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Filter feeders increase sedimentation of titanium dioxide: The case of zebra mussels

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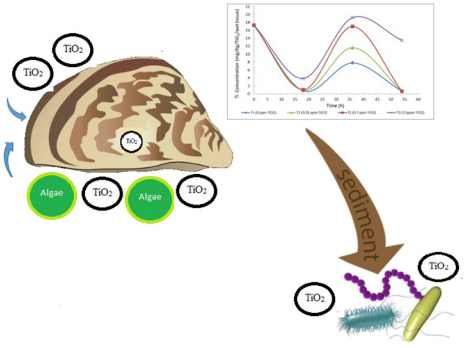
HIGHLIGHTS

- The depuration of zebra mussels occurs progressively with a rate around 5.6 mg Ti/day
- Mussels retain Ti at a concentrations of 3.5 ppm but lower concentration trigger complete Ti excretion
- Ti can be captured by zebra mussels freely dissolved or attached to particles of organic material (algae).
- Zebra mussel can retain Ti as a function of the TiO_2 concentration

GRAPHICAL ABSTRACT



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ABSTRACT

Titanium dioxide particles (TiO_2) are widely used to produce whitens (titanium white) and different class of nanomaterials (semiconductors, photo catalysts and nanotubes). Nanomaterials are excellent adsorbents and catalysts with a wide range of applications. However, these are reported to induce biological and genetic alterations among several invertebrate groups. Invasive species such as zebra mussels can be used as model organisms to study the behavior of particles and nanoparticles (NPs) due to their wide distribution; mussels have been extensively used for monitoring water pollution. In the present study, TiO_2 particles were dispersed and added to a *Chlorella* culture to emulate a natural scenario. To study the reaction of zebra mussels to different TiO_2 concentrations, they were fed with 0.35, 0.7 and 3.5 mgTiO_2/L of the suspension for 3 days and the titanium was measured in the water column, mussels and sediments with ICP-AES. Zebra mussels obtained from the Port of Quebec had up 61.62 mg Ti/kg wet tissue at the time of capture. After 10 days of depuration, they had from 0.23 to 16.28 mgTi/kg wet tissue. Mussels accumulated TiO_2 after 36 h of exposition as a function of TiO_2 concentration, but mussels did not present significant mortality due to TiO_2 toxicity until concentrations higher than 0.7 ppm. A second set of experiments was run to understand the TiO_2 pathway attached to microalgae vs free TiO_2. Results indicated that mussels accumulated slightly more Ti when it was mixed with microalgae. However, the statistical difference was non- significant. A 100 times higher accumulation of Ti in sediments was identified when mussels are present. Thus, it was concluded that the sedimentation of TiO_2 is enhanced by the zebra mussels' filtration activity.

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1. Introduction

Zebra mussels (*Dreissena polymorpha*) are bivalves from the Azov, Black and Caspian seas (Ricciardi et al., 1996). These were worldwide introduced during the 80′s via ballast water of trading ships and spread in different places, such as St. Lawrence River (Yoo et al., 2014), Ebro River in Castejón (Morales et al., 2013), River Rhine, Danube River, Euphrates River basin, and Kuban River among others (GISD, 2014).

The filter-feeding system of zebra mussels allows them to uptake freely dissolved or suspended chemical contaminants across its gill membrane. When the chemical contaminants are associated with particles such as algae or suspended sediments, these are desorbed during the gut passage and then these are assimilated into the mussel tissue or excreted as feces (Bruner et al., 1994). Hence, mussels have been considered as "suitable sentinel organisms" for aquatic monitoring of chemical contaminants, such as polychlorinated biphenyls (PCBs), dichloro diphenyl trichloroethane (DDT), benzo(a) pyrene (BaP), hexachlorobiphenyl (HCBP) and titanium dioxide particles (TiO_2) (Bruner et al., 1994; Couleau et al., 2012). When zebra mussels filter large quantities of water can produce then the accumulation of chemical contaminants which can be transported directly to zebra mussel predators or deposited in sediments by contaminated feces from zebra mussels (Kwon et al., 2006). It depends of the exposure time, chemical contaminants concentration and exposure route (Palais et al., 2012). Thus, detritivores such as amphipods and chironomids which inhabit in the sediment may ingest highest concentration of chemical contaminants inducing the transport and bio-magnification process through the food chain because detritivores are prey for different fish species (Perez-Fuentetaja et al., 2015).

In view of the widespread use of TiO_2 and their environmental release into fresh water, Couleau et al. (2012) investigated the sub lethal effects of TiO_2 NPs on the immune system cells (hemocytes) of *Dreissena polymorpha*. After 24 h of in vivo exposure to TiO_2 NPs at different concentrations, they observed the internalization of TiO_2 NPs into hemocytes and the consequent immunological responses. However, it is known that there are changes in the transport and behavior of NPs when these are exposed to different media such as fresh or seawater, because depending with the TiO_2 concentration and the presence of organic material such as microalgae, homoagglomeration (NPs coalesce or clump together with other NPs) or heteroagglomeration (NPs are adsorbed onto cells) process may occur and to influence the bioaccumulation or sedimentation process in aquatic environments (Sendra et al., 2017). Thus, this study has examined the rerouting of TiO_2 by *Dreissena polymorpha* for exposure experiments mediated by *Chlorella* microalgae at different TiO_2 concentrations and exposition time from 24 to 72 h. These results contribute for the understanding of the behavior of how these particles are transported, bio-accumulated or expelled to the sediments by *Dreissena polymorpha* as a function of TiO2 concentration, time and the presence or absence of *Chlorella* microalgae.

2. Material and methods

2.1. Chemicals and standards

Nitric acid (Trace metal™ grade, 67-70% Fisher Scientific, Ontario, Canada), per-chloric acid (reagent grade 67-71% Fisher Scientific, Ontario, Canada). Standards for metal analysis (SCP Science, plasmaCAL, Quebec, Canada). HPLC grade water was prepared in the laboratory using milli-Q/Millli-Ro Milli pore system (Milford, Massachusetts, USA). Titanium dioxide (TiO_2) powder (reagentPlus® grade, 99–100%, Sigma-Aldrich, Ontario, Canada). Commercial fertilizer (Miracle-Gro® with 5% N and 30% P, Quebec Canada) was used to promote Chlorella algae growth.

2.2. Zebra mussels (Dreissena polymorpha) conditioning

Around two thousand specimens of zebra mussel (*Dreissena polymorpha*) were collected at the old Quebec port, St Lawrence River, (46° 49′ 20.2614″ N 71° 12′ 43.8264″ W) in early spring 2014 and also in December 2015. The mussels attached on submerged cords and wires of the Port of Quebec yacht marina were carefully and manually collected to avoid damage of the bivalves and transported to the laboratory in a plastic container.

The mussels were cleaned with de-chlorinated tap water and transferred into five 20 L glass fishbowls. They were acclimated and starved for 10 days in aerated water at 15 ± 1 °C to reduce the background content of TiO_2 to the minimum. The water was daily changed, later 10 days the mussels were separated into groups of 250 mussels per treatment with a size and weight average of 2 ± 0.27 cm and 1.4 ± 0.41 g, respectively.

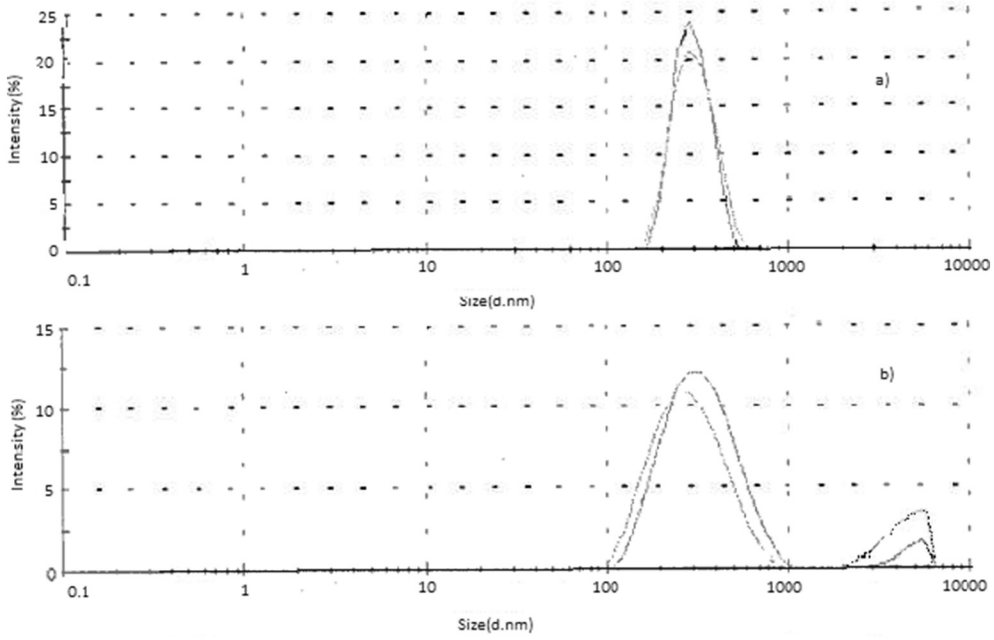

Fig. 1. Particle size of TiO_2 in the solutions a) stock and b) TiO_2 + algal *mix complex*.

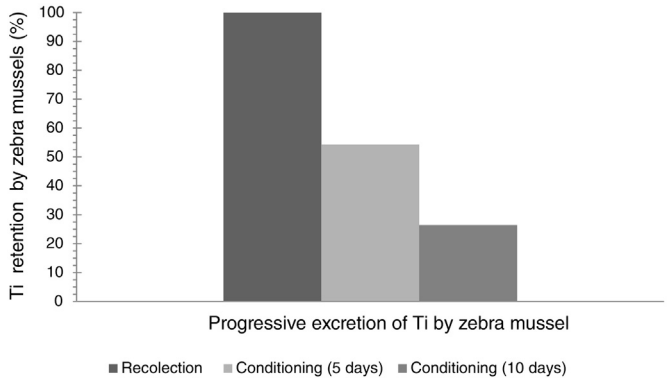

Fig. 2. Representation of the residual retention of Ti in soft tissue of zebra mussels from the day of capture until the 10th day of acclimation.

2.3. Chlorella algae monoculture

In order to facilitate ingestion of TiO_2 by mussels, a monoculture of *Chlorella* was used to induce adsorption of TiO_2 particles. 5 L *Chlorella* suspension was placed in a glass fishbowl for four days at 22 ± 1 °C. The suspension was exposed to natural light and aeration to maintain algal suspension and oxygen availability. About 6 g of commercial fertilizer and 5 L of chlorine-free tap water were added every 48 h to promote the algal growth. In a second experiment, *Chlorella* powder was used directly to make Chlorella algae suspension without stimulation of its growth.

2.4. Stock solution of TiO_2

500 mL of stock solution at 120 ppm of TiO_2 was prepared with milli-Q water. The solution was sonicated for 50 min to maximize homogeneous dispersion (sonication 5 s/5 s on/off at 40 kHz, ultrasonic homogenizer Autotune 750 W, Cole-Parmer Instruments, Vernon Hills, Illinois, US). Then, the particle diameter was measured by using a nanosizer-zetasizer (model Nano-ZS, Malvern, Canada). For this analysis, 100 μL of TiO_2 stock solution were placed in a chamber and 900 μL of milli-Q water were added to complete a volume of 1000 μL. The particle diameter was measured and it was from 100 to 400 nm (mean: 290 nm) due to fast TiO_2 agglomeration.

2.5. TiO_2–algal mix complex

Chlorella algae monoculture and the stock solution were transferred toward a glass bowl to produce a TiO_2–algal complex suspension at a concentration of 5 ppm. This solution was aerated to mix and allow adsorption of TiO_2 particles on Chlorella. After that, the TiO_2–algal mix solution was used to prepare the treatments evaluated in this study.

2.6. Experimental design

Preliminary, three treatments with TiO_2 concentration of 0.35, 0.7 and 3.5 ppm (treatment 1, 2 and 3 respectively) were prepared. To do so, a volume of 0.35, 0.7 and 3.5 L of the algae-TiO_2 complex suspension was introduced in the glass bowls and the volume was completed with chlorine-free tap water to achieve 5 L of solution by treatment. Additionally, a witness treatment containing 0.35 L of *Chlorella* algae monoculture free of TiO_2 and 1.5 L of chlorine-free tap water was used as control to determine residual excretion by the mussels that could interfere with the measurements. Then, 250 mussels were placed in each bowl. The temperature in the room where the experiment was made was kept at 15 ± 1 °C. The photoperiod was set for 8 h light and 16 h dark. All the treatments were conditioned with aerators throughout the overall experiment to maintain sufficient oxygen level and simulate environmental turbulence. Every18h, 36 and 54 h samples of around 30 g of mussels were drawn and immediately frozen. The rest of mussels and the bowls were washed three times at waterjet. Then, new treatment solutions with their respective concentration were prepared and placed in each bowl to maintain initial conditions. The experiment was stopped at 54 h of evaluation.

A second experiment was made to understand the TiO_2 decantation pathway attached to microalgae vs free TiO_2. For that, the same conditions used in the first experiment were repeated but, in this case, only 3.5 ppm of TiO_2 was used in duplicates, to achieve good filtration activity and low toxicity for the mussels. Then, 250 mussels were placed in each bowl. The temperature and the photoperiod in the room were kept at 15 ± 1 °C and set for 8 h light and 16 h dark similar to first experiment. All the treatments were conditioned with aerators throughout the experiment. Every 24 h, 48 and 72 h samples of around 30 g of mussels were drawn and immediately frozen. The rest of mussels and the bowls were washed three times with pressured water to remove all potentially attached pathogenic bacteria. Then, new treatment solution with the same concentration was prepared and placed in each bowl to maintain initial conditions. The experiment was stopped at 72 h of

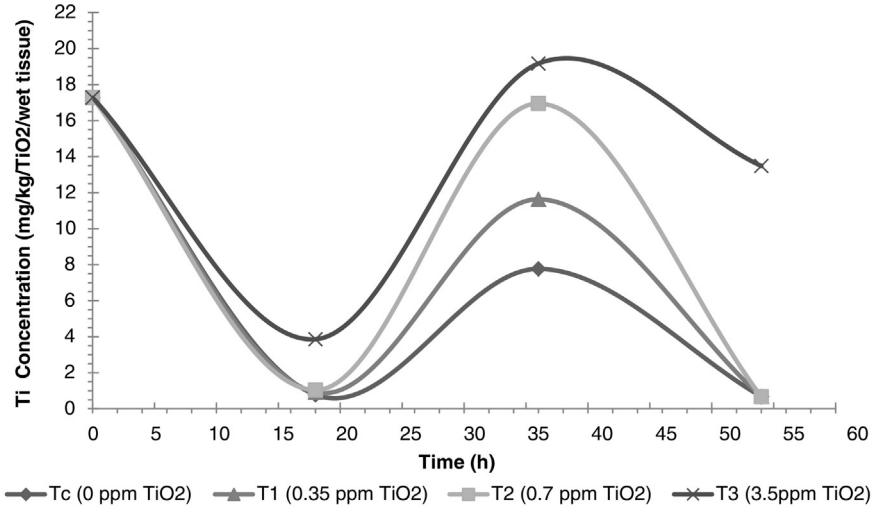

Fig. 3. Behavior of Ti in wet tissue of zebra mussel under different TiO_2 concentration and exposure time.

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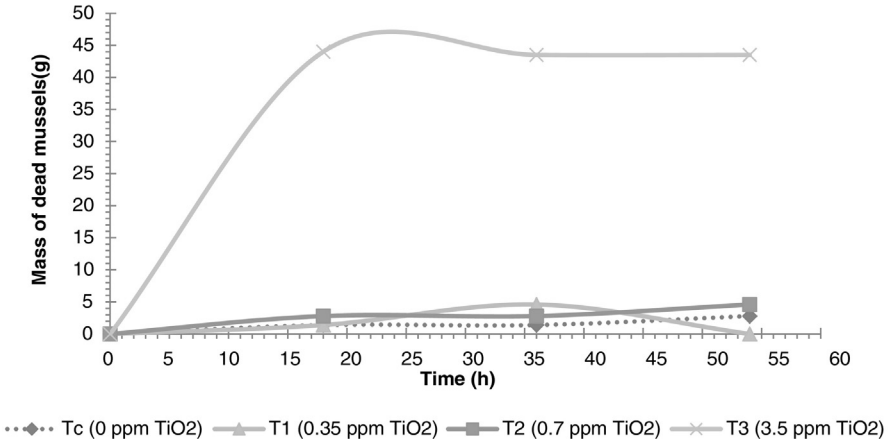

Fig. 4. Mortality of zebra mussel exposed at different concentration of TiO_2.

evaluation. For Ti quantification the same process of sampling conditioning and analysis was applied.

2.7. Sampling for Ti quantification

At time zero and every 18 or 24 h during the whole experiment, samples of the suspension, mussels and sediments were collected from each treatment. Samples from each treatment consisted in 50 mL of the suspension taken from the surface, 50 mL taken from the bottom (sediments). Samples were stored in polypropylene tubes of 50 mL (Fisherbrand™, FisherScientific, Quebec, Canada). Samples of 30 g of mussels per treatment were simoultaneously taken and stored in plastic bags for analysis of titanium (Ti) content. When the experiment was completed, the quantification of Ti was carried out using inductively coupled plasma-atomic emission spectrometry (ICP-AES VISTA, Canada).

2.8. Quantification of Ti by ICP-AES

Before the Ti quantification, the samples were digested as follows:

Liquid samples: 2 mL of liquid samples were transferred to Teflon tubes. Then, 4 mL of nitric acid and 2 mL of perchloric acid was added. The Teflon tubes were closed and placed in the autoclave to digest the samples. The digestion was made at temperature of 121 ± 1 °C and pressure around 17 psi for 4 h.

Sediments: The samples were filtered to collect the sediments. Then, the sediments were dried at 65 °C in an oven until constant weight. The sediments weight was registered at the nearest 0.01 mg and the samples were transferred to Teflon tubes to make the digestion such as previously explained for liquid samples.

Mussels: The tissue of the mussels was removed from their valves. Wet tissues of mussels were dried at 65 °C in an oven until constant weight. Dry samples (0.2 g) were placed in the Teflon tubes to make the digestion similar to this made for liquid samples. Two reagent blanks were prepared with the same digestion procedures as described for the control. The digested samples were then filtered and transferred to falcon tubes of 50 mL (Fisherbrand™, FisherScientific, Quebec, Canada) and MilliQ water was added completing a volume of 50 mL. After that, 20 mL of the conditioned samples were used to quantify the Ti concentration by ICP-AES.

Before Ti quantification, ICP-AES calibration standards of Ti ranging from 0.02 to 10 ppm were prepared using SCP Science, PharmaCAL standards. The standards were prepared by using HNO_3 (10%). All standard and sample solutions were spiked with internal standard (IS) Yttrium (Y) 1 ppm. The spiking IS solution was taken from a single element stock solution of Y 1000 ppm. The final Ti concentration in dry weight was calculated after reading in the spectrophotometer. The operating parameters of the equipment were: Concentric glass nebulizer; nebulizer flow (1 L/min) cyclonic spray chamber; torch (quartz torch-single

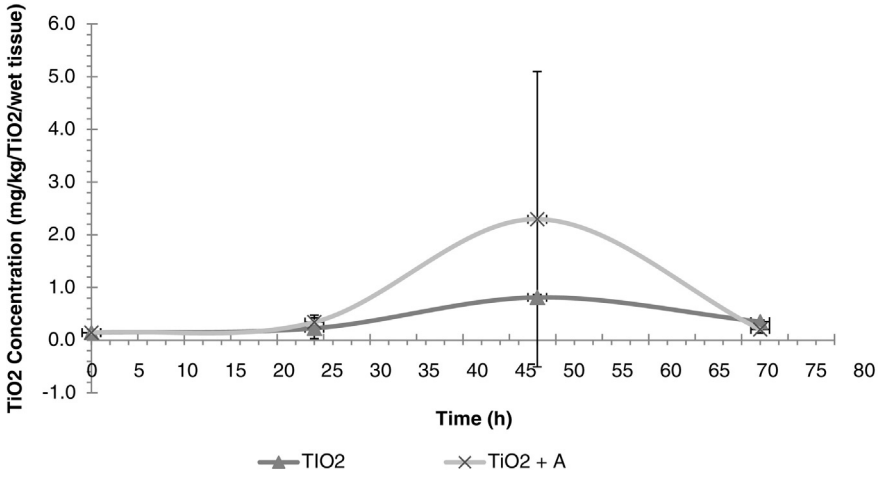

Fig. 5. Ti content in zebra mussel tissues exposed to TiO_2 suspension with and without algae.

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slot); injector (glass); power (1300 W); plasma view (axial); plasma flow (15 L/min); auxiliary flow (2.25 L/min); sample uptake rate (0.6 mL/min); processing mode (pick area); calibration (linear through zero); background correction (spectral background correction); integration Time (30 s); sample tubing (samples and standards white/white, internal standard orange/white; replicates (3)). The detection limit of the instrument was 0.01 ppm.

2.9. Statistical analysis

An one way ANOVA was made to evaluate the significance of the observed differences between treatments. After that, a Tukey's Test for Non-additivity was made to evaluate the interaction of the data and to evaluate if the observed effect of time and Ti concentration in mussels and sediments was significant. This analysis consider a two-factor axb factorial design that has only $n = 1$ replicate for each of the ab treatment combinations (time and concentration). The data analysis was made by using statistical software SAS version 9.4.

3. Results and discussion

3.1. Characterization

3.1.1. Zebra mussels

The mussels collected from St. Lawrence River corresponding to the species, *Dreissena polymorpha* had a size distribution with a mode at 2 cm (mean: 2.03 ± 0.27 cm). The mean weight was 1.4 ± 0.41 g with a mode at 1.3 g. Characteristics of the collected mussels matched with the description of zebra mussel in the nonindigenous aquatic species database (USGC-NAS, 2014).

3.1.2. TiO_2 particle size

Fig. 1a-b presents the particle size of TiO_2 in the solutions stock and algae -TiO_2 mix suspension.

The mean of the particle diameter in the stock suspension was around 290 nm, while in the algal-TiO_2-complex two picks were identified. These two picks suggested that in the algal-TiO_2- complex, homo-agglomeration (NPs coalesce or clump together with other NPs) and hetero-agglomeration (NPs are adsorbed onto algae) could occur in same time in the suspension used. Thus, the first pick represents the unattached-algae particles and the second from the TiO_2- algae complex at around 5500 nm (Fig. 1a-b). Couleau et al. (2012) reported a tendency of TiO_2 NPs to agglomerate in water and settle to the bottom of beakers. They explained that the suspensions used in their experiment increased the size of the NP as a function of the NP concentrations. Also, Wigginton et al., (2007) reported that NPs with different sizes can form agglomerates (>1 μm immediately form agglomerates after their release into the environment). Such observations support the present results of agglomeration of TiO_2 particles. The longer the particles are mixed in a natural matrix, the higher the possibility of agglomeration with diverse materials and get "hidden" by a heterogeneous crown. It is difficult to predict or simulate therefore natural agglomerates in experimental settings.

Table 1
Analysis of variance to evaluate the effect of TiO_2 concentration and exposure time in the Ti concentration in the wet tissue of zebra mussel assuming no-interaction. The significant values are highlighted in bold.

Source	Degrees of freedom	Sum of squares	Quadratic mean	F Value	Pr > F
Model with non-interaction	5	444.42	88.88	7.29	**0.0157**
Error	6	73.16	12.19		
TiO_2 concentration	3	154.90	51.63	4.23	0.0629
Time	2	289.53	144.76	11.87	**0.0082**
Tukey's Test for Non-additivity					
TiO_2 concentration	3	154.90	51.63	3.70	0.0967
Time	2	289.53	144.76	10.36	**0.0167**
Interaction	1	3.32	3.32	0.24	0.6467

Table 2
Analysis of least squares means to evaluate the significant difference among treatments with different TiO_2 concentration. The significant values are highlighted in bold.

i/j	Treatment 1	Treatment 2	Treatment 3	Treatment Control
	Pr > F			
Treatment 1		0.5605	**0.0296**	0.6788
Treatment 2	0.5605		0.0679	0.3337
Treatment 3	**0.0296**	0.0679		**0.0169**
Treatment Control	0.6788	0.3337	**0.0169**	

3.2. Ti retention-excretion by zebra mussel

The initial and progressive retention of Ti measured in the wet tissue of collected mussels is indicated in Fig. 2. The 100% corresponds to the initial Ti concentration in mussels which was around of 62 mgTi/kg wet tissue. This value was 10 times higher than previously reported for the species (Bourgeault et al., 2015) and can be attributed to the exposition level of chemical contaminants in the period (summer 2014) and place in which mussels were collected. Because, it is known that in summer mussels tend to accumulate more contaminants due to the increase of filtration rates (Palais et al., 2012). Also, it was observed that after 5 and 10 days of mussels conditioning in chlorine-free tap water, the concentration was progressively decreasing to 54 and 26% respectively comparing to initial Ti concentration in mussels (Fig. 2). Thus, the rate of Ti deposition was around 5.6 mg/day in the first 5 days, but after 5 days the rate of Ti deposition decreased to 3.4 mgTi/day. This behavior can be explained due to that during conditioning zebra mussels were not fed and it could expose them to nutritional stress which decreased their metabolic activity (Palais et al., 2012).

After 10 days of conditioning, the Ti concentration in mussels was around 16.3 mgTi/kg wet tissues. This concentration was established as the initial concentration at time zero when the mussels were exposed to different treatments of TiO_2 (0.35, 0.7 and 3.5 ppm) during 3 days. Results of the Ti concentration calculated in the wet tissue of mussels at different time are showed in Fig. 3. Results suggested that mussels had the capacity to adsorb and eliminate quickly the Ti adsorbed in the first 18 h, because Ti did not stay in the solution neither in mussels. However, after 36 h, mussels adsorbed Ti as a function of the TiO_2 concentration used by treatment and then it was excreted before 54 h (Ti concentration was very close to zero), except in the case of treatment 3 (3.5 ppm). According with the behavior showed in Fig. 4 the rate of Ti excretions was decreasing in time. Because the second dose of Ti given to mussel at 18 h was excreted after 36 h except for treatment 3. It indicates that mussel tissues reach a threshold in which a depuration mechanism is activated but can be limited at high concentration of Ti (treatment 3). Bourgeault et al. (2015) reported that zebra mussels are able to eliminate completely the Ti after 24 h of exposition of TiO_2 at 1 ppm. However, the results in the present study indicate that it will depend of the Ti concentration and exposure time. Taking into account the low Ti concentration present in rivers, the route of Ti transport from mussels through the food chain is mainly through feces deposition and not through mussel predators. On the

Table 3
Analysis of least squares means to evaluate the significant difference among treatments exposed at different exposure time. The significant values are highlighted in bold.

i/j	18 h	36 h	54 h
	Pr > F		
18 h		**0.0038**	0.4382
36 h	**0.0038**		**0.0096**
54 h	0.4382	**0.0096**	

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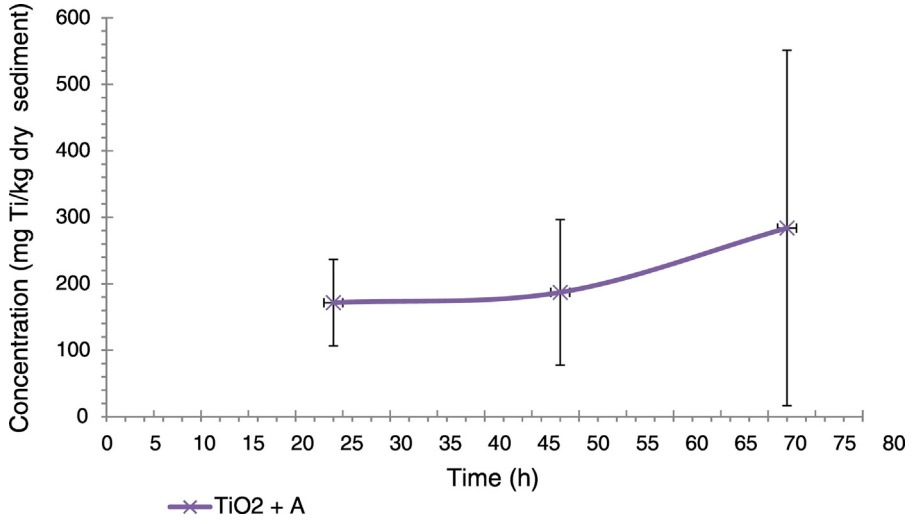

Fig. 6. Ti content in sediments as a function of exposure time of mussels to TiO_2.

other hand, during the experiment it was observed that around 43.67 g/day ±0.24 of mussels were dead (Fig. 5) in the treatment 3 (TiO_2 concentration of 3.5 ppm). This mortality was higher respect to other treatments in which the mass of dead mussels was only from 1.87 to 3.4 g/day. Considering the fact that the metal detoxification and the maintenance of detoxification mechanisms might be energetically expensive, the TiO_2 concentration used in the treatment 3 could affect the homeostasis of the organisms and also their metal detoxification capacity. Thus the increased energetic cost by stress expression and finally produce the mussels mortality (Smolders et al., 2004; Voets et al., 2009).

To evaluate the significance of the filtration activity of mussels on the TiO_2 decantation, Tables 1–3 show the results from the analysis of variance (ANOVA). The least squares means evaluate the effect of TiO_2 concentration and exposure time in the wet tissue of zebra mussel (Montgomery, 2001). The Pr > F value in Table 1 indicates that the model assumes no-interaction and therefore, it can estimate the difference among the treatments at a significance level $\alpha \leq 0.05$ and that the observed effects can be attributed to the evaluated variables and not to the interaction. To corroborate this observation a Tukey's test for non-additivity was performed. The results are showed in Table 1 indicating that the main differences among treatments can be attributed to the exposure time and not to the changes among the TiO_2 concentrations evaluated in the experiment ($\alpha \leq 0.05$). Thus, to elucidate each one of the difference between the treatments a least squares means test was made and results are showed in Table 2, which show that the observed difference among the treatments was significant only between the treatment 1 and 3; 2 and 3; and control and 3 at an significant level $\alpha \leq 0.05$. Table 3 presents the differences among the exposition time. It can be seen that the main changes in the Ti concentration in the mussel tissues were significant between 18 and 36 h; and also between 36 and 54 h. Thus, it can be concluded that only at TiO_2 concentration of 3.5 ppm, a significant difference in Ti concentration in the wet tissues of zebra mussels can be identified and that the main changes occur around 36 h of TiO_2 exposition. On other hand, a similar statistical test was made to evaluate the effect of TiO_2 concentration and exposure time on the mass of dead mussel. Results showed a significant effect only between the treatment with a TiO_2 concentration of 3.5 ppm and the other treatments ($Pr > F = 0.0001$). In this case, the exposure time has no effect on the mass of dead mussel ($Pr > F = 0.8428$).

As complement to the understanding of Ti retention-excretion behavior by zebra mussels, a second set of experiments was run to understand the TiO_2 pathway attached to microalgae vs free TiO_2. In this experiment mussels were exposed in a solution at 3.5 mg/L TiO_2 with and without microalgae. Results showed in Fig. 5 indicated that mussels accumulated less TiO_2 when it was unattached to microalgae. However when the statistical difference was evaluated by ANOVA, results showed that this difference was not significant at a level $\alpha \leq 0.05$ where the Pr > F value was of 0.592 and 0.993 for the treatment (with algae and without algae) and the exposure time respectively. Thus, the Ti suspended in water or attached to microalgae seems to be equally catchable by zebra mussels. However, it can be seen that the standard deviation at an exposure time of 48 h was higher than the other times. Thus other factors could influence the Ti concentration identified in the tissue of mussels. Because it is know that the particle type in the environment, the particle size and the state of the TiO_2 particles can affect the behavior and transport of TiO_2 in the ecosystems, these parameters should be taken into account when zebra mussels are used.

The Ti in sediments was quantified, but in the treatment without algae, no sedimentation was observed and this did not allow the corresponding determination in an appropriate way. Thus, only the Ti concentration present in the sediments from the treatment with TiO_2-algae was evaluated and results are showed in Fig. 6. It can be seen that the Ti concentration in sediments was around of 100 times higher with respect to the TiO_2 concentration present in the mussel's tissue, this indicates that an important accumulation of Ti occur in sediments and the main route of Ti transport are the mussels through their filtration activity to feces deposition. A slight increase of Ti concentration in sediments as a function of exposure time after 50 h was observed. However, due to the standard deviation were high, conclusion cannot obtained respect to the increase of Ti as a function of exposure time.

4. Conclusion

Zebra mussels (*Dreissena polymorpha*) showed almost complete Ti detoxification capacity when they were exposed to TiO_2 concentration lower to 3.5 ppm. However, when mussels were exposed to a TiO_2 concentration of 3.5 ppm, the detoxification capacity of mussels was limited and then Ti was also accumulated in their tissues. On the other hand, it was observed that zebra mussels adsorbed Ti freely dissolved or suspended in organic matter present in the environment and that the main way of Ti magnification from zebra mussels was through the Ti accumulated in sediments.

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Declaration of interest

The authors declare that they have no conflict of interest.

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