluted in ether and centrifuged, then resuspended in ethanol. The particles were then surface
functionalized by reacting enediol containing molecules with the gem-titanols of the titanium dioxide
surface. Additional descriptions of the synthesis, characterization of surface coating and dialysis of
excess ligand and solvent are available.

Particle Characterization

TiO$_2$ nanoparticles were characterized for size, $\zeta$ potential, crystal structure and sedimentation
rate. The size of the TiO$_2$ NPs were characterized by dynamic light scattering, transmission electron
microscopy (TEM) and x-ray diffraction (XRD) spectroscopy. Dynamic light scattering sizes were collected
on a Zetasizer (Nano ZS, Malvern) with particles suspended in Chriesbach River water which was
recorded after particle size stabilized, as determined from repeated particle size measures. This
suspension was subsequently used as the exposure medium (composition given in SI Table 2). For TEM
analysis, particles suspended in deionized water were drop deposited on carbon coated copper Formvar
grids. TEM analysis was performed on a CM30 (FEI) operated in bright field mode with an acceleration
voltage of 300 kV. Images were collected by a CCD camera attached to the TEM. XRD spectra were collected using Co k radiation (X’Pert Powder diffractometer with XCelearator detector, PANalytical, Almelo, The Netherlands). For exposure and suspension characterization TiO$_2$ nanoparticle suspensions were prepared by dilution and shaking of a 10 mg L$^{-1}$ stock suspension to the desired concentration.$^{13}$ The $\zeta$-potential of the TiO$_2$ nanoparticles was determined on a Zetasizer (Nano ZS, Malvern), with the particles suspended by shaking in Chriesbach river water.$^{13}$ TiO$_2$ nanoparticle sedimentation rates were determined by measuring the change in transmission of the suspension at 260 nm over 8 hours. TiO$_2$ nanoparticles were suspended at 10 mg L$^{-1}$ in four technical replicates and the transmission was measured at 0, 0.5, 1, 2, 4, and 8 hours. The sedimentation rate was calculated from the slope of the regression of the line of $\ln(C/C_0)$ vs time and the sedimentation rate was represented as the sedimentation rate constant $k_{sed}$ [h$^{-1}$] ± SD.$^{13}$

**Nematode Identification and Culturing**

The nematode was isolated from the Chriesbach River on the campus of Eawag in Duebendorf, Switzerland. Nematodes were grown in batch cultures in aerated water from the Chriesbach River (SI Table 2) amended with protozoa pellet and wheat seeds (Carolina Biological Supply, Burlington, NC), hemoglobin (Calbiochem, Darmstadt, Germany) and cholesterol (Alfa Aesar, Karlsruhe, Germany). The nematode was identified by DNA barcoding at the University of Bielefeld. The large and small subunit 18 rRNA were sequenced and the nematode was determined to be the bacteria feeding nematode *Plectus aquatilis*. The full sequences can be found in the SI. *Plectus aquatilis* has a life cycle of 14 days$^{15}$ and range in length from 500-1,000 µm and 10 µm in diameter as adults. As a result of the long life cycle, it is not possible to obtain a synchronized culture of *Plectus aquatilis*. This latter aspect results in a test population of varying sizes which we consider representative of the types of populations that will be exposed in the environment. The varying sizes are, on the other hand, a source of variability between technical replicates.

**Exposure Conditions**
For exposure experiments, nematodes were separated from growth medium on sucrose gradients as described in the SI. For accumulation studies, approximately 2,000 nematodes per 1 mL replicate, determined by counting, were exposed to TiO₂ nanoparticles (doped with 1% Nb) at concentrations ranging from $1.3 \times 10^{-7}$ to $1.3 \times 10^{-3}$ M (0.01-100 mg L⁻¹) in Chriesbach river water for 24 hours. Chriesbach river water was used as the exposure medium as more representative of environmental conditions than exposures conducted in artificial exposure media. After uptake, nematodes were depurated by first washing away excess TiO₂ NPs, then nematodes were fed for 24 hours in clean medium, then washed again, digested and TiO₂ NP were quantified. For kinetic uptake and elimination studies, using either Nb-doped or $^{51}$V labeled TiO₂ NP, nematodes were exposed for 2, 4, 8, 16, and 24 hours to nanoparticles, after which the nematodes were washed and then depurated for 2, 4, 8, 16 and 24 hours by feeding on the growth medium described above. To determine the extent to which nanoparticles attached to the exterior of the nematodes, at each exposure concentration and time point, nematodes were fixed with formaldehyde and exposed and analyzed as described above.

**Digestion of Nematodes and Quantification of TiO₂ Nanoparticles**

TiO₂ exposed nematodes were digested in an Ultraclav Microwave using nitric acid, hydrogen peroxide (Merck, Darmstadt, Germany) and hydrofluoric acid (Fluka, Steinhein, Germany). To ensure the quality of titanium analysis, the NIST standard reference material typical diet (reference number 1548a) containing titanium dioxide at a concentration of 4.7 mg kg⁻¹ was used. Previous reports have suggested use of alternative digestion chemicals such as ammonium sulfate/sulfuric acid¹⁶ or sodium hydroxide¹⁷ for dissolution of titanium dioxide. However, use of ammonium sulfate/sulfuric acid resulted in rupture of the digestion vessels, while sodium hydroxide or nitric acid alone did not completely dissolve the standard reference material and resulted in reduced concentrations of titanium (SI Figure 1). Dissolved titanium and niobium concentrations were quantified by isotopes $^{50}$Ti and $^{93}$Nb with an Element II ICP-MS (Thermo Fischer Scientific Inc., Bremen, Germany).
For nematodes exposed to $^{48}\text{V}-\text{TiO}_2$, TiO$_2$ NP concentrations were determined by dissolution of the nematodes in Ultima Gold scintillation cocktail (Perkin Elmer, Waltham, MA) and the $^{48}\text{V}$ was quantified by liquid scintillation on a Tricarb scintillation counter (Perkin Elmer, Waltham, MA).

**Kinetic Modeling of TiO$_2$ NP Uptake and Elimination**

A one compartment model was used to determine uptake and elimination rates, as has been previously applied to describe elimination of metal nanoparticles$^{18,19}$. According to this conceptualization, TiO$_2$ NP taken up by the nematode are instantly distributed throughout the organism and accumulation is described by the following equation:

$$\frac{dC_{\text{int}}(t)}{dt} = k_{\text{in}} C_{\text{w}}(t) - k_{\text{out}} C_{\text{int}}(t)$$

Where $C_{\text{int}}(t)$ is the internal concentration of TiO$_2$ NP in the nematode (kg TiO$_2$ (kg nematode)$^{-1}$), $k_{\text{in}}$ is the uptake rate constant from the water (L kg$^{-1}$ hr$^{-1}$), $C_{\text{w}}(t)$ is concentration of TiO$_2$ NP in the water (kg L$^{-1}$), $k_{\text{out}}$ is either the total (i.e. fast and slow) elimination rate constant ($k_{\text{out1}}$) for the first 26 h of exposure or the body (i.e. slow) elimination rate constant ($k_{\text{out2}}$) after 26 h of exposure for the nematode (hr$^{-1}$).

Uptake and elimination rate constants were fit to measured concentrations of TiO$_2$ NPs by minimizing the sum of squares between measured and modeled concentrations using the Levenberg–Marquardt algorithm. All simulations were conducted with Model Maker software, version 4.0, developed and published by Cherwell Scientific Ltd. (Oxford, UK).

**RESULTS AND DISCUSSION**

**Characteristics of TiO$_2$ NP as powder and in a natural freshwater.**

In powder form, niobium doped TiO$_2$ NP had a diameter of 31 nm$^{12}$ and $^{48}\text{V}$ labeled TiO$_2$ and surface functionalized TiO$_2$ NP had a diameter of 21 nm and 3.3 nm, respectively, as determined from XRD spectra.$^{13}$ When suspended in water from the Chriesbach River (composition in SI Table 2), a small stream in Duebendorf, Switzerland, most particles aggregated to 800-5400 nm in diameter, with the exception of rutin-coated TiO$_2$ NP, which remained 68 nm in diameter (SI Figure 2 and ref.$^{13}$). All nanoparticles had a $\zeta$-potential of $-17 \pm 1$ mV in Chriesbach River water.$^{13}$ This increase in TiO$_2$ NP size
and reduced surface charge resulted in sedimentation rates varied with the surface coating of the TiO$_2$ NP. Rutin coated NP were most stable with a sedimentation rate of 0 h$^{-1}$ for rutin-coated TiO$_2$ NP and 1,3-benzenedisulfonic acid coated NP were the least stable with a sedimentation rate of 0.27 h$^{-1}$.

Sedimentation from the exposure suspension is an acknowledged problem in NP toxicology studies as the exposure concentration is expected to increase with increasing exposure time. As real environmental waters contain both dissolved and particulate materials not found in idealized lab exposure solutions, using environmental waters as exposure medium will greatly complicate nanoparticle exposure as particles agglomerate and sediment from the exposure medium. However, such exposures more accurately reflect what will happen during an environmental release of nanoparticles.

Steady-state is reached within 24 hours and elimination is biphasic.

An initial uptake experiment was conducted to determine the exposure time needed to reach a steady-state concentration of TiO$_2$ NPs in the nematode (Figure 1A), meaning the concentration where the amount of TiO$_2$ associated with the nematodes remained constant. Nematodes were exposed for 24 hours, then washed and the TiO$_2$ NP content associated with the nematodes (i.e. attached and internalized) was quantified. Over the course of 16 hours of exposure, the TiO$_2$ NP concentration associated with the nematode increased, then between 16-24 hours of exposure, a constant TiO$_2$ NP concentration was observed (Figure 1A). Subsequently, uptake and elimination rates were determined by exposing nematodes to TiO$_2$ NPs for 24 hours followed by elimination on TiO$_2$ NP-free media containing food for 24 hours (Figure 1 B-C). This approach to determine bioaccumulation has been previously applied to nanoparticle exposures in both aquatic and sediment based systems. The rate of uptake varied between biological replicates, but a steady-state concentration in the nematode was attained within 24 hours of exposure. When the nematodes were washed and placed in clean medium, they eliminated between 40 and 60 % of the TiO$_2$ NP in the first two hours, after which the elimination rate decreased significantly (Figure 1 B-C). When this exposure data were modeled using single first order rate constants in a simple one compartment model, consisting of a nematode compartment and...
single uptake and elimination rates, the model did not satisfactorily describe the elimination phase of
the exposure (SI Figure 3). When a second elimination rate was added, the modeled elimination rates
more closely matched the experimental data (Figure 1 B-C). Data from the first 26 hours were modeled
to derive uptake ($k_{in}$) and elimination ($k_{out1}$) rates ($r^2 >0.90$). Data for 26-48 hours after exposure were
used to calculate the second slow elimination rate ($k_{out2}$, Fig. 1B), unless this elimination rate was not
significantly different from zero (f-test p>0.05; Fig. 1C). The observation of two elimination regimes
likely results from the internalization of TiO$_2$ NPs into two distinct fractions. The second much slower
elimination phase is consistent with elimination of TiO$_2$ NP trapped in stomach villi or internalized
beyond the gut lumen of the nematode, where the elimination rates are expected to be significantly
reduced. Alternatively, for particles that exhibit greater dissolution potential, others have proposed that
the need to use two elimination rates results from the differing elimination behavior of dissolved ions
and intact nanoparticles. However, as TiO$_2$ NPs exhibit very low dissolution potential under
physiological conditions, the observation of two elimination regimes must result from differences in
internalization of the nanoparticles and not differences in elimination rates for ions and particles.

Uptake and the first fast elimination ($k_{out1}$) rate varied by up to a factor of 26 between experiments,
while the error associated with these rates was typically less than 20% of the measured value (Table 1).
The slow elimination rate ($k_{out2}$) was not determined for experiment 1C, as the elimination rate was not
different from zero. The elimination rates modeled from data in experiment 1A, which is based on the
uptake phase only, indicates that elimination during the initial 24 hour uptake exposure is a combination
of the slow and fast elimination regimes. The mass remaining in the nematode after elimination varied
significantly from $1 \times 10^{-4}$ to $3 \times 10^{-3}$ kg TiO$_2$ (kg nematode)$^{-1}$ and this difference likely results from
natural variation in nematode sizes, resulting from use of non-synchronized nematode cultures.

Equilibrium partitioning approach is not valid for TiO$_2$ NP.

To assess the suitability of an approach based on equilibrium partitioning, for which different
external exposure concentrations should have no influence on the resulting bioconcentration factors,
nematodes were exposed to concentrations ranging from $1.3 \times 10^{-7}$ to $1.3 \times 10^{-3}$ M niobium-doped TiO$_2$
The nematodes were exposed to TiO$_2$ NP for 24 h after which they were rinsed with NP-free exposure medium and fed on TiO$_2$ NP-free medium for 24 h. TiO$_2$ NP content associated with the nematodes (attached and internalized) was then quantified. Nematodes were fed on clean medium after exposure to allow for the quantification of TiO$_2$ NP internalized by the nematode, and not simply TiO$_2$ NP retained in the intestinal tract. To account for attachment of TiO$_2$ NP to the exterior of the nematodes, control nematodes were first fixed with formaldehyde, then exposed to TiO$_2$ NP as described above. The mass of TiO$_2$ NPs attached to nematodes in killed control experiments ranged from 19 to 49% of the mass of TiO$_2$ associated with live exposed nematodes (Figure 2A). The attached mass of TiO$_2$ NPs was subtracted from the mass in live exposed nematodes to arrive at the mass TiO$_2$ NPs internalized. The decrease in the relative amount of TiO$_2$ NP attached to the nematode at higher exposure concentrations results from saturation of favorable attachment sites for TiO$_2$ NPs and therefore a smaller relative amount of TiO$_2$ NPs attached to the nematode. At exposure concentrations ≥ 1.3 x 10$^{-6}$ M, a linear relationship between exposure concentration and mass taken up was observed, while at lower exposure concentrations the relative internalization of TiO$_2$ NP increased (Figure 2B). This indicates that a single concentration factor does not control the internalization of TiO$_2$ NPs by the nematode.

From the kinetic model results, BCFs were determined by dividing the intake rate ($k_{in}$) by the initial elimination rate, $k_{out}$, an approach that does not require the assumption of equilibrium partitioning. The BCFs ranged from 24 ± 3.4 to 600 ± 97 L kg$^{-1}$ (Table 2). When the steady-state portion of the nematode uptake data were applied to an equilibrium partitioning paradigm ($c_{organism}$ divided by $c_{water}$), BCFs ranged from 9.7 ± 7.0 to 590 ± 47 L kg$^{-1}$ (Table 2). The BCF variability observed for kinetic models likely results from the biological variability inherent in using a culture of nematodes at varying life stages. Using the equilibrium partitioning approach for the different exposure concentrations (Figure 2B), the BCFs were relatively constant between 48 ± 5.1 and 91 ± 7.8 L kg$^{-1}$ for exposure concentrations ≥1.3 x 10$^{-5}$ M, while the BCF increased up to 5.1 (± 3.2) x 10$^3$ L kg$^{-1}$ at the lowest exposure concentration (Table 2). For exposures at 1.3 x 10$^{-4}$ M, there is good agreement between the BCF modeled based on
uptake and elimination rates (24 ± 3.4 to 600 ± 97 L kg⁻¹) and the BCF determined using equilibrium partitioning (48 ± 5.1 L kg⁻¹). As it is well established that the concentration of nanoparticles in suspension influences the nanoparticle aggregation and sedimentation rates, the range of measured BCFs may be larger than what is reported here. At higher TiO₂ nanoparticle concentrations, greater aggregation and sedimentation rates are generally expected than is expected at lower TiO₂ nanoparticles, which would lead to greater nematode exposure concentrations (at the higher TiO₂ nanoparticle concentrations) and therefore a larger BCFs, than what is calculated here based on dosed TiO₂ nanoparticle concentrations. The BCFs determined by application of an equilibrium partitioning approach, based on Figure 2B, accounted for the mass of TiO₂ NP attached to the nematode by subtracting the mass of TiO₂ NP attached to killed controls. If the BCFs were instead determined without accounting for elimination and attachment of TiO₂ NP to the nematode, the BCFs would be more than double the calculated value above. This doubling results from elimination accounting for removal of 40-60% of the TiO₂ content associated with the nematode (Figure 1B and C) and attachment accounting for 19-49% of the associated TiO₂ content (Figure 2A). However, these higher values would still be within the range of the kinetically modeled BCFs (Table 2). The BCFs determined from kinetic models (Table 2) are also in good agreement with the BCFs reported for Danio rerio and are a factor of 10-100 less than those reported for Daphnia magna, where the exposure included elimination, but did not account for TiO₂ NP attachment. The higher BCFs for Daphnia magna likely result from a more efficient particle scavenging mechanism for Daphnia magna than for Danio rerio or Plectus aquatilis, although particle attachment cannot be ruled out. If an equilibrium partitioning approach were applicable to TiO₂ NP bioconcentration, the BCF would remain constant across all exposure concentrations. Since the BCF increases for the lowest exposure concentrations, application of an equilibrium partitioning approach to describe TiO₂ NP-nematode interactions is inappropriate. Additionally, for the partitioning approach to be valid, the nanoparticles must be homogenously distributed in the exposure media, which does not apply to TiO₂ NP.

Surface coatings greatly influence the TiO₂ NP – nematode interactions.
To further develop parameters that describe TiO$_2$ NP-nematode interactions, nematodes were exposed to 1.3 x 10$^{-3}$ M surface functionalized TiO$_2$ NP for 24 hours, then depurated by feeding on TiO$_2$ free medium for 24 hours, after which the TiO$_2$ NP content associated with the nematodes was quantified. Killed control exposures were used as described above to determine the fraction of TiO$_2$ NP attached to the nematode and by subtraction, the fraction of TiO$_2$ NP internalized by the nematode. The contribution of nanoparticle attachment to the nematode relative to the internalized concentration varied greatly with surface coatings, with the difference between the positively charged galloycyanine-coated particles attaching to the killed control nematode and the live exposed nematodes, being not statistically significant, $p>0.05$ (Figure 3A). TiO$_2$ NPs with environmentally relevant surface coatings that were stabilized by steric mechanisms (rutin and tannic acid) were taken up to a lesser extent than particles that had coatings with ionizable functional groups or hydrophobic properties (Figure 3A).

To rationalize the kinetic interactions of TiO$_2$ NPs and the nematodes, correlations between associated or internalized TiO$_2$ NP and TiO$_2$ NP particle properties, namely agglomerate size, $\zeta$-potential, and sedimentation rate, were examined (Figure 3 B and SI Figure 4). Because of the high variability, alizarin red coated TiO$_2$ NP were not included in this analysis. Poor linear correlations were observed between TiO$_2$ NP content associated or internalized by the nematodes and TiO$_2$ NP $\zeta$-potential or TiO$_2$ NP size, $r\leq 0.42$ (Figures SI 4 A, B, D, and E). A better correlation was observed for TiO$_2$ NP internalization and TiO$_2$ NP sedimentation rate, $r=0.75$ (Figures SI 4C). However, the strongest correlation was observed between TiO$_2$ associated (i.e. attached and internalized) with the nematode and the TiO$_2$ NP sedimentation rate, $r=0.99$ (Figure 3 B). The increased nematode association for TiO$_2$ NPs with greater sedimentation rates likely results from bottom dwelling nematodes being exposed to greater concentrations of rapidly sedimenting TiO$_2$ NP. The fact that the correlation between sedimentation rate and TiO$_2$ NP association with the nematode was stronger than sedimentation rate and TiO$_2$ NP internalization shows that TiO$_2$ NP association is the greater determinant of exposure than internalization alone. For correlations between TiO$_2$ NP association and particle sedimentation rates, catechol and benzene disulfonic acid coated particles were determined to be outliers of the linear
regression by the Grubb’s test. Catechol coated particles, which are more hydrophobic and are not
stabilized by electrostatic or steric mechanisms, and therefore more likely to attach to environmental
surfaces, were more readily associated with the nematode than would be expected based on the
observed sedimentation rate. Benzene disulfonic acid coated particles, which have two strongly ionized
sulfonic acid groups and are therefore more electrostatically stabilized, were less readily associated with
the nematode than would be expected based on their sedimentation rate. Overall, the stability of the
particles in suspension determines the extent to which TiO$_2$ NPs are associated or internalized by the
nematode.

Acknowledgements

The authors would like to thank Florian Altermatt for nematode isolation and help with setting up the
culture and Walter Traunspurger for identifying the nematode; and Markus Niederberger and Florian
Heilitag for help synthesizing the surface functionalized TiO$_2$ nanoparticles. The authors are grateful for
fruitful and lively discussions with Hannah Schug, Heike Hildebrand, Stefan Schymura and Karsten
Franke. This study was financially supported by the German Federal Ministry of Education and Research
within the NanoNature initiative (project NanoTrack, support code 03X0078A). The authors acknowledge
financial support from QNano Transnational Access Program (JRC TAF-13, JRC TAF-119) for synthesizing
the 48V-TiO$_2$. 
### Table 1) Uptake and elimination rate constants for the nematode *Plectus aquatilis* exposed to TiO$_2$ nanoparticles

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Rate constant</th>
<th>Error$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1A</td>
<td>$k^b_{in}$</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>$k^c_{out 1}$</td>
<td>0.075</td>
</tr>
<tr>
<td>Experiment 1B</td>
<td>$k^b_{in}$</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>$k^c_{out 1}$</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>$k^c_{out 2}$</td>
<td>0.0065</td>
</tr>
<tr>
<td>Experiment 1C</td>
<td>$k^b_{in}$</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>$k^c_{out 1}$</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>$k^c_{out 2}$</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$standard deviation, $^b$Uptake rate constant in L kg$^{-1}$ h$^{-1}$, $^c$elimination rate constant in h$^{-1}$, ND stands for not determined because second elimination rate was not different from zero. Experiment name corresponds to data in Figure 1. # - elimination rate constant determined in the absence of a depuration phase. $K_{out 1}$ is the total (i.e. fast and slow) elimination rate constant for the first 2 h of elimination and $K_{out 2}$ is the body (i.e. slow) elimination rate constant after 2 h of elimination.
Table 2) BCFs (L kg⁻¹) for TiO₂ NPs exposed nematode *Plectus aquatilis* determined using kinetic modeling and equilibrium partitioning approaches.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>TiO₂ NP (M)</th>
<th>BCF (L kg⁻¹)</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetic Modeling ((k_{in} x k_{out}^{-1}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig.1A</td>
<td>1.3x10⁻⁴</td>
<td>120</td>
<td>27</td>
</tr>
<tr>
<td>Fig.1B</td>
<td>1.3x10⁻⁴</td>
<td>24</td>
<td>3.4</td>
</tr>
<tr>
<td>Fig.1C</td>
<td>1.3x10⁻⁴</td>
<td>600</td>
<td>97</td>
</tr>
<tr>
<td>Equilibrium Partitioning ((C_{organism} x C_{water}^{-1})) based on steady-state concentrations derived from Kinetic Modeling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fig. 1A</td>
<td>1.3x10⁻⁴</td>
<td>94</td>
<td>45</td>
</tr>
<tr>
<td>Fig. 1B</td>
<td>1.3x10⁻⁴</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>Fig. 1C</td>
<td>1.3x10⁻⁴</td>
<td>590</td>
<td>47</td>
</tr>
<tr>
<td>Fig. 1B Elimination</td>
<td>1.3x10⁻⁴</td>
<td>9.7</td>
<td>7.0</td>
</tr>
<tr>
<td>Fig. 1C Elimination</td>
<td>1.3x10⁻⁴</td>
<td>360</td>
<td>66</td>
</tr>
<tr>
<td>Equilibrium Partitioning ((C_{organism} x C_{water}^{-1})) based on measured concentrations (Fig.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.3x10⁻⁷</td>
<td>5100</td>
<td>3200</td>
<td></td>
</tr>
<tr>
<td>1.3x10⁻⁶</td>
<td>310</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1.3x10⁻⁵</td>
<td>91</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>1.3x10⁻⁴</td>
<td>48</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>1.3x10⁻³</td>
<td>71</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1) Uptake and elimination of $1.3 \times 10^{-4}$ M TiO$_2$ NPs (1x10$^{-5}$ kg L$^{-1}$) in the nematode *Plectus aquatilis*: A) TiO$_2$ content in nematodes exposed for up to 24 h to niobium doped TiO$_2$ NPs; B) TiO$_2$ content in nematodes exposed for 24 h to $^{48}$V labeled TiO$_2$ NP after which they were washed and depurated on particle-free growth medium for 24 h; C) TiO$_2$ content in nematodes exposed for 24 h to niobium doped TiO$_2$ NPs, after which they were washed and depurated on particle-free growth medium for 24 h. Points are measured values (n=3 ± standard deviation), lines are calculated with uptake and elimination rates shown in Table 1.
Figure 2) TiO$_2$ NP accumulation in nematodes exposed for 24 h followed by elimination in TiO$_2$ NP-free media for 24 h (n=3 ± standard deviation), A) fraction of TiO$_2$ nanoparticles attached to the nematode, determined from killed controls, relative to the total mass associated with the nematode, determined from live exposed nematodes. B) TiO$_2$ NP content in live exposed nematodes as a function of TiO$_2$ NP exposure concentration, determined by subtracting the mass of TiO$_2$ attached to killed control exposed nematodes. TiO$_2$ NP were niobium-doped.
Figure 3) Exposure of nematodes to 1.3x10^{-3} M TiO_2 NP with various surface functionalities (n=3 ± standard deviation). A) TiO_2 content in nematodes exposed to TiO_2 nanoparticles with various surface coatings after 24 h exposure followed by elimination for 24 h. Live exposed (dark bar) and killed control (grey bar) nematodes. B) Correlation between TiO_2 nanoparticle sedimentation rate and total TiO_2 content associated with nematodes. Outlying points, labeled and highlighted in red, were determined by Grubb’s test (see Table SI 3).

References


