Abstract

HOW DO MODIFIED NANOCLAYS ADVERSELY AFFECT AQUATIC SPECIES RELATIVE TO NATURAL NANOCLAY?

By

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Nanoclays represent a large class of modified nanomaterials (NMs) (i.e. nanoscale particles from 1 to 100 nm) that has received great attention from the scientific and industrial communities. This material has been widely incorporated into conventional polymers in order to improve their barrier properties, thermal and mechanical resistance, and to reduce their costs. Currently, some nanoclays are also useful in water treatment and pollution control for removing toxic chemicals from water supplies. However, due to the increased frequency of industrial and environmental applications of the modified nanoclays, their release into the environment will be inevitable in the next decade, especially in the aquatic ecosystems. Thus, many concerns have been raised about the lack of information and potential adverse effects of modified nanoclays relative to natural nanoclay already present in the environment. This dissertation has investigated the physicochemical characterization of natural nanoclay (Na^+ montmorillonite) and two modified nanoclays (Cloisite® 30B and Novaclay^{TM}), and their potential toxicity on algal population growth of *Chlamydomonas reinhardtii*, on survivorship and body growth of *Daphnia magna* and *Chironomus dilutus*, and on survivorship, body condition, and liver tissues of *Gambusia holbrooki*. This study found that particle size of nanoclays was dependent on the nanoclay concentration in solution, which resulted in the formation of agglomerated particles at

lower concentrations and deagglomerated particles at higher concentrations due to particle-particle collisions. In addition, surface charge analysis showed that Novaclay ™ is more stable than natural nanoclay and Cloisite® 30B, making Novaclay™ more likely to remain as single particles rather than agglomerates in solution. Although, natural nanoclay and Novaclay ™ retained their platelet-type shape in aqueous medium, Cloisite® 30B transformed from a platelet-type shape in dry powder form to spherical particles when in solution. This study also found that natural nanoclay and modified nanoclays had important implications to aquatic life; however, there were differences in their toxicity due to nanoclay composition, concentration in media, particle size and shape, surface charge, and exposure time. In general, the toxicity of the three types of nanoclays to aquatic species rank as follows: Cloisite® 30B > Novaclay™ > natural nanoclay. Cloisite® 30B adversely affected more kinds of organisms than other nanoclay types. Cloisite® 30B, even at low concentrations, reduced growth rate of *C. reinhardtii*, *D. magna* survival, body growth of *C. dilutus* and caused liver tissue damage to *G. holbrooki*. Novaclay™ and natural nanoclay also induced histopathological changes on liver tissues of *G. holbrooki* at very low concentration. Conversely, Novaclay™ only reduced the survivorship of *D. magna* during chronic exposure at low and high concentrations, while natural nanoclay caused a decline in daphnid survival only at high concentration with acute exposure. None of the nanoclays affected the survivorship of *C. dilutus* or *G. holbrooki* during the duration of our study. We also found little effect of natural nanoclay and Novaclay™ on the body growth of *D. magna* and we were unable to assess the effects of Cloisite® 30B on the body growth of daphnids at higher concentrations, because all organisms died when exposed to Cloisite® 30B. In addition, none of nanoclays caused any effects on body condition of *G. holbrooki* after 14 days of exposure. The higher toxic effects of Cloisite® 30B may be associated with the presence of quaternary

ammonium compounds in its composition, which may cause oxidative stress in biological systems. While, the toxicity of Novaclay^{TM} and natural nanoclay is probably due to their high stability in aqueous medium that makes them more available for pelagic species (e.g.; daphnids and mosquito fish). Thus, we should be careful about the kinds of modified nanoclays that we introduce into aquatic environments, since they pose a threat to aquatic organisms and ecosystems processes.

HOW DO MODIFIED NANOCLAYS ADVERSELY AFFECT AQUATIC SPECIES RELATIVE TO NATURAL NANOCLAY?

A Dissertation

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CHAPTER 1: Physical and chemical characterization of natural and modified nanoclays in dry powder state and in aqueous solution

Abstract Nanoclays represent a class of natural and modified nanomaterials that have received attention from the industrial and environmental fields due to their potential use in conventional polymers, drug delivery systems, and water remediation. Nevertheless, studies that assess the physicochemical properties of nanoclays to advance our understanding of their potential toxicity are scarce. This study characterized the physicochemical properties of a natural nanoclay (Na^+ montmorillonite) and two modified nanoclays (Cloisite® 30B and Novaclay™) in dry powder state and in solution. To determine the elemental composition, surface charge, and particle size and shape of nanoclays, we used X-ray fluorescence spectrometer, dynamic light scattering, scanning electron microscopy and transmission electron microscopy, respectively. Our study found that all nanoclays show similar dominant elements, but they differ in the proportional abundances of these elements. Particle size was also dependent on the nanoclay concentration in solution, which resulted in the formation of agglomerated particles at lower concentrations and deagglomerated particles at higher concentrations due to particle-particle collisions. In addition, surface charge analysis showed that Novaclay™ is more stable than natural nanoclay and Cloisite® 30B, making Novaclay™ more likely to remain as single particles rather than agglomerates in solution. Although, natural nanoclay and Novaclay™ retained their platelet-type shape in aqueous medium, Cloisite® 30B transformed from a platelet-type shape in dry powder form to spherical particles when in solution. The information presented in this study helps

identify the physicochemical changes of nanoclays in solution, which contributes to our understanding of the mechanisms through which nanoclays affect biological systems.

Key words nanoclays; nanoparticle characterization; aquatic ecosystems; platelet and spherical shape.

Introduction

Natural nanoclays are nano-sized (<100 nm in diameter) clay minerals (2:1 layered silicates) produced from surface weathering of rocks. Nanoclays have received considerable attention in industrial fields due to their potential use in a variety of commercial products (e.g., polymer composites, sunscreens, and antibacterial), environmental applications (e.g., wastewater treatment), and low cost due to their high abundance in nature (Ellenbecker and Tsai 2011; Lee et al. 2005). However, natural nanoclays exhibit great variability in their chemical, mineralogical, and structural characteristics, which prevent their application in fields that require precise control of these properties. As a result, nanotechnology has been used to develop modified nanoclays with precise particle size, surface properties, and chemical composition.

While the benefits of modified nanoclays are well recognized, many concerns have been raised about their potential adverse effect on occupationally exposed workers, public health, and the environment (Hood 2004). From an environmental perspective, aquatic environments will likely receive modified nanoclays unintentionally from many sources; including production facilities through the release of nanoclays into rivers via untreated or treated wastewater (Gottschalk and Nowack 2011), and leaching of nanoclays from materials placed in landfills or discarded in inappropriate ways (Nowack and Bucheli 2007). Another mechanism of release of

modified nanoclays is during water treatment (Patel et al. 2006). Nanometric clay minerals can act as efficient sorbents to facilitate the removal of various pollutants during groundwater, surface water and wastewater remediation (Calabi Flood et al. 2009; Lee and Tiwari 2012). Studies have documented that the sorption of pollutants by modified nanoclays is superior to any other wastewater treatment technology, especially when the wastewater contains polychlorinated biphenyls (PCBs), phenols, heavy metals, oil, grease, or humic acid (Patel et al. 2006; Han Ko et al. 2007). Consequently, aquatic ecosystems will serve as a terminal sink for many types of nanoclay (Moore 2006).

Nevertheless, studies that evaluate and compare the toxicological effects of natural nanoclays and modified nanoclays on aquatic systems are scarce. Natural nanoclays have been part of our environment since the beginning of Earth's history so aquatic organisms have evolved in environments containing natural nanoclays (Handy et al. 2008). In fact, numerous studies have already reported some effects of natural nanoclays on aquatic species, as a result of human activities (e.g., agriculture, reservoirs construction) that contribute to increase the concentrations of clays in the environment (Kirk 1992; Levine et al. 2005). For example, Robinson et al. (2010) found that increased natural nanoclays clog the gut of *Daphnia magna*, reducing their growth and reproduction rate. In contrast, organisms have had a very brief evolutionary history with modified nanoclays and it is unknown whether modified nanoclays have a different effect on ecological systems than natural nanoclays.

Characterizing the physicochemical properties of nanoclays is an important initial step to advance our understanding of and ability to predict the potential effects of modified nanoclays on aquatic ecosystems. Indeed, information on surface charge, shape, state of dispersion, particle size and size distribution of different nanoclays may provide the basis for understanding their

biological effects (Powers et al. 2006). For example, Blake (2012) found that aggregated nanoclays in solution present minimal toxic effects on *Daphnia magna*, since they rapidly settle down to the sediment, reducing the time in which daphnids are exposed to nanoclays in the water column. Similarly, zebrafish embryos (*Danio rerio*) appear to be protected from other types of aggregated nanomaterials (NMs) (e.g., carbon nanotubes (CNTs), and metal oxide nanoparticles (ZnO)) that are too large in solution to pass through nanometer-size pores in the chorion (Cheng et al. 2007; Henry et al. 2007; Zhu et al. 2009). Though recent studies have collected some information about the physicochemical properties of NMs (Franklin et al. 2007; Zhu et al. 2010; Zhao and Wang 2011), only a few studies have attempted to report and discuss the implications of physicochemical properties of NMs (i.e. particle shape, reactivity, and agglomeration state) while in solution so as to be more relevant to aquatic species (Alagarasi 2011; Vajtai 2013). This study aims to characterize the physicochemical properties of a natural and two modified nanoclays in solution.

Material and methods

Materials Natural nanoclay (Na^+ montmorillonite, hydrophilic material) and conventional ammonium-modified nanoclay (Cloisite® 30B, hydrophobic material) were obtained from Southern Clay Products (TX, USA), while modified nanoclay without ammonium (Novaclay^{TM}, hydrophobic material) was obtained from Ioto International, located in Campo Magro, Paraná, Brazil. Cloisite® 30B is surface functionalized with MT2EtOH (methyl, tallow, bis-2-hydroxyethyl, quaternary ammonium chloride), while Novaclay^{TM} is synthesized with the addition of stearic acid of calcium (AMS-32^{TM}) within the interfacial lamella of the nanoclays but without ammonium compounds. Natural nanoclay does not have organic modifiers in its

structure. These three types of nanoclay were selected because 1) natural nanoclay (Na^+ montmorillonite) is very abundant in nature with a great potential to be used in the development of modified nanoclays and 2) both modified nanoclays will be likely used in the production of polymer nanocomposites, rheological modifier in paints, drug delivery systems and environmental remediation (Uddin 2008; Ellenbecker and Tsai 2011; Lee et al. 2005) which will likely result in their intentional or unintentional release into aquatic systems.

Stock solution preparation Nanoclays were received in dry powder form, which were weighed on an analytical mass balance, and then suspended in *Reconstituted Hard Water* medium (*RHW*) (U.S. Environmental Protection Agency, 2002) at the following concentrations: 0.01, 0.1, 1, 10, and 100 mg/L. *Reconstituted Hard Water* medium was chosen because it is a standard medium that is widely used in toxicological studies (Rosenkranz et al. 2009; Hall et al. 2009, Oberholster et al. 2011). We selected these concentrations because they represent ecologically relevant concentrations that span the range of values known to commonly occur in nature though concentrations could exceed 500 mg/L in some circumstances following heavy rain events (Kirk 1992; Robinson et al. 2010). To obtain homogeneous dispersion, all samples were initially stirred with a magnetic stirring device for 2 hours.

Characterization of nanoclays

a) Elemental composition of nanoclays

Elemental composition of nanoclays was obtained on a *PANanalytical Axios X-ray fluorescence spectrometer (XRF),* which was equipped with a 60 kV generator and a 4kW rhodium tube as the source of radiation. To determine the XRF measurements, each nanoclay powder (15g) was mixed with ethyl cellulose and wax (10 wt. %), and prepared to 40 mm diameter pressed pellets at high pressure (45,000 pounds) for analysis.

b) Surface charge (Zeta potential)

Surface charge (Zeta potential) of nanoclays in *RHW* medium was estimated by using Dynamic Light Scattering (DLS) (Malvern Instruments Zetasizer Nano ZS). Surface charge provides information about the stability of nanoclays in solution. Nanoparticles with zeta potential values greater than +25mV or less than -25mV are more likely to remain as single particles (rather than aggregate) in solution (i.e., have high stability) (Kochkina et al. 2015). To assess the surface charge of nanoclay in solution, we dispersed 1 mg of nanoclay/L in *RHW* medium. We only selected one of the five concentrations of nanoclays (1mg/L) to measure zeta potential because it likely provides the highest quality measurement data from DLS. Samples containing too low or high concentrations increase the chance of having noisy and distorted results, respectively (Brar and Verma 2011). We estimated zeta potential for each type of nanoclay three times.

c) Particle size and shape in dry powder state and in solution

Particle size and shape (e.g., spherical or non-spherical particles) are important metrics that may be associated with the potential toxicity and reactivity of NMs to aquatic biota (e.g., site(s) of deposition and detoxification mechanism of NMs, and biological responses (inflammatory process)). To characterize the size and shape of nanoclays in the dry state, we used Scanning Electron Microscopy (SEM, FEI Quanta 200). Specifically, a small amount of each nanoclay powder (~1g) was placed onto a clean support stub, coated with a very thin layer of copper by a sputter coater. Subsequently, we randomly selected 3 square regions on SEM images of the stub (center and edges of the image (~50 particles)) to obtain images of nanoclay particles. We then used ImageJ software to measure the size of 100 individual particles for each

nanoclay type. We used a standard classification scheme devised by Alagarasi (2011) to describe the shape of nanoclay particles on the grids.

Although, we measured the shape and size of nanoclays in powder form, it is also necessary to characterize them in the actual test media since potential physicochemical changes (i.e. agglomeration state) can occur while in solution. We characterized the shape and particle size distribution *(PSD)* of nanoclays in solution at each of five concentrations (0.01, 0.1, 1, 10, and 100 mg/L) with the assistance of Transmission Electron Microscopy (TEM, Philips CM12). In order to preserve the solution state of the nanoclay samples with the minimum of artifacts, we 1) stirred each solution for two hours, 2) placed a 10 µL sample of each solution onto a 300 mesh copper grid coated with Formvar film, 3) freeze the grid with liquid nitrogen (-30ºC), and 4) freeze-dried the grid for 15 minutes (Labconco freeze-drier, Labconco Corporation, Kansas City, MI, USA). We replicated this experiment two times. To describe the *PSD* we measured the diameter of 100 particles within each of 4 randomly selected regions in the TEM image using ImageJ software. We also used the classification scheme devised by Alagarasi (2011) to describe the shape of nanoclay particles on the grids.

Results and discussion

a) Elemental composition of nanoclays

The elemental composition of all three types of nanoclays is shown in Table 1. The dominant elements in all three types of nanoclays were oxygen (O) and silicon (Si) and together they accounted for, on average, 73% of the elements present. The proportional abundances of O and Si in Novaclay^{TM}, however, was less than that observed in Cloisite® 30B and natural

nanoclay. The proportional abundances of aluminum (Al), iron (Fe), and magnesium (Mg) was relatively high in each nanoclay type but the proportional abundance of Al was lower in Novaclay™ than in Cloisite® 30B and natural nanoclay. Other elements that ranked high in their proportional abundances included sodium (Na), calcium (Ca), and chlorine (Cl) but the different types of nanoclay varied markedly in the proportions of these elements that they contained. Most notably, the proportion of Ca and Cl was substantially higher in Novaclay™ than in the other nanoclays. Natural nanoclay also had more Na than either of the other two nanoclays but Novaclay™ had more Na than Cloisite® 30B. Novaclay™ did not contain any gallium (Ga) or niobium (Nb), but they were present in natural nanoclay and Cloisite® 30B at the same degree. Conversely, a small amount of barium (Ba) was only found in Cloisite® 30B and Novaclay™. Some elements were exclusive for natural nanoclay (Pb), Cloisite® 30B (F, Th), and Novaclay™ (Mn).

b) Surface charge (Zeta potential)

Though we found that all three nanoclays have moderate stability in solution (i.e., zeta potentials ranged from -11 mV to -21.4 mV), Novaclay™ is more stable ($\bar{x}$ ± 1 SD= -21.4 mV ±0.04) than natural nanoclay ($\bar{x}$ ± 1 SD= -18.7 mV ±0.05) which is more stable than Cloisite® 30B ($\bar{x}$ ± 1 SD= -11 mV ±0.06). Therefore, Novaclay™ is the least likely of our focal nanoclays to agglomerate or aggregate and most likely to be well dispersed in solution. In contrast, Cloisite® 30B and natural nanoclay are more likely to agglomerate, fall out of solution and deposit on sediments. These findings support our personal observation in laboratory, because we detected that both Cloisite® 30B and natural nanoclay seem to deposit on sediment faster than Novaclay™ after they were dispersed in solution.

c) Particle size and shape in dry powder and in solution

In the dry powder state, Cloisite® 30B had both a narrower particle size range (range: 2-35 nm) and smaller mean particle size ($\bar{x}$: 10 nm) than either natural nanoclay (range: 2-55 nm, $\bar{x}$: 21 nm) or Novaclay^{TM} (range: 2-52 nm, $\bar{x}$: 18 nm) which did not differ much from each other. All nanoclays have a platelet-type shape when in the dry powder state (Figure 1), so they are expected to similarly adhere to cells when in dry powder state (Verma et al. 2012).

The *PSD* of the different nanoclay types changed with the concentration of nanoclay present in solution (Figure 2 a-e). When the nanoclay concentration was low (0.01 mg/L), the *PSD* of each nanoclay type was multimodal but Cloisite® 30B **(CL)** had the largest range of particle sizes and had a larger mean particle size ($\bar{x}$=134 nm) than natural nanoclay **(NN)** ($\bar{x}$= 54 nm) and Novaclay^{TM} **(NOV)** ($\bar{x}$= 52 nm) (Figure 2a). The higher dominance of larger particles of Cloisite® 30B may be related to its lower stability in solution (-11mV) that contributes to the faster particle agglomeration at 0.01 mg/L, whereas natural nanoclay and Novaclay^{TM} have more stability in solution, favoring similar *PSD* at the same concentration. When the nanoclay concentration increased to 0.1 mg/L, however, the *PSD* for all nanoclay types was unimodal and all nanoclays had a similarly small mean particle size (NN*:* $\bar{x}$= 13 nm; CL: $\bar{x}$= 23 nm; and NOV: $\bar{x}$= 17 nm) and range of particle sizes (Figure 2b). The reduction in mean particle size as nanoclay concentration is increased can be explained if primary particles at lower concentrations (0.01 mg/L) agglomerate to form larger units by adhesion, but, due to increased particle-particle collisions in higher concentration (0.1 mg/L) the agglomerates tend to break down.

Indeed, previous studies show that physicochemical changes such as agglomeration and/or deagglomeration of NMs can occur due to variations in solution conditions (e.g., temperature, pressure, pH-value, viscosity, particle concentration, etc.) (Walters et al. 2013).

Specifically, after dispersing NMs in solution, they can remain as primary particles or form an assembly of agglomerate particles, which can be easily separated again into smaller agglomerates due to their weak attractive forces (van der Walls forces) (Jiang et al. 2009).

As the concentration of nanoparticles increased to 1 mg/L, the range of particle sizes increased for all nanoparticle types but more so for natural nanoclay (Figure 2c). Furthermore, the *PSD* for natural nanoclay exhibited several peaks while the *PSD* for modified nanoclays did not (Figure 2c). This resulted in an increase in the mean particle size of natural nanoclay and Cloisite® 30B (NN: $\overline{x}$= 80 nm; Cloisite® 30B: $\overline{x}$= 43 nm) but the average particle size of Novaclay^{TM} ($\overline{x}$= 20 nm) did not change much from that observed when the concentration was 0.1 mg/L. The greater increase in average particle size of natural nanoclay may be due to its larger particle size in dry powder, favoring natural nanoclay particles to collide, agglomerate and/or begin to form aggregates when in solution at 1 mg/L. In fact, increasing the nanoclay concentration is not only important to the formation of agglomerated particles, but also to the occurrence of aggregated particles in solution (Walters et al. 2013). Jiang et al. (2009) state that aggregates develop when primary or agglomerate particles begin to form a strong crystalline structure through the sintering process that is firmly fused together and harder to separate, which can explain the presence of larger particle size of nanoclays at 1 mg/L. Although, both agglomeration/deagglomeration and aggregation/disaggregation are held by distinct physical forces, they will certainly have a significant impact on observed toxicological responses, since particle size of NMs will determine their interactions with biological systems, including absorption, distribution, metabolism, and excretion (Oberdörster et al. 2005; Choi et al. 2007). For example, Bennett et al. (2012) reported that TiO_2 aggregates when exposed to light were partially disaggregated, releasing smaller particles in aqueous media that facilitated the TiO_2

transport and penetration into viable layers of pig skin. Indeed, smaller particles can directly pass through the cell membrane, as well as have higher surface areas, which increase their chemical reactivity and potential toxicity in biological systems (Roduner 2006; Sutariya and Pathak 2014). However, agglomerate particles may also cause toxic effects during long term exposures. Yang et al. (2008) found that single-walled carbon nanotubes do not seem to cause toxic effects on liver, spleen, and lungs during acute exposures, but, the accumulation and formation of agglomerates of NMs over time induce cytotoxic effects such as pulmonary interstitial fibrosis.

All three nanoclay types had a wide range of particle sizes and displayed multiple peaks in the *PSD* when nanoclay concentration increased to 10 mg/L (Figure 2d) but only natural nanoclay exhibited a substantial increase in mean particle size above that observed when the concentration of nanoclay was 1 mg/L (NN increase*:* 86 nm; CL increase: 6 nm; and NOV increase: 3 nm). Increasing nanoclay concentration to 100 mg/L resulted in a reduction in the maximum particle size exhibited by natural nanoclay but had little influence on the maximum particle size exhibited by Cloisite® 30B and Novaclay^{TM} (Figure 2e). Consequently, the average particle size of natural nanoclay decreased to 45 nm but the average size of Cloisite® 30B and Novaclay^{TM} particles was largely unaltered (CL: $\bar{x}$= 40 nm; NOV: $\bar{x}$= 22 nm). This result was again consistent with the trend observed for nanoclays at 0.1 mg/L, where higher nanoclay concentrations favor the collision rate among particles due to the stirring process, and consequently the agglomerates tend to dissociate into smaller particles. On the other hand, the dispersion state for Cloisite® 30B and Novaclay^{TM} seem to remain more stable at 10 and 100 mg/L, resulting in minimal changes in terms of particle size for both nanoclay types.

TEM images also showed that natural nanoclay and Novaclay^{TM} seem to maintain the platelet-type shape in solution, while Cloisite® 30B has oval-shaped particles in solution (Figure

3). Such difference in terms of shape may affect the potential toxicity of nanoparticles to aquatic species. Li et al. (2015) found that spherical NMs present the highest cell uptake rate when compared to other non-spherical nanoparticles (rod, silica, and disk). One reason for this is that non-spherical particles tend to be involved in a stronger membrane deformation and complicated rotations in order to be internalized by cells, resulting in slower cellular uptake rate. While, spherical particles are only involved in a minimum membrane bending energy barrier, favoring their fast entry into the cells. Alternatively, George et al. (2012) indicated that silver nanoplates were the most toxic form of NMs on both fish epithelial cell lines (e.g., cell death) and on zebrafish embryos (e.g., lack of embryo hatching) as opposed to nanospheres or nanorods. They assume that silver nanoplates have "sharp" edges on the surface, which favors them to break up cell membranes. Although, we are still far from fully understanding the relationship between physical shape of NMs and its contact with cell membranes, there is no doubt of its importance to better describe nanobio interactions.

Conclusion

Elemental composition, particle size, shape, and surface charge of nanomaterials play an important role in understanding the potential toxicity of NMs for human health and the environment. Our study has found that natural nanoclay and modified nanoclays differ in those particular physicochemical properties when analyzed in solution.

In fact, powdered natural nanoclay has the largest range of particle size and mean size particle than powdered versions of modified nanoclays but the *PSD* and mean particle size of each nanoclay type changed when placed in solution. Specifically, there were cyclic changes in

terms of *PSD* across the five solution concentrations that we examined. We suggest that under lower concentrations primary particles of nanoclays appear to agglomerate to form larger units by adhesion, due to weak physical interactions. However, as we increase the nanoclay concentration in solution, such agglomerates partially start to dissociate as a result of the particle-particle collisions during the stirring process, forming again smaller particles. Our results also showed that nanoclay particles seem to aggregate at high concentrations, which explains the occurrence of larger particle sizes and multiple peaks in the *PSD*.

In addition, the shape of Cloisite® 30B presented significant changes when it was dissolved in *RHW* medium. While, natural nanoclay and Novaclay^{TM} retain their platelet-type shape in solution, Cloisite® 30B is best described as spherical particles in solution. Previous studies have discussed the importance of identifying the correlation between particle shape and toxic effects of nanoclays on aquatic species. Indeed, the morphology of NMs may dictate its ability to adhere, and enter into specific cell types, as well as alter its time of residence inside the cell (Albanese et al. 2012).

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Table 1. Elemental composition analysis of three types of nanoclays (Natural nanoclay, Cloisite® 30B and Novaclay™) obtained on a PANanalytical Axios X-ray fluorescence spectrometer (XRF). This table lists the proportional abundance (%) of each element present in nanoclays. Number in parentheses indicate the rank order of the elemental composition for each nanoclay type.

Elements	Natural nanoclay	Cloisite® 30B	Novaclay™
O	47.81 (1)	48.65 (1)	42.83 (1)
Si	28.24 (2)	29.56 (2)	22.19 (2)
Al	10.60 (3)	11.64 (3)	7.61 (4)
Fe	5.06 (4)	6.24 (4)	6.56 (5)
Na	4.51 (5)	0.42 (6)	2.79 (6)
Mg	2.21 (6)	2.09 (5)	2.20 (8)
Ca	0.67 (7)	0.42 (6)	11.78 (3)
S	0.38 (8)	0.04 (13)	0.15 (11)
Cl	0.18 (9)	0.36 (7)	2.56 (7)
K	0.12 (10)	0.10 (10)	0.30 (10)
Ti	0.12 (10)	0.14 (8)	0.57 (9)
P	0.03 (11)	0.03 (14)	0.07 (15)
Sr	0.03 (11)	0.03 (14)	0.09 (13)
Zr	0.02 (12)	0.06 (11)	0.06 (16)
Zn	0.01 (13)	0.02 (15)	0.13 (12)
Ga	0.01 (13)	0.01 (16)	-
Pb	0.01 (13)	-	-
Y	0.004 (14)	0.01 (16)	0.01 (18)
Nb	0.004 (14)	0.004 (17)	-
F	-	0.11 (9)	-
Ba	-	0.05 (12)	0.08 (14)
Th	-	0.02 (15)	-
Mn	-	-	0.02 (17)

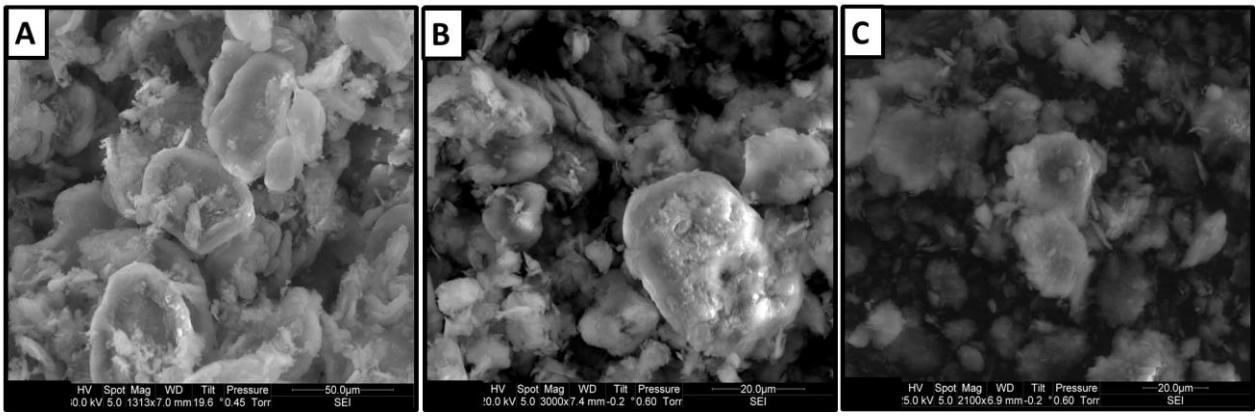
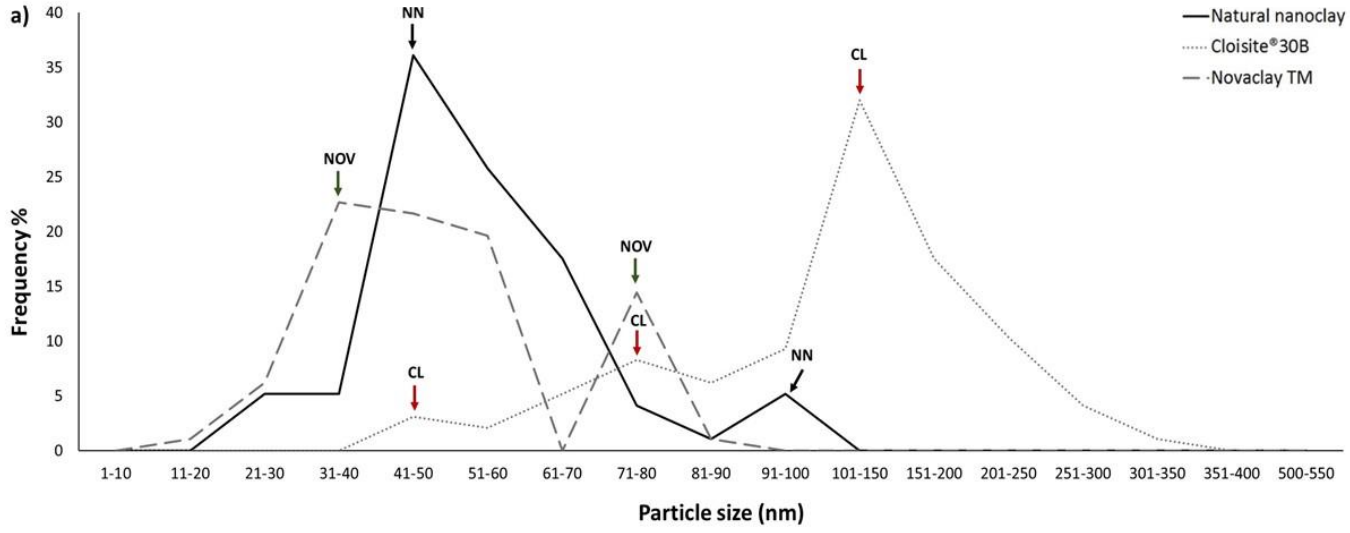
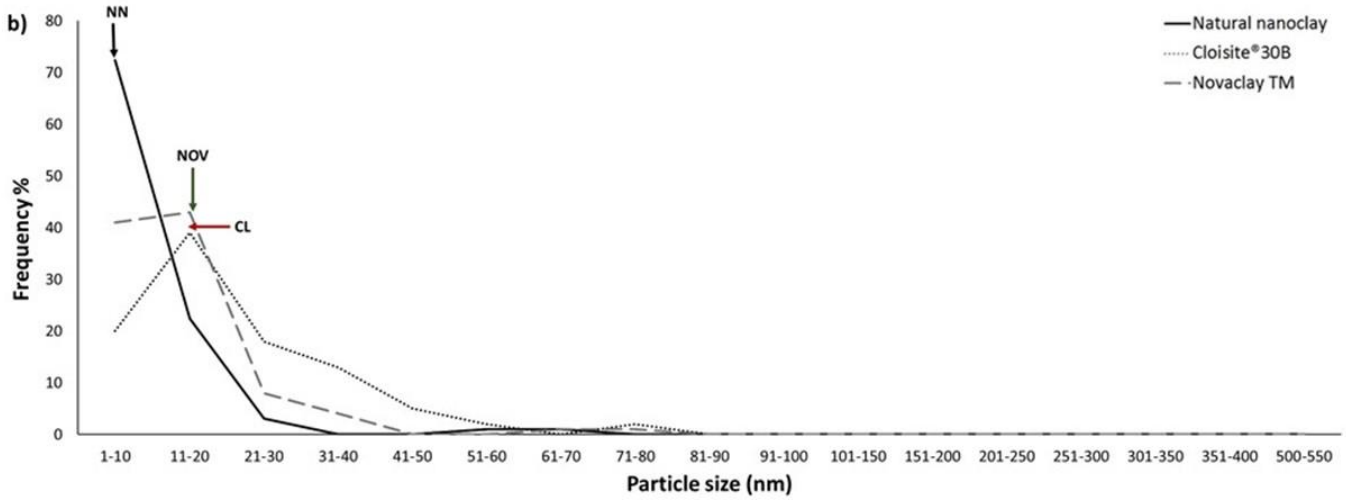
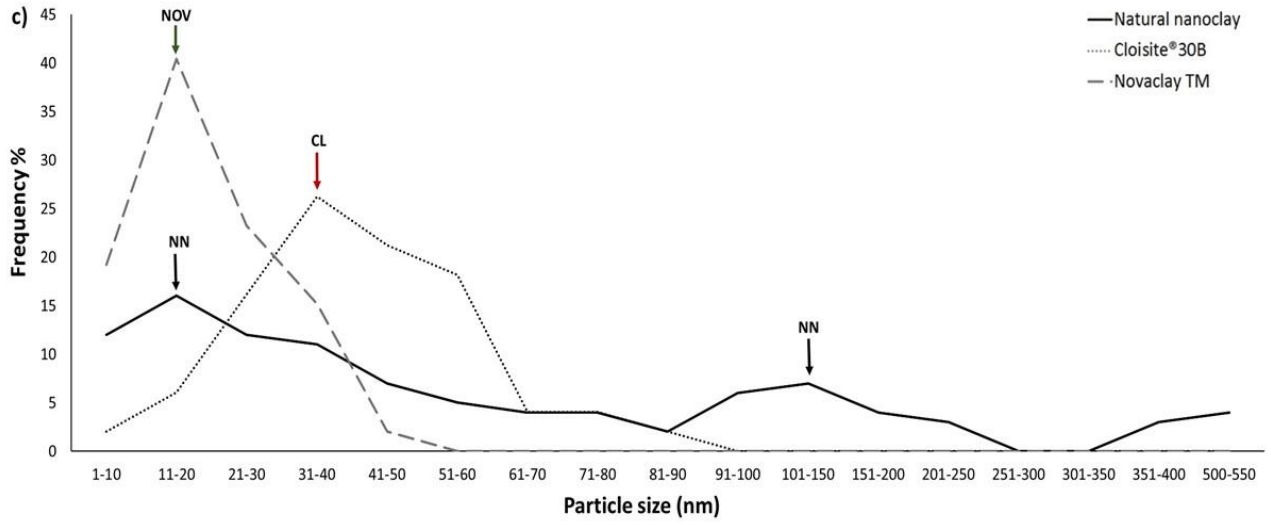
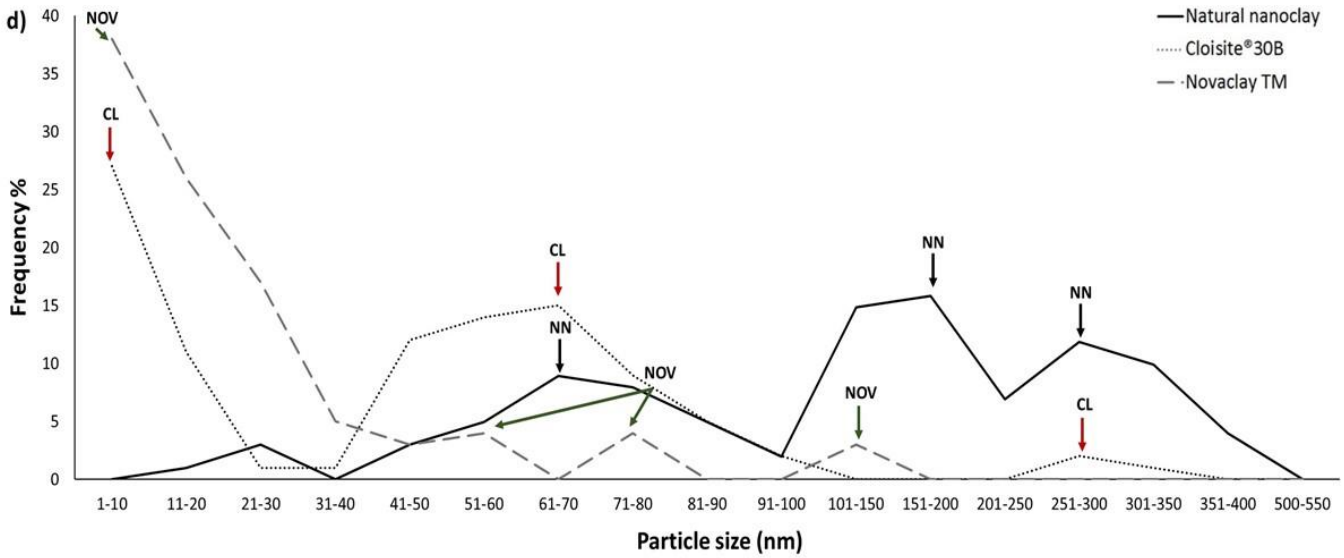


Figure 1. Scanning Electron Microscopy of three different types of nanoclays in dry powder state. **A)** Natural nanoclay (Na^+ montmorillonite), **B)** Conventional ammonium-modified nanoclay (Cloisite® 30B), and **C)** Modified nanoclay without ammonium (Novaclay^{TM})


a)
Natural nanoclay
Cloisite®30B
Novaclay TM
Frequency %
Particle size (nm)
NN
NOV
CL
NOV
CL
CL
NN
0
5
10
15
20
25
30
35
40
1-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
101-150
151-200
201-250
251-300
301-350
351-400
500-550


b)
Natural nanoclay
Cloisite®30B
Novaclay TM
Frequency %
Particle size (nm)
NN
NOV
CL
0
10
20
30
40
50
60
70
80
1-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
101-150
151-200
201-250
251-300
301-350
351-400
500-550


c)
Frequency %
Particle size (nm)
NOV
CL
NN
NN
Natural nanoclay
Cloisite®30B
Novaclay TM
0
5
10
15
20
25
30
35
40
45
1-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
101-150
151-200
201-250
251-300
301-350
351-400
500-550


d)
Frequency %
Particle size (nm)
NOV
CL
CL
NN
NOV
NOV
NN
NN
CL
Natural nanoclay
Cloisite®30B
Novaclay TM
0
5
10
15
20
25
30
35
40
1-10
11-20
21-30
31-40
41-50
51-60
61-70
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151-200
201-250
251-300
301-350
351-400
500-550

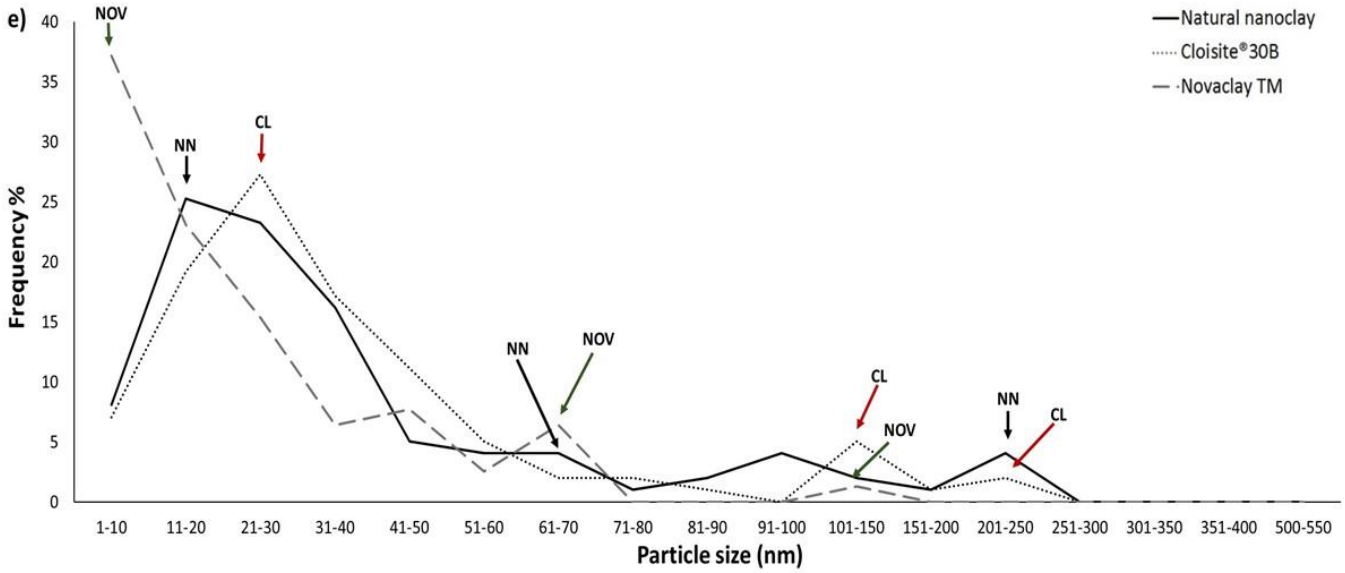

Figure 2. Particle size distribution of natural nanoclay, Cloisite® 30B, and Novaclay™ at five different concentrations: **(a)** 0.01 mg/L, **(b)** 0.1 mg/L, **(c)** 1 mg/L, **(d)** 10 mg/L, and **(e)** 100 mg/L. **NN:** natural nanoclay, **CL:** Cloisite® 30B, and **NOV:** Novaclay™.

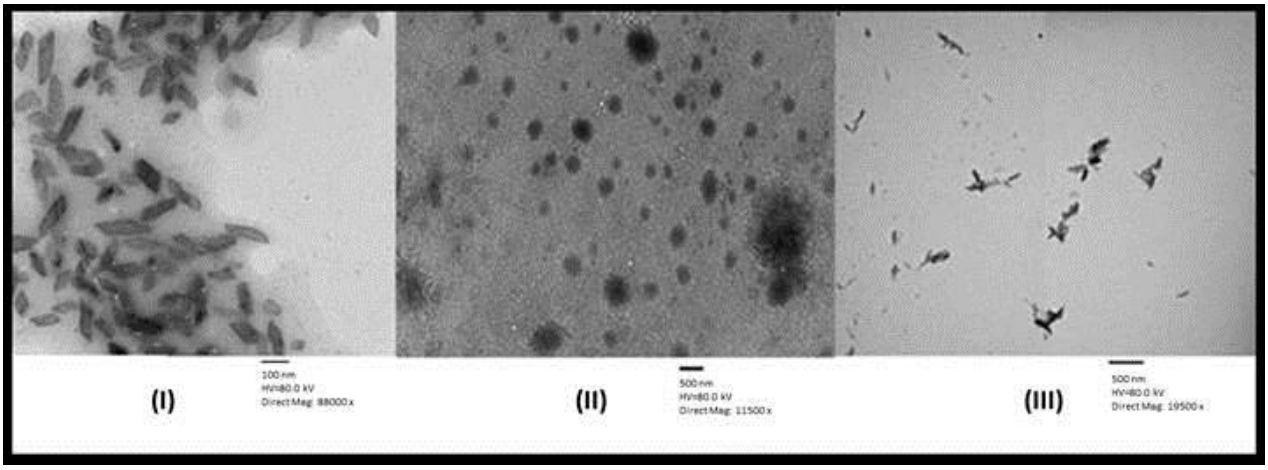

Figure 3. Transmission Electron Microscopy of three different types of nanoclays in solution. **I)** Natural nanoclay (Na^+ montmorillonite), **II)** Conventional ammonium-modified nanoclay (Cloisite® 30B), and **III)** Modified nanoclay without ammonium (Novaclay^{TM})

CHAPTER 2: Do modified nanoclays adversely affect *Chlamydomonas reinhardtii*, *Daphnia magna*, and *Chironomus dilutus* relative to natural nanoclay?

Abstract There is growing interest in the usage of modified nanoclays in industrial and environmental applications. Nevertheless, many concerns have been raised about their implications for aquatic ecosystems. This study investigated the potential toxicity of a natural nanoclay (Na^+ montmorillonite) and two modified nanoclays (Cloisite® 30B and Novaclay™) on population growth of *Chlamydomonas reinhardtii*, and on survivorship and body growth of *Daphnia magna* and *Chironomus dilutus*. Our results showed that Cloisite® 30B was the only nanoclay type that suppressed the algal population growth (NOAEL: 1 mgL^{-1} and LOAEL: 10 mgL^{-1}). Similarly, Cloisite® 30B affected the survivorship of *D. magna* after acute and chronic tests at lower concentrations (NOAEL: 0.1 mgL^{-1} and LOAEL: 1 mgL^{-1}). Novaclay™ only reduced the survivorship of *D. magna* during chronic exposure (NOAEL: 0.1 mgL^{-1} and LOAEL: 1 mgL^{-1}), while natural nanoclay caused a decline in daphnid survival only at 100 mgL^{-1} with acute exposure. Conversely, none of three types of nanoclays affected the survivorship of *C. dilutus*. We also found little effect of natural nanoclay and Novaclay™ on the body growth of *D. magna*, and we were unable to assess the effects of Cloisite® 30B on the body growth of daphnids at higher concentrations, because all organisms died when exposed to Cloisite® 30B. There was also evidence that Cloisite® 30B inhibited the body growth of *C. dilutus* (NOAEL: 10 mgL^{-1} and LOAEL: 100 mgL^{-1}). This study highlights that aquatic organisms are differentially susceptible to natural and modified nanoclays and most sensitive to Cloisite® 30B.

Consequently, nanoclays should be characterized thoroughly prior to their introduction into the environment.

Keywords: natural nanoclay, modified nanoclays, aquatic environment, ecotoxicology.

Introduction

Modified nanoclays have become an attractive class of nanomaterials (NMs) due to their wide use in several consumer products (Calabi Floody et al. 2009) and their potential use in wastewater treatment (Patel et al. 2006; Shirzad-Siboni et al. 2015). Nanometric clay minerals can act as efficient sorbents to facilitate the removal of various pollutants during groundwater, surface water and wastewater remediation. Indeed, modified nanoclays can enhance our efficacy to remove several pollutants (e.g., polychlorinated biphenyls (PCBs), phenols, and heavy metals) from wastewater when compared to other wastewater treatment technologies (Ko et al. 2007).

Despite the potential benefits that modified nanoclays offer society, concerns are also being raised about potential adverse effects of modified nanoclays on aquatic organisms and ecosystems processes (Hood 2004). In addition to the intentional release of modified nanoclays into aquatic environments for the removal of pollutants (Yuan and Wu 2007), modified nanoclays are expected to unintentionally enter aquatic ecosystems from production facilities through the release of nanoclays via untreated or treated wastewater (Gottschalk and Nowack 2011), and leaching of nanoclays from materials placed in landfills or discarded in inappropriate ways (Nowack and Bucheli 2007).

Previous studies have already reported that intense agricultural practices and the construction of reservoirs can enhance the concentrations of natural nanoclays in water bodies

that adversely affects aquatic life forms (Cuker 1987; Kirk 1992; MacIsaac and Rocha 1995; Levine et al. 2005). For example, Kirk and Gilbert (1990) found that increased concentrations of natural nanoclays caused large reductions in the population growth rate of four cladoceran species (*Bosmina longirostris*, *Ceriodaphnia dubia*, *Daphnia ambigua*, and *Daphnia pulex*). Though no studies have assessed the impact of modified nanoclays on aquatic organisms, recent studies have shown that some types of modified NMs affect properties of aquatic organisms (Oberdörster 2004). Lovern et al. (2007) reported that low concentrations of titanium dioxide (TiO_2) and fullerenes (nano-C_{60}) can cause zooplankton to change their behavior, become more vulnerable to predation, or die directly from exposure to the nanomaterials. The small size of modified NMs and their capacity to penetrate into biological structures can obstruct breathing through gills (Britto et al. 2012; Costa et al. 2012).

To date, however, we do not know whether modified nanoclays adversely affect aquatic organisms or whether the effect of modified nanoclays on aquatic organisms is any different than the effect of natural nanoclays. Such comparisons are essential to evaluate whether modified nanoclays represent a novel class of pollutant before they enter into the environment in large quantities. Furthermore, it is unknown whether different kinds of aquatic organisms (e.g., algae, pelagic herbivores or benthic herbivores) differ in their response to natural and modified nanoclays.

The goal of this study was to assess the potential toxicity of a natural nanoclay and two modified nanoclays on three aquatic sentinel species - a green algae (*Chlamydomonas reinhardtii)*, a freshwater crustacean (*Daphnia magna)*, and a freshwater benthic invertebrate (*Chironomus dilutus).* In addition to differences in their taxonomy, these organisms were chosen because of differences in their trophic position and microhabitat usage (pelagic versus benthic)

within an aquatic environment. The green algae *C. reinhardtii* is a primary producer and can respond quickly to changes in their environment (Rubinelli et al. 2002; Asamiziu et al. 2000; Wang et al. 2008). The freshwater crustacean *D. magna* is a pelagic filter feeder that consumes algae from the water column (Seda and Petrusek 2011). *C. dilutus* is a benthic insect that may be particularly vulnerable to nanomaterials since most types of nanomaterials tend to deposit and be adsorbed on sediment particles that settle to the bottoms of aquatic environments (Oberholster et al. 2011).

Material and methods

Materials

Natural nanoclay (Na^+ montmorillonite) and conventional ammonium-modified nanoclay (Cloisite® 30B) were obtained from Southern Clay Products (TX, USA), while modified nanoclay without ammonium (Novaclay^{TM}) was obtained from Ioto International (Campo Magro, Paraná, Brazil). Cloisite® 30B is surface functionalized with MT2EtOH (methyl, tallow, bis-2-hydroxyethyl, quaternary ammonium chloride), while Novaclay^{TM} is synthesized with the addition of stearic acid of calcium (AMS-32^{TM}) within the interfacial lamella of the nanoclays but without ammonium compounds. Natural nanoclay does not have organic modifiers in its structure. These three types of nanoclay were selected because 1) natural nanoclay (Na^+ montmorillonite) is very abundant in nature with a great potential to be used in the development of modified nanoclays and 2) both modified nanoclays will be likely used in the production of polymer nanocomposites, rheological modifier in paints, drug delivery systems and

environmental remediation (Uddin 2008; Ellenbecker and Tsai 2011; Lee et al. 2005) which will likely result in their intentional or unintentional release into aquatic systems.

Stock solution preparation

We created solutions of each nanoclay type within each of three types of media: 1) TAP medium (Gorman and Levine 1965), 2) Elendt M4 medium (Elendt 1990), and 3) *Reconstituted Hard Water* (U.S. Environmental Protection Agency 2002). These media were chosen because they are widely used in toxicological studies for algae, daphnids, and chironomids, respectively (Oberholster et al. 2011; Giannelli et al. 2012; Hoecke et al. 2009). We created solutions with nanoclay concentrations of 0, 0.01, 0.1, 1, 10, and 100 mg of nanoclay L^{-1}. We selected these concentrations because they represent ecologically relevant concentrations that span the range of values known to commonly occur in nature though concentrations could exceed 500 mg/L in some circumstances following heavy rain events (Kirk 1992; Robinson et al. 2010). To obtain homogeneous dispersion of nanoclays within solution, all stock solutions were initially stirred with a magnetic stirring device for 2 hours.

Chlamydomonas reinhardtii

We followed the guidelines of the Organization for Economic Co-operation and Development (OECD) 201 (2006) to assess the effects of nanoclays on the green algae *Chlamydomonas reinhardtii*. The wild-type (CC-125 137c mt+) of *C. reinhardtii* was obtained from the Chlamydomonas Genetics Center (Duke University, Durham, NC), and it was grown under controlled conditions (24º C, 16:8-h light: dark) in *TAP* medium (Gorman and Levine 1965) to a density of $5x10^5$ cells/ml. Cell density was determined by counting cells with a hemocytometer placed under a light microscope. We transferred 1 ml of media containing $5x10^5$

cells of *C. reinhardtii* to each of sixteen 250 ml beakers that differed in both the type and concentration of nanoclay particles present (0, 0.01, 0.1, 1, 10, and 100 mgL^{-1}) (16 treatments=3 types of nanoclay x 5 concentrations + 1 treatment with no nanoclays). We then estimated the abundance of algae present within each beaker after 0 and 72 hours of exposure by using a UV/Vis spectrophotometer to estimate the amount of chlorophyll *a* present. Estimates of chlorophyll *a* abundance is a good indicator of algal population size (Malek et al. 2011). We quantified algal population growth in each beaker as the log concentration of algae present after 72h of exposure divided by the log concentration of algae present at 0 hours of exposure. Each treatment was replicated once within each of 4 blocks to produce 4 estimates of algal population growth for each of the 16 treatments. A block consisted of one replicate of each treatment that were set up at the same time. Only one block was present at any given time but each block was initiated within 5 days of the completion of data collection in another block. All setup and sampling procedures were performed on a block-by-block basis to minimize the possible effects of temporal variability in methods.

Daphnia magna

Daphnia magna was obtained from Carolina Biological Supply Company (USA), and maintained continuously for 8 months under laboratory conditions (20 ± 2º C, 16:8-h light: dark). The culture medium (*Elendt M4*) was renewed three times a week, and daphnids were fed a constant amount of fresh algal culture ($5x10^5$ cells of *C. reinhardtii*/individual) daily.

a) Acute test (48h)

The effect of acute exposure to nanoclays on *Daphnia magna* was performed as 48h static renewal bioassays following OECD guidelines 202 (2004). Specifically, five daphnids

(<24h old) were raised in 100 ml beakers and directly exposed to one of the 16 treatments outlined above. We monitored the beakers daily to document survival of individuals, and dead individuals were removed. Death was determined by lack of movement or response to gentle prodding. Each daphnid was photographed at the beginning and end of the experiment to facilitate the measurement of body length (distance from the base of the tail spine to the base of the head). Length measurements were made with the aid of the Olympus SZX7 Stereo Light Microscope and cellSens software (Olympus Company). Average body growth for individuals within a beaker was estimated by subtracting the average length of all individuals in the beaker at the start of the experiment from the average length of all individuals in the beaker at the end of the experiment. *D. magna* were not fed during the 48h exposure to nanoclays. This experiment was repeated in four temporal blocks, as mentioned above, to produce 4 estimates of survivorship and body growth of *Daphnia magna* for each of the 16 treatments.

b) Chronic test (10 days)

The effect of chronic exposure to nanoclays on *D. magna* was conducted based on OECD guidelines 211 (1998). We used the same incubation conditions, concentrations, and nanoclay endpoints (e.g., survivorship and body growth) as in the acute tests, which allowed us to examine similar responses over a prolonged time period of 10 days. Daphnids were also photographed at the beginning (0h) and at the end of the experiment (10 d) to evaluate the effects of nanoclays on body growth. The culture medium was renewed every 48h, and daphnids were fed daily, using *C. reinhardtii* at a concentration of $5x10^5$ cells/individual. This experiment was also replicated once within each of four temporal blocks as in the acute tests.

Chironomus dilutus

The culturing of *C. dilutus* and their response to nanoclays followed U.S. Environmental Protection Agency (EPA) guidelines (2000). Chironomidae were obtained from the USEPA's Mid-Continent Ecology Laboratory (Duluth, Minnesota, USA), and 40 individuals were cultured in a 20L aquarium (23 ± 1º C, 16:8-h light: dark), and fed with fresh algae culture ($5x10^5$ cells of *C. reinhardtii*) and 4 mg of TetraMin® tropical fish food per 10 organisms daily. The aquarium was covered with mesh fabric to prevent the escape of emergent adults, which were gently siphoned with the aid of a dry aspirator flask.

We assessed the response of *C. dilutus* (8 d post hatch third instar larvae) to 10 days of exposure to nanoclays by placing five midges into each of sixteen 100 ml beakers that each varied in the type and concentration of nanoclays present (the same 16 treatments as identified above). Each beaker contained 25 ml of white quartz sand and 75 ml of solution. As with standard EPA protocol, we removed *C. dilutus* from their cases immediately prior to placing them into beakers to 1) allow us to measure the body length of each midge and 2) ensure direct exposure of midges to all test concentrations. We also measured the length of each individual after 10 days of exposure and calculated average body growth within a beaker by subtracting the average length of individuals at the beginning of the experiment from the average length of individuals at the end of the experiment. Survivorship was measured daily. Solutions within each beaker were replaced every 48 hours. This experiment was repeated in four temporal blocks as described above to produce 4 estimates of survivorship and body growth of *Chironomus dilutus* for each of the 16 treatments.

Data analysis

To determine the effects of three types of nanoclays on aquatic species, we conducted statistical tests with the aid of SAS Enterprise Guide 9.3 (SAS Institute INC, Cary, NC, USA). Our analyses all began with either general linear mixed models (one each for algal population growth rate, average body growth of *Daphnia magna* and average body growth of *Chironomus dilutus*) or generalized linear mixed models (one each for number of *Daphnia magna* that survived to the end of the experiment and the number of *Chironomus dilutus* that survived to the end of the experiment) using PROC Mixed and PROC GLIMMIX, respectively. All models included "treatment" as a fixed effect and "block" as a random effect. The generalized linear mixed models used a logit link function to assess survival as the log odds of surviving relative to dying and specified a binomial error distribution with variance components for all analyses. We then identified the NOAEL (No Observed Adverse Effect Level) and LOAEL (Lowest Observed Adverse Effect Level) for each type of nanoclay on each response variable by using Dunnet's procedure to compare responses in each treatment containing nanoclays to the responses observed in treatments lacking nanoclays. We also used orthogonal polynomial contrasts with our models to assess whether responses changed predictably with the dosage of each type of nanoclay present. We assessed up to fifth order polynomials but only present the statistical results of third order or higher contrasts if there is sufficient evidence to suggest they may be important. In most cases, the strength of statistical evidence to support a third order or higher contrast was very weak ($F_{1, 47} \leq 0.99$, $p \geq 0.325$). In addition, we compared the differences among nanoclay types within each concentration by performing pairwise comparisons via Fisher's LSD. Given the multiple comparisons made among treatments for each response variable, we adjusted

p values for each comparison to control for the False Discovery Rate (FDR) (Verhoeven et al. 2005).

Results and discussion

Chlamydomonas reinhardtii

We found very little evidence for a change in the population growth rate of *C. reinhardti* as the concentration of either natural nanoclay or Novaclay™ increased (linear trend analysis: $F_{1, 47} \leq 0.22$, $p \geq 0.642$; quadratic trend analysis: $F_{1, 47} \leq 1.22$, $p \geq 0.275$) but strong evidence that population growth rate of *C. reinhardti* was suppressed more by higher concentrations of Cloisite® 30B than lower concentrations of Cloisite® 30B (linear trend analysis: $F_{1, 47}=326.04$, $p<0.0001$; quadratic trend analysis: $F_{1, 47}=5.62$, $p=0.022$) (Figure 4). Consequently, we cannot report a NOAEL and LOAEL for either natural nanoclay or Novaclay™ on *C. reinhardti* population growth. The population growth rate of *C. reinhardti* did not respond to Cloisite® 30B at levels below 1 mgL^{-1} (the NOAEL for Cloisite® 30B) but was reduced by 17% when concentrations increased to 10 mgL^{-1} (the LOAEL for Cloisite® 30B) and by 71% when concentrations increased to 100 mgL^{-1} (Figure 4).

Previous studies have reported that the physicochemical properties (e.g., particle size, surface charge, shape, and state of dispersion) of nanomaterials may provide important information to better understand their toxic effects on biological systems (Powers et al. 2006).

Our preliminary study (Tullio et al., Chapter 1) has showed that the three types of nanoclays differ in some of their physicochemical properties when they are in solution. For example, Cloisite® 30B is the most likely to agglomerate/aggregate due to its lowest stability in

solution (zeta potential: -11 mV) than natural nanoclay (zeta potential: -18.7 mV) and Novaclay^{TM} (zeta potential: -21.4 mV), which may favor the faster agglomeration/aggregation of Cloisite® 30B particles to the cell wall of *C. reinhardtii.* The attachment of Cloisite® 30B particles to the cell wall of *C. reinhardtii* can result in important adverse effects on algal population growth including: 1) inhibition of the photosynthetic activity by shading, 2) agglomeration of unicellular algae that can reduce the mass transfer of gas and nutrients in the media, 3) precipitation of algal cells in nanoclay-algal flocs, and 4) competition between algae and nanoclay particles for phosphorus uptake (Avnimelech et al. 1982; Cuker et al. 1990; Cuker 1993; Choi et al. 2014).

Aruoja et al. (2009) have reported that TiO_2 nanomaterials aggregates inhibited the population growth rate of *Pseudokirchneriella subcapitata* as a result of aggregation and shading. Other authors have also reported that the agglomeration of nanomaterials to algal cells appears to be an important mechanism of toxicity for algae species, since cell walls of algae species only allow the passage of small molecules (5 to 20 nm) limiting the entrance of large aggregated nanomaterials into cells (Fleischer et al. 1999; Madigan et al. 2003; Navarro et al. 2008). However, the degree of contact between algae cells and nanoclays can be associated with both algae and nanoclay concentration. In other words, higher nanoclay concentrations and/or the larger algal cell size (i.e., length, width and/or surface area) enhance the encounter rate and consequently the nano-bio interactions (Guenther and Bozelli 2004). Thus, we suggest that high concentrations of Cloisite® 30B particles (i.e. 10 and 100 mgL^{-1}) favored the formation of Cloisite® 30B-algal cells agglomerates that reduced the light available to the entrapped algal cells, inhibiting their algal population growth.

Particle shape is also an important metric that can be associated with the potential toxicity and reactivity of Cloisite® 30B to aquatic biota. Cloisite® 30B particles have oval-shaped particles in solution, which may facilitate the cell uptake rate of nanoclays when compared to other non-spherical particles, such as platelet-type shape found in natural nanoclay and Novaclay^{TM}. Li et al. (2015) explain that non-spherical particles tend to be involved in a stronger membrane deformation and complicated rotations in order to be internalized by cells, resulting in slower cellular uptake rate. While, spherical particles are only involved in a minimum membrane bending energy barrier, favoring their fast entry into the cells.

In addition, Cloisite® 30B is synthetized with a quaternary ammonium compound (QAC), which may have contributed to the higher toxic effects of Cloisite® 30B on algal population growth when compared to the other two nanoclay types. QACs are cationic surfactants widely used as an organic modifier in modified nanoclays for wastewater remediation, because they act as efficient sorbents to facilitate the removal of several pollutants (e.g., oil, grease, heavy metals, polychlorinated biphenyl (PCBs), and humic and fulvic acids) from water supplies (Xue et al. 2013). The structure of QACs usually contains at least one hydrophobic hydrocarbon chain linked to a positively charged nitrogen atom, and other alkyl groups which are mostly short-chain substituents such as methyl and benzyl groups (Zhang et al. 2015). Although, the QACs structure facilitates the adsorption affinity of modified nanoclays onto a variety of materials, it may induce delayed maturation, decreased growth rates, and reduced survivorship in aquatic species even under low concentrations (Lewis and Morris 1986; Adams and Bealing 1994). Jing et al. (2012) reported that different types of QACs inhibited the algae growth rate of *Chorella pyrenoidosa* and *Scenedesmus quadricauda*, because QACs and the cell walls of algae cells present opposed surface charges, favoring the attractive interaction

between the positively charged "head" of nitrogen atoms and negatively charged algal cell walls. Previous studies have documented that such nano-bio interactions can cause oxidative stress in biological systems (Nel et al. 2009; Bhattacharya et al. 2010; Oukarroum et al. 2012). Oxidative stress occurs when reactive oxygen species (ROS) disturb the balance between oxidative pressure and antioxidant defense, resulting in cell injury by attacking DNA, proteins, and membranes (Moore 2006). ROS can be formed via radicals, chemicals on the particle surface, or as a result of the interaction between nanomaterials and cellular components (Nel et al. 2006; Karlsson et al. 2009). Maisanaba et al. (2014) showed that Cloisite® 30B induced the generation of intracellular reactive oxygen species on the human intestinal cell line Caco-2, causing cell damage. On the other hand, Sharma et al. (2010) found that Caco-2 exposed to filtered suspensions of Cloisite® 30B (i.e. samples containing only the ammonium compound added to the clay as an organic modifier) induced the DNA-strand breaks in cells but without the production of ROS. Though those studies have demonstrated some evidence about the potential of modified nanoclays to produce ROS on human cell lines, there is still no information about the mechanisms through which all three nanoclays present in this study can induce the formation of ROS on aquatic life. Thus, future studies are required to elucidate the ability and mechanisms of modified nanoclays to cause oxidative stress in aquatic species.

In summary, our results indicate that Cloisite® 30B shows the highest toxic effects on algal population growth, and increasing the concentration of Cloisite® 30B in the water poses a greater threat to *C. reinhardti* than does increasing the concentration of either natural nanoclay or Novaclay^{TM} for the range of concentrations considered in this study.

Daphnia magna

a) Acute test (48h) and Chronic test (10d)

Our results indicated a negative relationship between *Daphnia magna* survivorship and Cloisite® 30B during short and long term exposures (linear trend analysis: $F_{1, 47} \geq 25.13$, $p<0.0001$; quadratic trend analysis: $F_{1, 47} \geq 31.74$, $p<0.0001$; tertiary trend analysis: $F_{1, 47} \geq 7.01$, $p \leq 0.011$) (Figure 5 (i-ii)). Cloisite® 30B severely reduced the survivorship of *Daphnia magna* at very low concentration (NOAEL: 0.1 mgL^{-1} and LOAEL: 1 mgL^{-1}) for both acute and chronic tests (Figure 5 (i-ii)). All *D. magna* died when exposed to water with 10 mgL^{-1} of Cloisite® 30B. Zhu et al. (2009) also found that nanoscale ZnO (*n*ZnO) caused 100% mortality of *Daphnia magna* at 10 mgL^{-1}, which shows that daphnids are very sensitive to different types of nanomaterials at similar concentrations.

In addition, the microcrustacean *D. magna* showed an increased sensitivity to Cloisite® 30 B (LOAEL: 1 mgL^{-1}) than *C. reinhardtii* (LOAEL: 10 mgL^{-1}) in our study. We suggest that *D. magna* shows lower LOAEL when exposed to Cloisite® 30B due to the following factors: i) increased turbidity caused by the addition of nanoclays in media that reduces the light attenuation, and consequently decreases the amount of photosynthesizing algae (food for zooplankton), ii) rapid agglomeration/aggregation of Cloisite® 30B particles to the cell walls of *C. reinhardtii*, as discussed above, which reduces the quality and availability of food for daphnids, iii) difficulty of depuration of nanoclays from the gut lines of *D. magna*, and iv) different exposure routes of Cloisite® 30B to *D. magna* via water exposure and food ingestion (i.e. daphnids can uptake nanoclays from algae also exposed to nanoclays in media) (Kirk and Gilbert 1990; Zhao and Wang 2011; Ji et al. 2011).

García et al. (2001) also reported that *Daphnia magna* is very sensitive to QACs. Acute toxicity tests on swimming capacity of *Daphnia magna* indicated EC_{50} (i.e. concentration necessary to give half maximal response) values ranging from 0.1 to 1 mgL^{-1} for different types of QACs (alkyl trimethyl ammonium halides (ATMAC C12-16) and alkyl dimethyl ammonium halides (BAC C12-16). Although the mode of action of QACs has not been systematically documented for most of the aquatic organisms, including zooplankton, studies have suggested that the toxicity of QACs can be attributed to their binding to the organisms' cell membrane altering the phospholipid bilayer, thereby causing cell membrane disruption and protein denaturation, leading to cell death (Zhang et al. 2015; Pérez et al. 2009; Canadian Council of Ministers of the Environment 1999).

Survival of *D. magna* only decreased appreciably with an increase in the concentration of Novaclay^{TM} during chronic exposure (linear trend analysis: $F_{1, 47}$=10.42, p=0.002; quadratic trend analysis: $F_{1, 47}$=0.01, p=0.936; tertiary trend analysis: $F_{1, 47}$=12.32, p=0.001) but not acute exposure (linear trend analysis: $F_{1, 47}$=0.99, p=0.326; quadratic trend analysis: $F_{1, 47}$=0.12, p=0.735; tertiary trend analysis: $F_{1, 47}$=0.69, p=0.411) (Figure 5 (i-ii)). The NOAEL and LOAEL for the effects of chronic exposure to Novaclay^{TM} on the survivorship of *D. magna* were 0.1 mgL^{-1} and 1 mgL^{-1} (Figure 5ii). This result shows the importance of conducting long-term studies to better understand the effects of modified nanoclays on aquatic ecosystems. Indeed, the effects of Novaclay^{TM} seems to vary among aquatic species (i.e. no effects observed on *C. reinhardtii*), and exposure duration. The higher toxicity of Novaclay^{TM} on the survivorship of *D. magna* when compared to *C. reinhardtii* is likely due to its higher stability in the medium (zeta potential= -21.4 mV) (Tullio et al., Chapter 1), which favor Novaclay^{TM} particles to be well

dispersed and stabilized in aqueous solution, making them more bioavailable and potentially dangerous to highly mobile pelagic species, especially after continuous exposure.

The chronic effects of different types of nanomaterials on *Daphnia magna* can be a result of feeding depression due to the accumulation of particles in the digestive tract of the daphnids even though filter feeder organisms usually feed on larger particle size (0.4 to 40 µm) than nanometric scale particles (Geller and Müller 1981; Zhao and Wang 2011; Völker et al. 2013). For example, Rosenkranz et al. (2007) have reported that 20 nm polystyrene nanoparticles can move from the digestive tract into other parts of daphnids which indicates that small sized particles may also be consumed by filter feeder organisms and potentially lead to accumulation of nanoparticles and/or toxic effects. In fact, large-bodied cladocerans such as *Daphnia* are generalist filter feeding organisms, so they do not discriminate between food particles and suspended particles, resulting in the ingestion of significant amounts of nanomaterials (single and/or agglomerated particles) present in water column that can clog the intestinal tract and reduce the ability of uptake and process the food (Kirk 1992; Zhu et al. 2009; Robinson et al. 2010).

In addition, Novaclay^{TM} shows a small mean particle size ($\overline{x}$ = 20 nm) when in solution at 1 mgL^{-1} (Tullio et al., Chapter 1) that may have facilitated the ingestion of Novaclay^{TM} particles by *D. magna*. Adams et al. (2006) state that the ingestion and/or accumulation of nanomaterials is likely size-dependent, especially for filter-feeding organisms. Studies have already indicated that smaller particles are more easily ingested by *D. magna*, because large particles are too difficult to process and they tend to aggregate into flocculent masses, making them mostly unavailable for daphnids uptake (Ebert 2005; Roduner 2006).

Conversely, the survivorship of *D. magna* only changed with an increase in the concentration of natural nanoclay when under acute exposure at 100 mgL^{-1} (the LOAEL for natural nanoclay) (linear trend analysis: $F_{1, 47}$=7.78, p=0.008; quadratic trend analysis: $F_{1, 47}$=1.65, p=0.205; tertiary trend analysis: $F_{1, 47}$=5.35, p=0.025) but not chronic exposure (linear trend analysis: $F_{1, 47}$=3.99, p=0.052; quadratic trend analysis: $F_{1, 47}$=1.07, p=0.306; tertiary trend analysis: $F_{1, 47}$=6.34, p=0.015) (Figure 5 (i-ii)). This is believed to have occurred due to the presence of high concentrations of natural nanoclay (100 mgL^{-1}) in solution that clog the gut of *D. magna*, leading to mortality of daphnids. Several studies have already reported the toxic effects of natural nanoclay on the survivorship of *Daphnia,* as a result of the intense agricultural practices and the construction of reservoirs that enhance the concentrations of natural nanoclays in the environment (Kirk 1992; Levine et al. 2005). In reality, we observed that both acute (t_{24}=0.003, p=0.029) and chronic (t_{24}=0.016, p=0.130) exposure to natural nanoclay caused *D. magna* survival to decrease as the concentration of natural nanoclay increased but the survival of *D. magna* in the presence of natural nanoclay did not drop below that observed in the absence of nanoclays when the concentration of natural nanoclay present during chronic exposure was 100 mgL^{-1}. Chronic toxicity tests of natural nanoclay did not show a dose dependent decrease in survival of *D. magna*, because daphnids can likely purge natural nanoclay from their gut, increase their feeding rates, and recover during each water exchange (Robinson et al. 2010). As is standard protocols (OECD 202 (2004); OECD 211 (1998)), *D. magna* was only fed with *C. reinhardtii* during chronic tests, which may also have contributed to reduce the toxicity of natural nanoclay. Indeed, natural nanoclay shows larger mean particle size and moderate stability in solution (Tullio et al., Chapter 1) that make it more likely to agglomerate and/or attach to algae cells, fall out the solution and deposit on sediments, decreasing the concentration of natural

nanoclay in water column and consequently reducing its toxicity on *Daphnia magna* (Ribeiro et al. 2014).

Furthermore, increasing the concentration of natural nanoclay had negative effects on the growth of *D. magna* under both acute and chronic (linear trend analysis: $F_{1, 47} \leq 12.04$, $p \geq 0.001$) exposure but acute exposure caused *D. magna* to begin growing more slowly at lower concentrations than did chronic exposure (Figure 6 (i-ii)). Despite these trends, the growth of *D. magna* under the highest concentrations of natural nanoclay in our study (with either acute or chronic exposure) did not statistically differ from that observed when no nanoclays were present ($t_{18} \leq 2.23$, $p \geq 0.207$) (Figure 6 (i-ii)). Thus, we cannot report a LOAEL for the effect of natural nanoclay on *D. magna* growth. Robinson et al. (2010) also observed that there was no significant decrease in body growth of *Daphnia* when exposed to suspended clays for 24h, which suggests that daphnids present a shift of more energy into growth after exposure.

On the other hand, there was a positive relationship between daphnia body growth and Novaclay™ at 0.01 mgL^{-1} for acute ($t_{18}=3.6$, $p=0.017$) test but not chronic exposure ($t_{18}=0.1$, $p=1$) (Figure 6 (i-ii)). Rellstab and Spaak (2007) state that clays can positively affect daphnid growth, since organic matter and bacterial biofilm can adsorb to the clay surface, providing a complementary nutrition to filter feeders organisms. Novaclay™ also presents higher amount of calcium in its composition when compared to natural nanoclay and Cloisite® 30B (Tullio et al., Chapter 1), which may have enhanced the body growth of *D. magna* at very low concentrations of Novaclay™ with acute exposure. In fact, daphnids are heavily calcified organisms that moult frequently, so that their calcium demand is very high as a result of their need to maintain their protective shells and carapaces, and grow (Orr et al. 2005; Jeziorski and Yan 2006). Thus, we suggest that the presence of calcium at very low levels in media may have favored daphnids to

grow and store calcium while moulting. As with natural nanoclay, we do not have sufficient statistical evidence to report a LOAEL for Novaclay^{TM} on *D. magna* growth (Figure 6 (i-ii)).

Changes in the concentration of Cloisite® 30B was associated with changes in *D. magna* growth under both acute (linear trend analysis: $F_{1, 47}$=7.05, p=0.011; quadratic trend analysis: $F_{1, 47}$=7.49, p=0.009) and chronic (linear trend analysis: $F_{1, 47}$=17.22, p=0.0001; quadratic trend analysis: $F_{1, 47}$=22.26, p<.0001; tertiary trend analysis: $F_{1, 47}$=29.34, p<.0001) exposure. Under both types of exposure, a slight increase in concentration (above 0 mgL^{-1}) of Cloisite® 30B increased *D. magna* growth, as observed in Novaclay^{TM}, but further increases in concentration caused a slight decline in growth that leveled off as the concentration increased until no *D. magna* could survive higher concentrations of Cloisite® 30B (Figure 6 (i-ii)).

This study indicates that all three types of nanoclays show adverse effects on *Daphnia magna*; however, there are differences in their toxicity related to nanoclay composition, concentration in media, particle size, surface charge, and exposure time. Modified nanoclays seem to have higher toxic effects on survivorship of *Daphnia magna* than natural nanoclay at very low concentrations mainly due to the Cloisite® 30B composition (i.e. presence of QACs) and high stability of Novaclay^{TM} particles in aqueous medium that makes them more available for filter feeders organisms (Tullio et al., Chapter 1).

Chironomus dilutus

None of three types of nanoclays affected the survivorship of *C. dilutus* after 10 days of exposure (linear trend analysis: $F_{1, 47}\leq$2.14, p≥0.150; quadratic trend analysis: $F_{1, 47}\leq$0.53, p≥0.471) (Figure 7). Previous studies have shown that chironomids are more tolerant to the exposure of the different types of nanomaterials (e.g., nano titanium dioxide ($nTiO_2$), nano zinc oxide (nZnO), and nanosilver (nAg)) than algae and daphnids (Tomilina et al. 2014; Yoo-Iam et

al. 2014; Álvarez-Manzaneda et al. 2017). *C. dilutus* are sediment-dwelling organisms that live under conditions of accumulation of organic and inorganic compounds in the silt covering the bottom of waterbodies, so that they often consume different particle sizes (single and/ or agglomerated particles) or sediment particles with high organic carbon content as well as elevated chemical concentrations that may determine the higher tolerance of chironomidae larvae to the toxic effects of pollutants, including nanoclays (Oberholster et al. 2011; Harkey et al. 1994).

On the other hand, our results found strong evidence that Cloisite® 30B inhibits the body growth of chironomids (t_{24}=4.26, p=0.003) under very high concentration (NOAEL: 10 mgL^{-1} and LOAEL: 100 mgL^{-1}) (linear trend analysis: $F_{1, 47}$=38.59, p<0.0001) (Figure 8). Similar results were observed by Oberholster et al. (2011) and Karouna-Renier and Zehr (1999) when chironomids were exposed to seven different types of nanomaterials and macroscale chemical pollutants, respectively. The reduction in chironomid body growth as the concentration of Cloisite® 30B increased could have direct influence in their ability to reproduce, since the organisms tend to mature at smaller sizes in the presence of Cloisite® 30B particles (Oberholster et al. 2011).

We found no effects of natural nanoclay (linear trend analysis: $F_{1, 47}$=1.32, p=0.256; quadratic trend analysis: $F_{1, 47}$=1.21, p=0.277; tertiary trend analysis: $F_{1, 47}$=0.09, p=0.761; quartic trend analysis: $F_{1, 47}$=0.55, p=0.461; quintic trend analysis: $F_{1, 47}$=3.96, p=0.053) and Novaclay™ (linear trend analysis: $F_{1, 47}$=1.73, p=0.195; quadratic trend analysis: $F_{1, 47}$=1.33, p=0.255) on body growth of *C. dilutus* (Figure 8).

Conclusions

Although, our results show that different kinds of organisms (e.g., algae, pelagic herbivores or benthic herbivores) differed in their response to natural and modified nanoclays, we can indicate that, in general, the toxicity of the three types of nanoclays to aquatic species ranks as follows: Cloisite® 30B > Novaclay^{TM} > natural nanoclay. Cloisite® 30B adversely affected all three aquatic species, while Novaclay^{TM} and natural nanoclay only affected *Daphnia magna*.

This study took an important first step in assessing and comparing the potential toxic effects of a natural nanoclay and two modified nanoclays on aquatic environments. In view of our findings, there is still a need to further investigate the dynamic series of interactions between nanoclay surfaces and cellular components, since those interactions can determine whether the nanoclays are bioavailable and can participate in biocompatible or bioadverse interactions.

Currently, there is also no information about the trophic transfer of nanoclays in aquatic food webs, which could be an important mechanism of transport and fate of nanoclays. The aquatic species selected in this study are well suited to assess the trophic transfer of different types of nanoclays, since they either belong to different trophic levels or differ in their microhabitat usage (i.e. water column versus sediment).

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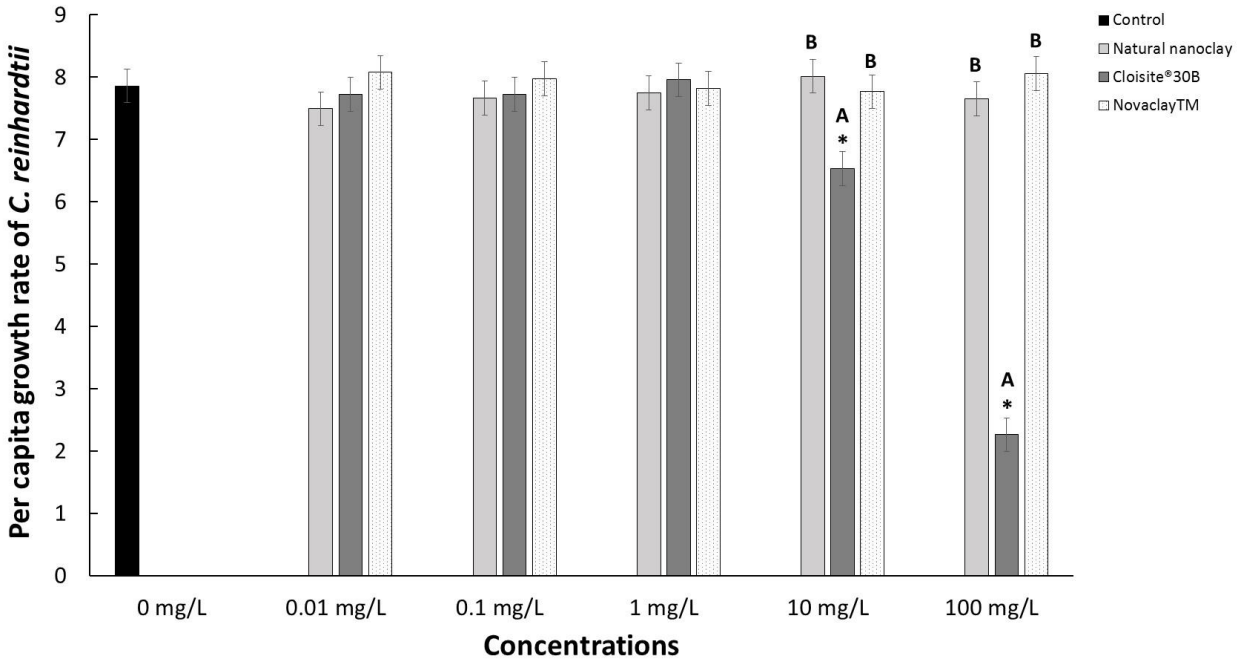

Figure 4. Least square mean estimates ± standard error (SE) of population growth rate of *Chlamydomonas reinhardtii* when exposed to three types of nanoclays using a general linear mixed model (PROC Mixed). (*****) Treated groups statistically different than control by using Dunnet's procedure ($p<0.05$). Different letters (**A, B**) above bars within each concentration identify which nanoclay types differ from each other in their effects on algal growth rate at that particular concentration by performing pairwise comparisons. Adjusted p-values for each group comparison were generated using the False Discovery Rate (FDR).

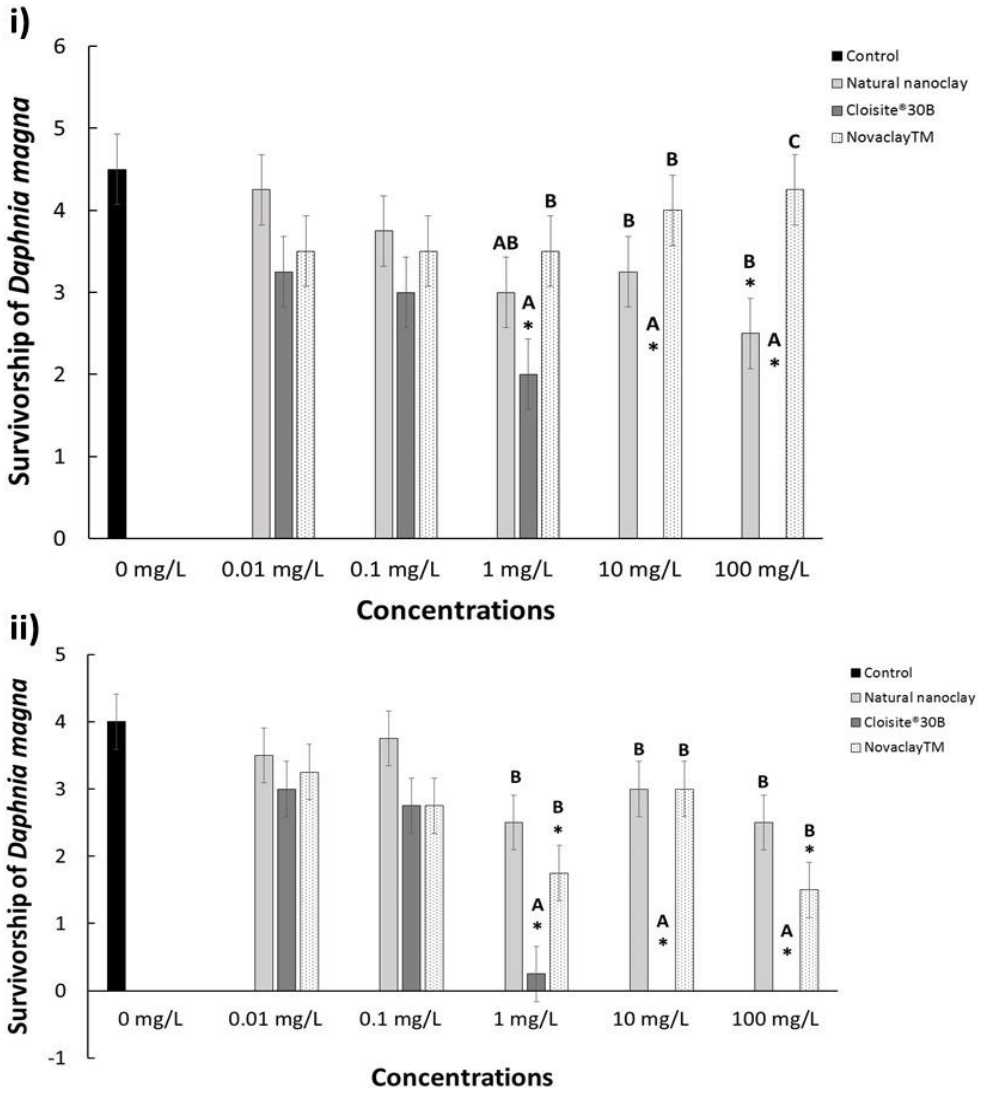

Figure 5. Least square mean estimates ± standard error (SE) of survivorship of *Daphnia magna* when exposed to three types of nanoclays for both acute **(i)** and chronic **(ii)** tests using a generalized linear mixed model (PROC GLIMMIX). (*****) Treated groups statistically different than control by using Dunnet's procedure ($p<0.05$). Different letters (**A, B**) above bars within each concentration identify which nanoclay types differ from each other in their effects at that particular concentration, while (**AB**) above bars within each concentration show which nanoclay types do not differ from each other in their effects on the survivorship of *Daphnia magna* at that particular concentration. Adjusted p-values for each group comparison were generated using the False Discovery Rate (FDR).

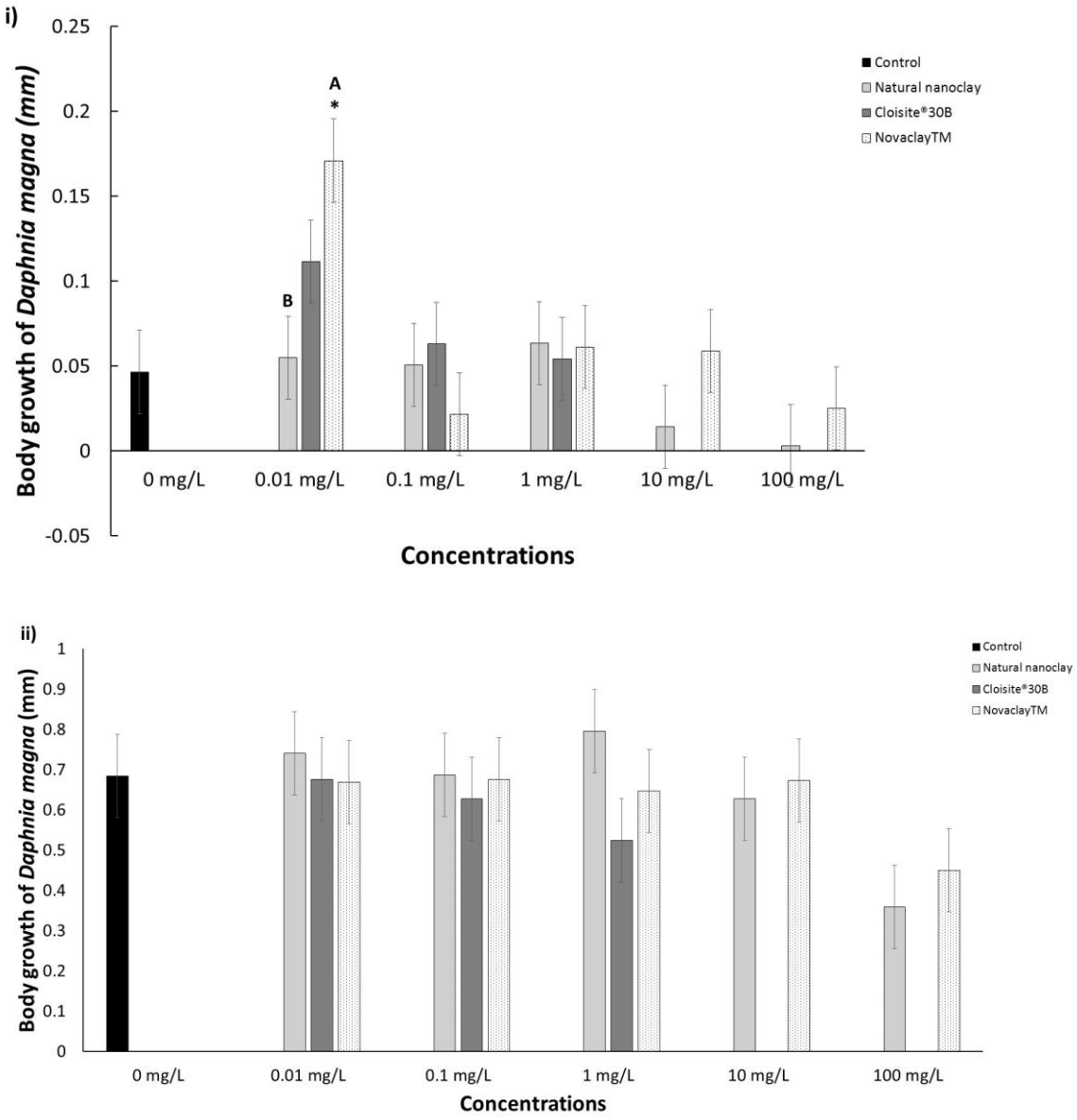

Figure 6. Least square mean estimates ± standard error (SE) of body growth of *Daphnia magna* when exposed to three types of nanoclays for both acute **(i)** and chronic **(ii)** tests using a general linear mixed model (PROC Mixed). (*****) Treated groups statistically different than control by using Dunnet's procedure ($p<0.05$). Different letters (**A, B**) above bars within each concentration identify which nanoclay types differ from each other in their effects on daphnia body growth at that particular concentration by performing pairwise comparisons. Adjusted p-values for each group comparison were generated using the False Discovery Rate (FDR).

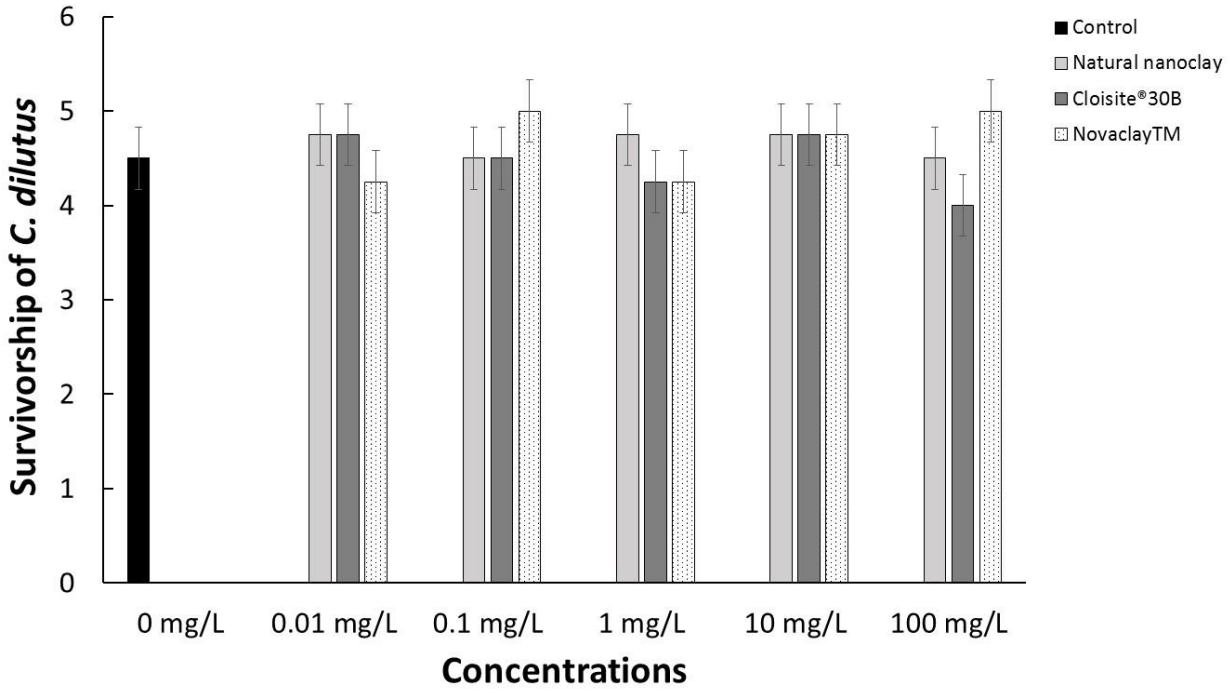

Figure 7. Least square mean estimates ± standard error (SE) of survivorship of *Chironomus dilutus* when exposed to three types of nanoclays using a generalized linear mixed model (PROC GLIMMIX).

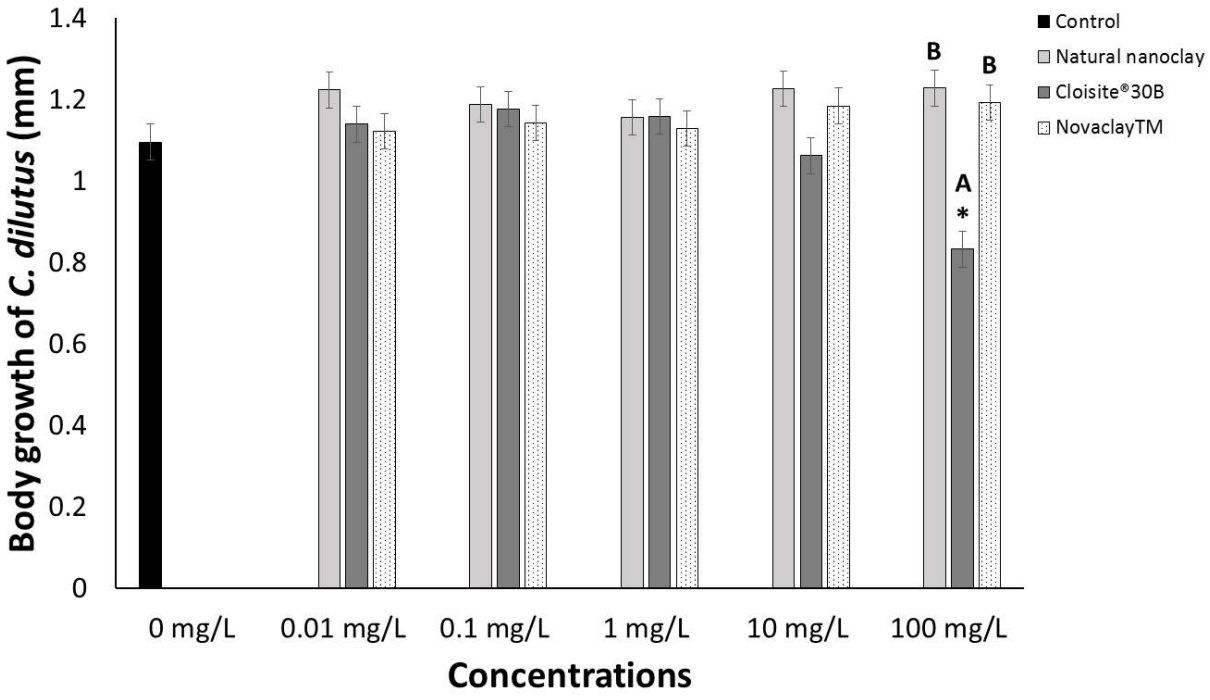

Figure 8. Least square mean estimates ± standard error (SE) of body growth of *Chironomus dilutus* when exposed to three types of nanoclays using a general linear mixed model (PROC Mixed). (*****) Treated groups statistically different than control by using Dunnet's procedure ($p<0.05$). Different letters (**A, B**) above bars within each concentration identify which nanoclay types differ from each other in their effects on *C. dilutus* body growth at that particular concentration by performing pairwise comparisons. Adjusted p-values for each group comparison were generated using the False Discovery Rate (FDR).

CHAPTER 3: Effects of natural and modified nanoclays on mosquito fish (*Gambusia holbrooki*)

Abstract Modified nanoclays have been developed for a variety of industrial and environmental applications, so they are expected to be detected in aquatic environments. Nevertheless, there are uncertainties whether the release of modified nanoclays adversely affect aquatic organisms relative to natural nanoclays already present in the environment. This study investigated the potential toxicity of a natural nanoclay (Na^+ montmorillonite) and two modified nanoclays (Cloisite® 30B and Novaclay^{TM}) on survivorship, body condition, and the liver tissues of *Gambusia holbrooki* after 14 days of exposure. Our results showed that none of the three types of nanoclays affected the survivorship and body condition of mosquito fish, while they did induce histopathological changes on liver tissues of *G. holbrooki* at very low concentration (LOAEL: 0.01 mgL^{-1}). The effects of nanoclays on the circulatory, regressive and degenerative changes of mosquito fish varied among nanoclay types. Although, Novaclay^{TM} caused circulatory changes on hepatic tissues of *G. holbrooki* (LOAEL: 0.01 mgL^{-1}), both natural nanoclays and Cloisite® 30B showed little effect on that particular tissue damage. In contrast, the three types of nanoclays induced regressive and degenerative changes on liver tissues of mosquito fish under low concentrations (LOAEL: 0.01 mgL^{-1}). This study clearly reveals that natural and modified nanoclays have important implications to aquatic life. Consequently, the widespread use of nanoclays in several applications arises great concern about their safety for humans and aquatic organisms.

Keywords: natural nanoclays, modified nanoclays, aquatic environment, histopathological analysis, ecotoxicology.

Introduction

Nanotechnology has been considered one of the most promising areas for the development of science and technology (Batley and McLaughlin 2010). For example, modified nanoclays are being developed for their usage in drug development, production of stronger and lighter materials, and water remediation (Patel et al. 2006; Newberry and Uldrich 2010). Modified nanoclays are expected to enter aquatic environments by leaching out of products with nanoclays, deliberate introduction of nanoclays to natural water bodies to facilitate remediation of contaminated water, or through the release of nanoclays via untreated or treated wastewater (Gottschalk and Nowack 2011; Handy et al. 2008). Consequently, it is inevitable that modified nanoclays will ultimately be found in the environment.

Though there are many possible benefits that derive from the production of modified nanoclays, it is important to investigate whether these new materials will have negative consequences for natural ecosystems before significant exposure occurs. Information about the toxicological and pathological risks of modified nanoclays to aquatic biota are essential to the development of policy that will be necessary to prevent or mitigate the potential impacts of modified nanoclays. One of the critical issues that must be addressed is determining the extent to which the release of modified nanoclays in the environment adversely affect aquatic ecosystems relative to natural nanoclays that are already present in the environment.

Previous studies have already identified that an increase in the concentration of natural nanoclays can adversely affect aquatic organisms. For instance, Greig et al. (2005) found that high quantities of fine clay particles in water can coat the surface of salmon eggs, which reduces their rate of oxygen consumption. Vinyard and O' Brien (1976) found that high concentrations of suspended clays in the water enhances turbidity which decreases the ability of visually searching zooplanktivorous fish to locate and kill their prey. Most recently, Tullio et al. (Chapter 2) reported that an increase in the concentration of natural nanoclays can adversely affect the survival probability of *Daphnia magna* though the nanoclays had no effect on the population growth rates of *Chlamydomonas reinhardtii* and survival probability of *Chironomus dilutus*.

With one exception (Tullio et al., Chapter 2), there has been no work investigating the effects of modified nanoclays on the performance of individuals and how these effects compare to those produced by increasing the concentration of natural nanoclays. This is unfortunate as other kinds of modified nanoparticles (nano-TiO_2, nano-C_{60}, nano-Ag) are known to have harmful effects on aquatic organisms (George et al. 2012; Lovern et al. 2007). Previously, we (Tullio et al., Chapter 2) found that increases in the concentration of modified nanoclays (Cloisite® 30B and Novaclay^{TM}) can have negative effects on the performance of algae, zooplankton and chironomids; however, there are differences in the toxicity of modified nanoclays due to their unique physicochemical properties when in solution (Tullio et al., Chapter 1). Furthermore, the effects of modified nanoclays often differed from that elicited by natural nanoclay (Tullio et al., Chapter 2). Though this prior work tells us something about the potentially adverse effects of modified nanoclays, it is unknown what the effects of natural and modified nanoclays are on the performance of vertebrate species that live in aquatic environments. Freshwater fish species are ideal sentinels for obtaining nanotoxicity information

from the cellular level to the whole animal level, which can be extrapolated to humans and other vertebrates (Bai et al. 2010).

Histopathological investigations of fish organs, especially of the liver, represent a useful tool to assess the effects of nano-scale particles on the body organs of aquatic organisms (Abarghoei et al. 2016; Monfared et al. 2015). In fact, recent studies have reported severe pathological effects (e.g., hepatitis-like injury, cells with pyknotic nuclei, hepatocyte enlargement, ballooning degeneration, and loosened liver parenchyma) in liver tissues when rainbow trout (*Oncorhynchus mykiss*) and medaka (*Oryzias latipes*) were exposed to different types of nanomaterials such as nano-copper (*n*Cu) and nano-silver (*n*Ag), respectively (Al-Bairuty et al. 2013; Wu and Zhou 2013). The selection of fish liver as an appropriate biomarker for nanomaterials toxicity is due to its important role in the metabolism and the excretion of toxic substances from the fish body (Federici et al. 2007; Handy et al. 2011; Suganthi et al. 2015).

The objective of this study was to assess the potential toxicity of a natural nanoclay and two modified nanoclays on the survivorship, body condition, and on the liver tissues of a freshwater fish (*Gambusia holbrooki)*. *Gambusia holbrooki* is an abundant and widely distributed species across the United States of America (Pyke 2005). This species is a suitable sentinel species due to the fact that: i) it has a small body size and is adaptable to controlled laboratory conditions, ii) it responds in a similar way to pollutants as mammals, iii) presents similarities in its organs physiology to other vertebrates making it possible to compare health effects in humans, iv) it can indicate how vertebrate organisms may differ in their responses to nanoclay exposure compared to algae, filter feeders, and benthic species in terms of survivorship and development of aquatic species, and v) represents higher trophic levels in aquatic systems,

which contribute to investigate the propensity of bioaccumulation of nanoclays through trophic transfer (Caliani et al. 2009; Jagoe et al. 1996; Nunes et al. 2015).

Material and methods

Materials

Natural nanoclay (Na^+ montmorillonite) and conventional ammonium-modified nanoclay (Cloisite® 30B) were obtained from Southern Clay Products (TX, USA), while modified nanoclay without ammonium (Novaclay^{TM}) was obtained from Ioto International (Campo Magro, Paraná, Brazil). Cloisite® 30B is surface functionalized with MT2EtOH (methyl, tallow, bis-2-hydroxyethyl, quaternary ammonium chloride), while Novaclay^{TM} is synthesized with the addition of stearic acid of calcium (AMS-32^{TM}) within the interfacial lamella of the nanoclays but without ammonium compounds. Natural nanoclay does not have organic modifiers in its structure. These three types of nanoclay were selected because 1) natural nanoclay (Na^+ montmorillonite) is very abundant in nature with a great potential to be used in the development of modified nanoclays and 2) both modified nanoclays will be likely used in the production of polymer nanocomposites, rheological modifier in paints, drug delivery systems and environmental remediation (Uddin 2008; Ellenbecker and Tsai 2011; Lee et al. 2005) which will likely result in their intentional or unintentional release into aquatic systems. The physicochemical characterization of all three types of nanoclays has been described by Tullio *et al.* (Chapter 1).

Stock solution preparation

Nanoclays were received in dry powder form, which were weighed on an analytical mass balance, and then suspended in *Reconstituted Hard Water* at the following concentrations: 0.01, 0.1, 1, 10, and 100 mgL^{-1}. We selected these concentrations because they represent ecologically relevant concentrations that span the range of values known to commonly occur in nature though concentrations could exceed 500 mg/L in some circumstances following heavy rain events (Kirk 1992; Robinson et al. 2010). To obtain homogeneous dispersion, all samples were initially stirred with a magnetic stirring device for 2 hours.

Study animals

Adult mosquito fish (*Gambusia holbrooki*) were obtained from Carolina Biological Supply Company, Burlington, North Carolina, USA, and acclimated to laboratory conditions in a 40 L glass aquarium filled with *Reconstituted Hard Water* (continuous aeration, temperature 20± 1ºC, pH: 7.1, photoperiod 16h:8h, and total hardness as $CaCO_3$ 180 mgL^{-1}) as proposed by OECD guidelines 204 (1984) (approved by the East Carolina University Animal Care and Use Committee). *G. holbrooki* were fed with TetraMin® tropical fish food once daily (*ad libitum*).

Experimental design

We assessed the survival, condition factor and histopathology of mosquitofish placed into sixteen 20 L aquaria that had either no nanoclays or one of the three types of nanoclays at one of five concentrations (0.01, 0.1, 1, 10, and 100 mgL^{-1}) which produced 16 treatments (3 types of nanoclays x 5 concentrations + 1 treatment with no nanoclays). After acclimation, five adult mosquito fish (3 ♀ & 2 ♂) were placed into each aquarium for 14 days. During exposure, 80% of media was renewed every 48h with re-dosing after each change. No signs of pain or discomfort

(increased resting or rapid swimming, loss of righting reflex, and unusual gill ventilation) were observed for *Gambusia holbrooki* during the experiment. Fish were fed with TetraMin® tropical fish food once daily (*ad libitum*) and number of surviving fish was assessed each day. We recorded the average fork length (distance from the tip of the snout to the end of the middle caudal fin rays) and average body weight of males and females within each aquaria at both the beginning and end of the experiment (0 and 14 days of exposure) to allow us to estimate the average condition factor (CF) of each sex within aquaria at the start and end of the experiment. Average condition factor of fish of a particular sex within aquaria was estimated as proposed by Monfared et al. (2015):

$$CF = \text{average of body weight (g)} / \text{average of fork length}^3\ \text{(cm)} \times 100$$

After 14 days of exposure, all fish in the 20 L aquaria were euthanized by immersion in MS-222 ($250 mg L^{-1}$). This experimental process was repeated three times (each time is referred to as a "block") and there was one replicate aquaria of each treatment type within each block. Each block was initiated within 10 days of the completion of data collection in another block. All setup and sampling procedures were performed on a block-by-block basis to account for the possible effects of temporal variability in methods.

Preparation of tissue samples

Liver tissues were removed from one male fish from each aquarium and fixed in a 10% neutral buffered formalin, dehydrated in a graded series of ethanol (70, 95, and 100%), cleared in Slide Brite xylene substitute (Newcomer Supply), infiltrated in paraffin (PureAffin x, Newcomer Supply) using a Tissue Tek 2000 tissue processor, and embedded using a Leica EG 1150 embedding center. Tissue blocks were sectioned at 5 µm with a Leica 2025 Microtome, and then

stained with Hematoxylin-Eosin to evaluate their general tissue morphology after nanoclay exposure. Pregnancy generates ultrastructural changes in liver tissues (hypertrophy of nuclei and nucleoli, and changes in liver cell size) that coincide with specific phases of ovarian activity and are indicative of vitellogenin synthesis in the liver rather than responses to pollutants (Aida et al. 1973; Guraya 1986). However, these changes could interact with the treatments to induce high variation in our focal endpoints, which would alter the interpretation of the pathological effects of nanoclays. Consequently, female fish were excluded from histopathological analysis because several were pregnant at the end of the experiment.

We looked for histopathological alterations (e.g., blood cell aggregation with increased number of Kupffer cells, hemosiderin deposition, hepatocytes vacuolization, and cell death) within liver tissue in 5 randomly selected sections of one male fish from each aquarium. The observations were made at 40 x magnification using an Olympus BX 41 microscope equipped with an Olympus digital camera. We used a modified version of the protocol proposed by Bernet et al. (1999) to 1) classify different types of tissue damage (i.e., "circulatory, regressive and degenerative changes" sensu Bernet et al. 1999), 2) quantify the degree of damage within each type of tissue damage ("a" scores sensu Bernet et al. 1999), and 3) an organ tissue damage index that combines information about the different types of tissue damage together while recognizing that some types of tissue damage are more important than others. The different types of tissue damage that we assessed were (Table 2):

i) Circulatory disturbances: pathological conditions of blood and tissue fluid flow (e.g., increased blood cell aggregation with increased number of Kupffer cells and hemosiderin deposits), which are easily reversible and can be only caused as a result of altered organism metabolic status rather than a direct effect of contaminant exposure;

ii) Regressive changes: processes that result in a functional reduction or loss of an organ (e.g., hepatocytes vacuolization), which can be reversible, depending on the severity and extent of the alteration, and

iii) Degenerative changes: histopatological alterations (e.g., cell death) that have the highest importance factor, because they are usually irreversible and their persistence may cause partial or total loss of organ function as a result of the direct effect of the contaminant exposure.

We quantified the severity of a particular type of tissue damage observed in a slide by dividing the viewing space on a slide into 9 equal sized squares (3 x 3) and determining how many of those squares displayed the kind of tissue damage being assessed. Degenerative changes (e.g., cell death) were only considered if there was complete fragmentation of the cell membrane for at least 25% of a square. Severity of damage (i.e., the "a" score) was scored as 0 (no observed injuries in any square); 2 (1 to 3 squares with injuries); 4 (4 to 6 squares with injuries); and 6 (7 to 9 squares with injuries). We then determined the organ tissue damage index (I_{org}) for each slide by

$$I_{org.} = \sum_{alt.} (\boldsymbol{a} \times \boldsymbol{w})$$

where: "alt" refers to a particular type of tissue damage; *a* is the severity of damage associated with a particular type of tissue damage (0 to 6); *w* = importance factor (1 to 3) to recognize that some types of damage are more important than others (Bernet et al. 1999). Degenerative changes were considered to be most important (and scored an importance factor of 3) and circulatory disturbances was least important (and scored an importance factor of 1). Regressive changes were considered to be of intermediate importance (and scored an importance factor of 2). The importance factors identified for each type of tissue damage were defined by Bernet et al. 1999. The total organ tissue damage index and the degree of tissue damage for each type of tissue

damage for animals in a particular aquarium were estimated based on the average scores across the 5 sections from each male.

Data analysis

To determine the effects of three types of nanoclays on *G. holbrooki*, we conducted statistical tests with the aid of SAS Enterprise Guide 9.3 (SAS Institute INC, Cary, NC, USA). Our analyses all began with general linear mixed models (one each for the condition factor index measured at the start and end of the experiment, for the average of the organ tissue damage index, and for the average of the degree of tissue damage for each type of tissue damage) using PROC Mixed. All models included "treatment" as a fixed effect and "block" as a random effect. The model for the analysis of condition factor also included the effect of sex and the interaction between sex and treatment to determine if the condition factor varied with treatment differently for each sex. For the degree of regressive and degenerative tissue damage, we excluded the data from the control groups, because all individuals in the control treatment received a score of 0 (i.e.; there was no variation in the control group). To facilitate the comparison of regressive and degenerative changes in the treatment group to that observed in the control group and to better satisfy assumptions pertaining to homogeneous variances we recalibrated "a" scores for regressive and degenerative changes. Specifically, we added 1 to the "a" score for each aquarium and then took the log of this value. This approach rescales the "a" score such that a value of 0 represents no damage detected and the parameter estimates for this score in other treatments can be compared to a score of 0 (i.e., the constant score observed in the control group). Total organ index exhibited some variability in all treatments but the extent of heterogeneity varied among treatment groups. We log transformed the total organ index to better satisfy the assumption of homogeneous variances. To represent the results for the regressive and degenerative changes,

and the total organ tissue damage index graphically, the LS mean estimate and its corresponding standard error were back transformed which resulted in asymmetrical error bars given the log transformation.

For all models, we identified the NOAEL (No Observed Adverse Effect Level) and LOAEL (Lowest Observed Adverse Effect Level) for each type of nanoclay on each response variable by using Dunnet's procedure to compare estimates in each treatment containing nanoclays to the estimates observed in treatments lacking nanoclays. We also used orthogonal polynomial contrasts with our models to assess whether responses changed predictably with the dosage of each type of nanoclay present. We assessed up to fifth order polynomials but only present the statistical results of third order or higher contrasts if there is sufficient evidence to suggest they may be important. In most cases, the strength of statistical evidence to support a third order ($F_{1, 31} \leq 2.87$, $p \geq 0.101$) or higher contrast was weak ($F_{1, 31} \leq 3.25$, $p \geq 0.082$). In addition, we compared differences among nanoclay types within each concentration by performing pairwise comparisons via Fisher's LSD. Given the multiple comparisons made among treatments for each response variable, we adjusted p values for each comparison to control for the False Discovery Rate (FDR; Verhoeven et al., 2005). Survival data was not included in the statistical analysis, because we did not observe mortality for *G. holbrooki* after nanoclay exposure, except for two aquaria containing natural nanoclays and Cloisite® 30B at the highest concentration (100 mgL^{-1}).

Results

Nanoclays had very little impact on *G. holbrooki* survival. Survivorship of mosquito fish was 100% in all but two of the 48 times that we assessed survival. When mortality occurred there

was 1) two fish that died in a single aquarium containing the highest concentration of natural nanoclays or 2) one fish that died in a single aquarium containing the highest concentration of Cloisite® 30B. Consequently, mortality was 0% in all treatments except for the treatment with highest concentration of natural nanoclays (13.3% mortality) and the treatment with the highest concentration of Cloisite® 30B (6.7% mortality).

Condition factor did not vary appreciably among individuals of different sexes ($F_{1, 32}$=1.35, p=0.254) or among treatments ($F_{15, 30}$=1.07, p=0.424) at the start of the experiment. We also did not observe any differences in condition factor between sexes ($F_{1, 32}$=0.53, p=0.472) or among treatments ($F_{15, 30}$=1.16, p=0.355) at the end of the experiment and there is little evidence to suggest that differences in the condition factor across treatments at the beginning of the experiment was different for male and female fish ($F_{15, 32}$=1.44, p=0.190). Furthermore, neither the condition factor of males or females at the end of the experiment differed predictably as the concentration of any type of nanoclay increased (linear trend analysis: $F_{1, 31}\leq0.94$, $p\geq0.340$; quadratic trend analysis: $F_{1, 31}\leq0.52$, $p\geq0.480$). Although, condition factor index assumes that heavier fish of a given length are in better condition, our results of condition factor suggested that *G. holbrooki* from all treatments did not vary with nanoclay concentration, showing similar heath status. Thus, we cannot report a LOAEL for the effect of nanoclays on the condition factor of *G. holbrooki*.

Histopathological alterations in the liver parenchyma of fish affected by nanoclays included: circulatory changes (blood cell aggregation with increased number of Kupffer cells and hemosiderin deposits), regressive changes (hepatocyte vacuolization), and degenerative changes (cell death) (Figure 9 to 13). Though, the majority of the fish from the control group did not

show any signs of histopathological changes (Figure 9), there was one individual fish that presented minor circulatory disturbances (blood cell aggregation) in liver tissues (Figure 14).

Our results showed little effects of both natural nanoclays and Cloisite® 30B on circulatory disturbances in mosquito fish liver tissue as their concentrations increased (linear trend analysis: $F_{1, 31} \leq 1.35$, $p \geq 0.254$; quadratic trend analysis: $F_{1, 31} \leq 0.67$, $p \geq 0.418$) but increasing the concentration of Novaclay^{TM} induced greater amounts of circulatory disturbances to the hepatic tissue of *G. holbrooki* at very low concentrations (LOAEL: 0.01 mgL^{-1}) ($F_{1, 31}=0.94$, $p=0.340$; quadratic trend analysis: $F_{1, 31}=0.01$, $p=0.916$; tertiary trend analysis: $F_{1, 31}=2.13$, $p=0.155$; quaternary trend analysis: $F_{1, 31}=3.02$, $p=0.092$; quintic trend analysis: $F_{1, 31}=4.15$, $p=0.05$) (Figure 14). Novaclay^{TM} appeared to have a stronger effect on the amount of circulatory damage when the concentration was between 0.01 mgL^{-1} and 1 mgL^{-1} but then had little effect at a concentration of either 10 mgL^{-1} or 100 mgL^{-1} compared to both nanoclay types (Figure 14).

On the other hand, all types of nanoclay in our study enhanced the degree of regressive ($t_{29} \geq 2.07$, $p \leq 0.04$) and degenerative ($t_{29} \geq 9.26$, $p < .0001$) changes relative to that observed in the absence of nanoclays (i.e., 0 mgL^{-1}) and these effects were observed at the lowest concentration of nanoclays that we considered (LOAEL: 0.01 mgL^{-1}) (Figure 15 and 16). The frequency of both types of tissue damage in *G. holbrooki* was not associated with nanoclay type or concentration (linear trend analysis: $F_{1, 31} \leq 0.58$, $p \geq 0.453$; quadratic trend analysis: $F_{1, 31} \leq 0.87$, $p \geq 0.360$; tertiary trend analysis: $F_{1, 31} \leq 2.87$, $p \geq 0.101$; quaternary trend analysis: $F_{1, 31} \leq 3.25$, $p \geq 0.082$); however, an increase in the concentration of Novaclay^{TM} seemed to induce increased degenerative changes in mosquito fish than did increasing the concentration of natural nanoclay at 1 mgL^{-1}(Figure 16).

Furthermore, the presence of any nanoclay in the water caused 4-7 times the amount of liver tissue damage for the "I_{org}" than for when nanoclays were not present (Figure 17; weight of evidence that the difference between LS mean estimate in a particular treatment and that observed in the control was $t_{16} \geq 4.43$, $p \leq 0.004$ for all treatments). Consequently, the LOAEL for the three nanoclay types is represented by the lowest concentration of nanoclays implemented in this study (LOAEL: 0.01 mgL^{-1}). Though the degree of liver tissue damage did change as the concentration of any type of nanoclay increased (linear trend analysis: $F_{1, 31} \leq 5.66$, $p \geq 0.024$; quadratic trend analysis: $F_{1, 31} \leq 6.09$, $p \geq 0.020$; tertiary trend analysis: $F_{1, 31} \leq 7.99$, $p \geq 0.008$; quaternary trend analysis: $F_{1, 31} \leq 13.94$, $p \geq 0.001$; quintic trend analysis: $F_{1, 31} \leq 38.83$, $p < 0.0001$) this was largely attributed to the presence of nanoclays as nanoclay concentration did not explain much variation in the amount of liver tissue damage (linear trend analysis: $F_{1, 29} \leq 0.54$, $p \geq 0.470$; quadratic trend analysis: $F_{1, 29} \leq 0.12$, $p \geq 0.731$). Liver tissue damage did not differ statistically among nanoclay types at any of the concentrations that we assessed ($t_{16} \leq 1.56$, $p \geq 0.956$).

Discussion

Our study revealed that neither the survival nor body condition of *G. holbrooki* was affected by any of the types of nanoclays that we examined. This works bolsters the idea that *G. holbrooki* is, at least in some ways, resistant to the effects of pollutants (e.g., organic wastes, pesticides, and heavy metals) (Cherry et al. 1976; Lloyd et al. 1986; Willis and Ling 2000). Though, we should be cautious when using survival and body condition estimates from our short-term experiments (i.e. 14 days) because these fish can live to an average age of 1.5 years. Our assessment only occurred over 2.5% of their whole life time and in the wild these fish might be exposed over their entire lifetime. Others have also found that the survival and body condition

of other species of fish are also rather resistant to the influence of nanoparticles in general. For example, the survival of embryos of zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) do not appear to be affected by exposure to coated nanomaterials (*n*TiO_2, *n*ZnO, *n*Fe_2O_3, and *n*CeO_2) and nano-titanium dioxide (*n*TiO_2), respectively (Felix et al., 2013; Paterson et., 2011). Ramsden et al. (2009) also found that the body condition of rainbow trout (*Oncorhynchus mykiss)* was not adversely affected by nano-titanium dioxide (*n*TiO_2). Assessment of how survival or body condition of individuals respond to pollutants may be insufficient, however, to predict longer term effects of pollutants on ecosystems health (Forbes et al. 2006; van der Oost et al. 2003). In fact, there is a need to include early warning indicators in ecotoxicological tests such as sensitive biomarkers (e.g., physiological, biochemical, and histopathological parameters), because they can provide more information about the toxicity of pollutants before sublethal effects (e.g., body growth and reproductive potential) and lethal effects on individuals occur (Handy and Depledge 1999).

Recent studies have found that nano-titanium dioxide, nano-gold, nano-silver do not impact survivorship or body condition of fish over the timelime studied, but caused histopathological lesions in liver tissues which reduced organ function and consequently the fish's health (Federici et al. 2007; Monfared et al. 2015; Truong et al. 2012). Each of the three types of nanoclays we studied caused histopathological changes on liver tissues for all five concentrations of nanoclays even though they did not impact body condition or survival after only 14 days of exposure.

Mosquito fish treated with the three nanoclay types showed increased blood cell aggregation with Kupffer cells (liver-specialized macrophages). Kupffer cells are classified as scavenger cells responsible for destruction, detoxification, or recycling of endogeneous and

exogeneous materials (Agius and Roberts 2003; Kolios et al. 2006). Other authors have also reported the presence of Kupffer cells as an indication of injured hepatocytes due to exposure of liver tissue to nanomaterials. For example, Ostaszewska et al. (2016) found the presence of Kupffer cells in the liver of Siberian sturgeon (*Acipenser baerii*) when exposed to both nano-silver (*n*Ag) and nano-copper (*n*Cu), and the frequency of liver-specialized macrophages was dose-dependent. Abdelhalim and Jarrar (2011) demonstrated that male Wistar-Kyoto rats exposed to nano-gold caused an increase in the occurrence of Kupffer cells in the hepatic tissue of the rat. These findings confirm the importance of Kupffer cells in hepatic tissues in scavenging of different types of nanomaterials; however, when activated Kupffer cells also produce signaling molecules (e.g.; cytokines) that promote inflammatory responses necessary to remove the foreign molecules, which generates alterations in the hepatic cytoplasm (Jaeschke et al. 2002). As a result of that, nanomaterials may interact with enzymes and other hepatic proteins that may affect the antioxidant response and generate reactive oxygen species (ROS), resulting in oxidative stress in the liver tissues (Ostaszewska et al. 2016).

Nanoclays also induced the formation of hemosiderosis (a form of circulatory disturbance) in *G. holbrooki*. Previous studies have shown the presence of hemosiderosis in fish livers due to their exposure to pollutants. For instance, Bowser et al. (1990) reported the presence of hemosiderosis in brown bullheads (*Ictalurus nebulosus*) exposed to PCB and heavy metals in Hudson River, New York. Abarghoei et al. (2016) also observed hemosiderosis in liver tissues of goldfish (*Carassius auratus*) after their exposure to silver nitrate ($AgNO_3$) at 0.05 and 0.1 ppm, while nano-silver (*n*Ag) particles did not cause the same pathology in goldfish. Hemosiderosis occurs after excessive destruction of red blood cells with liberation and deposition of iron within an organ (e.g.; lungs, kidneys, livers) due to hemorrhage from trauma, chronic congestion,

parasitic infections, and exposure to toxic chemicals in humans and vertebrate animals (Khan and Nag 1993). This abnormal condition is characterized by the presence of a yellow-brown pigment called hemosiderin, which is usually found in interstitial macrophages in lungs of humans (Nursel Türkmen et al. 2008), renal cortex in kidneys of humans (Suzukawa et al. 1993), and Kupffer cells of liver and melanomacrophage centers in fish (Thiyagarajah et al. 1998). Hepatic hemosiderin deposition in humans may occur in a variety of diseases such as hematologic disorders (e.g., transfusions), chronic viral hepatitis, cirrhosis, and anemia (Batts 2007). Other authors have also described the potential correlation between hemosiderosis and anemia in vertebrate animals as a result of the exposure to organic pollutants in the environment. Khan et al. (1992) suggested that the presence of hemosiderin deposits in liver of winter flounder from polluted sites in Port Harmon was causing anemia in fish, since hemosiderin is a product of red blood cells degradation that has been filtered out by the lymphoid-macrophage system. Similarly, *Gambusia holbrooki* presented moderate hemosiderosis in liver tissue when exposed to three types of nanoclays in this study, suggesting that natural nanoclays and modified nanoclays may be inducing anemia in freshwater fish species at very low concentrations (0.01 mgL^{-1}).

We also observed that exposure of mosquito fish to nanoclays caused an increase in the degree of hepatocyte vacuolation. Many studies have shown that vacuolations of hepatocytes is a common response associated with exposure to toxicants and nanomaterials (Cengiz and Unlu 2006; Mishra and Mohanty 2008; Rajkumar et al. 2016; Saraiva et al. 2015). Fanta et al. (2003) and Velmurugan et al. (2007) observed vacuolation of hepatocytes in catfish (*Corydoras paleatus*) and mrigal carp (*Cirrhinus mrigala*) after exposure to the pesticide methyl parathion and lambda-cyhalothrin, respectively. Linhua et al. (2009) and Lee et al. (2012) also indicated a

similar finding in juvenile carp (*Cyprinus carpio*) exposed to nano-titanium dioxide (nTiO$_2$) at concentrations ranging from 10 to 200 mgL^{-1}. In addition, nuclear vacuolation of hepatocytes can be a regressive process resulting from nonalcoholic fatty liver disease (NAFLD) in humans, which is mainly related to obesity, diabetes, and drug-induced liver disease (Hübscher 2006). The formation of vacuoles in hepatocytes with fatty vacuolation was highly observed in obese patients by Luyckx et al. (1998). Davidson and Eastham (1966) also described pathological lesions in the liver of two individuals after acetaminophen overdose, which included hepatocyte vacuolation accompanied by fulminating necrosis. In reality, the formation of vacuoles in hepatocytes is mainly due to the abnormal accumulation of large (macrovesicular) or small (microvesicular) intracytoplasmic fat droplets in liver cells, which can be an important indicator of early stages of necrosis in humans and vertebrate animals (Reddy and Rao 2006).

Although, the methodology used in this study to assess histopathological changes in liver tissue (e.g.; Hematoxylin-Eosin stain) did not allow us to characterize and differentiate apoptosis and necrosis processes, we were able to identify cell death (i.e. large areas that contain cells with complete membrane fragmentation) without identifying its mechanism. The histopathological alterations in *G. holbrooki* induced by nanoclays, including cell death, suggest that nanoclays induce the formation of ROS, leading to oxidative stress and disturbance to biological systems. Indeed, circulatory, regressive, and degenerative changes in liver tissues have been linked to the formation of ROS due to the interaction between nanomaterials and cellular components, causing oxidative stress that may be responsible for DNA damage (e.g.; chromosomal fragmentation, DNA strand breakages, and induction of gene mutations) and cell death (apoptosis and necrosis) (Houglum et al. 1997; Jaeschke et al. 2002; Khanna et al. 2015; Lee et al. 2012). Rajkumar et al. (2016) found that freshwater fish (*Labeo rohita*) treated with nano-silver (nAg) caused increased

concentration of ROS leading to oxidative stress, which was related to the histopathological changes such as formation of vacuolation and necrosis in liver tissues. Nevertheless, further studies evaluating the molecular mechanisms of nanoclay toxicity on biological systems are required to better understand their mode of action on humans and aquatic species.

Though all three types of nanoclays induced toxic effects on the hepatic tissue of *G. holbrooki*, Novaclay™ and Cloisite® 30B seem to have higher toxicity on the circulatory disturbances of the hepatic tissues of mosquito fish at lower and higher concentrations, respectively. Previously, we (Chapter 1) reported that the three types of nanoclays differ in their physicochemical properties when they are in solution, which can explain variation in the toxicity among nanoclays. For example, Novaclay™ (zeta potential: -21.4 mV) is more stable than natural nanoclay (zeta potential: -18.7 mV) and Cloisite® 30B (zeta potential: -11 mV), therefore Novaclay™ is the most likely to be well dispersed and remain as single particles in solution when compared to both nanoclay types at all five concentrations. Consequently, Novaclay™ particles can be more bioavailable and potentially dangerous to highly mobile pelagic species such as mosquito fish. Other authors have already described that nanometric scale particles can obstruct breathing through gills and/or be absorbed from the water, causing severe damage in biological structures such as liver tissues (Chen et al. 2011; Britto et al. 2012, Costa et al. 2012). Novaclay™ particles also induced toxic effects on daphnia survival due to their high stability in solution, but only after continuous exposure (Tullio et al., Chapter 2). In contrast, natural nanoclays and Cloisite® 30B particles are more likely to agglomerate/aggregate, fall out the solution and deposit on sediments, making them less likely to encounter and interact with *G. holbrooki*. In addition, we suggest that the exposure of *G. holbrooki* to Novaclay™ under high concentrations may have activated the detoxification mechanism, minimizing the harmful

impacts and insults of Novaclay^{TM} particles on mosquito fish. Clemente et al. (2013) have reported that high concentrations of nano-titanium dioxide (nTiO$_2$) may have activated detoxification routes or antioxidant mechanisms in freshwater fish (*Piaractus mesopotamicus)*, inhibiting the effects of nanomaterials and contributing to the lack of a dose-response relationship.

Conversely, Cloisite® 30B seem to have higher toxic effects on liver tissues of mosquito fish at the highest concentration (100 mgL^{-1}), which is likely due its composition. Sharma et al. (2010) have already found that the organic modifier (MT2EtOH) of Cloisite® 30B remains in the water after filtering the suspensions of Cloisite® 30B and that this organic modified can induce DNA-strand breaks in the human intestinal cell line Caco-2. Thus, we suggest that the organic modifier (MT2EtOH) only present in Cloisite® 30B particles is being released in aqueous solution, enhancing the toxicity of Cloisite® 30B on hepatic tissues of mosquito fish.

Conclusion

Our prior work (Tullio et al., Chapter 2) and the work reported in this chapter clearly demonstrate the effects of natural and modified nanoclays to aquatic life. Cloisite® 30B adversely affected *Chlamydomonas reinhardtii*, *Daphnia magna*, *Chironomus dilutus*, and *Gambusia holbrooki*, while natural nanoclays and Novaclay^{TM} only affected *Daphnia magna*. In contrast, all three types of nanoclays showed toxic effects on *Gambusia holbrooki*. Differences in the toxicity of different nanoclays likely derive from the known variation in the physicochemical properties of the nanoclays (Tullio et al., Chapter 1) and also to differences among organisms in their sensitivity to nanoparticles on the basis of their microhabitat usage (water column versus sediment). Modified nanoclays seem to have higher toxic effects on all

four aquatic species than natural nanoclays, however, aquatic species appear to be least sensitive to Novaclay™ compared to Cloisite® 30B. Thus, the widespread use of nanoclays in a variety of commercial products and environmental applications raises great concern about their safety for human and aquatic life.

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Table 2. Description of the histopathological changes analyzed in liver tissues of *Gambusia holbrooki* when exposed to three different types of nanoclays at six different concentrations (0, 0.01, 0.1, 1, 10, and 100 mgL^{-1}).

Reaction patterns	Alterations	Description	Liver diseases in humans associated with similar alterations in fish species
Circulatory changes (altered blood flow) (w=1)	**Increased number of Kupffer cells**	Kupffer cells (liver-specialized macrophages) are responsible for destruction, detoxification, or recycling of endogenous and exogenous materials (Ostaszewska et al. 2016)	Viral hepatitis, steatohepatitis, alcoholic liver disease, intrahepatic cholestasis, and liver fibrosis (Kolios et al. 2006).
	Hemosiderin pigment	Yellow/brown pigment and granular, and it usually appears intracellularly in Kupffer cells and hepatocytes (Kanel and Korula 2005).	Alcoholic and nonalcoholic fatty liver disease, chronic viral hepatitis, and anemia of chronic disease (Batts 2007; Hudacko and Theise 2011).
Regressive changes (w=2)	**Hepatocytes vacuolization**	Hepatocytes that contain a large well-defined single rounded vacuole within each cell, and their nucleous and cytoplasm are displaced (Thoolen et al. 2010).	Viral hepatitis, diabetes mellitus, nonalcoholic fatty liver disease (Nayak et al. 1996; Aravinthan et al. 2012; Chaundhary et al. 2014).
Degenerative changes (w=3)	**Cell death**	Large areas that contain cells with complete membrane fragmentation (Fink and Cookson 2005).	Most of the liver diseases in humans (Jaeschke and Gujral 2004; Guicciardi et al. 2013).

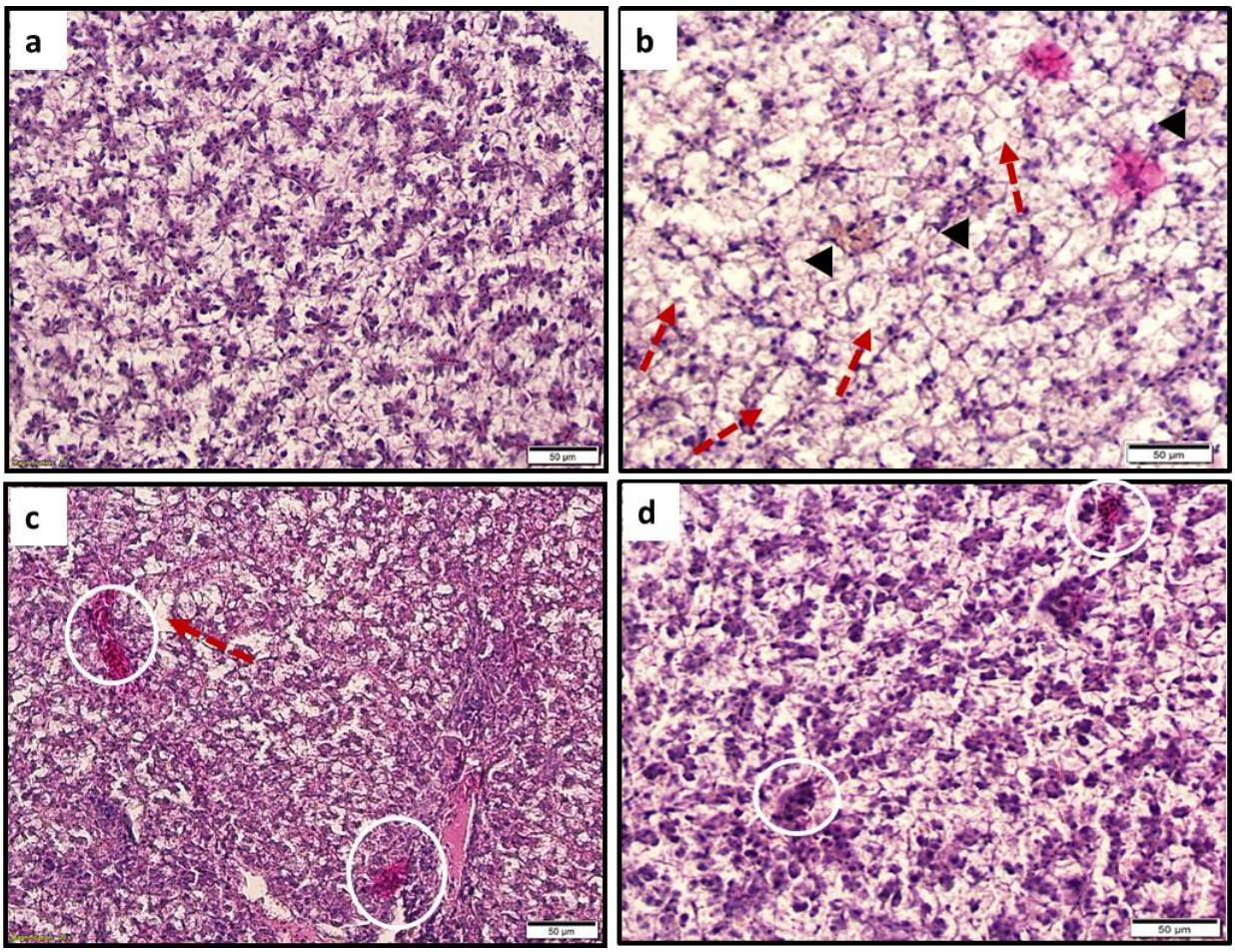

Figure 9. Liver light microscopy of histological sections of *Gambusia holbrooki* exposed to three different types of nanoclays at 0.01 mgL^{-1} (**a:** control (0 mgL^{-1}); **b:** natural nanoclay; **c:** Cloisite® 30B; **d:** Novaclay™). Blood cell aggregation with increased number of Kupffer cells (*white marked circularly*), hemosiderin (*black arrowhead*), cell death (*red dashed arrow*); x 20 magnification and scale bars = 50µm.

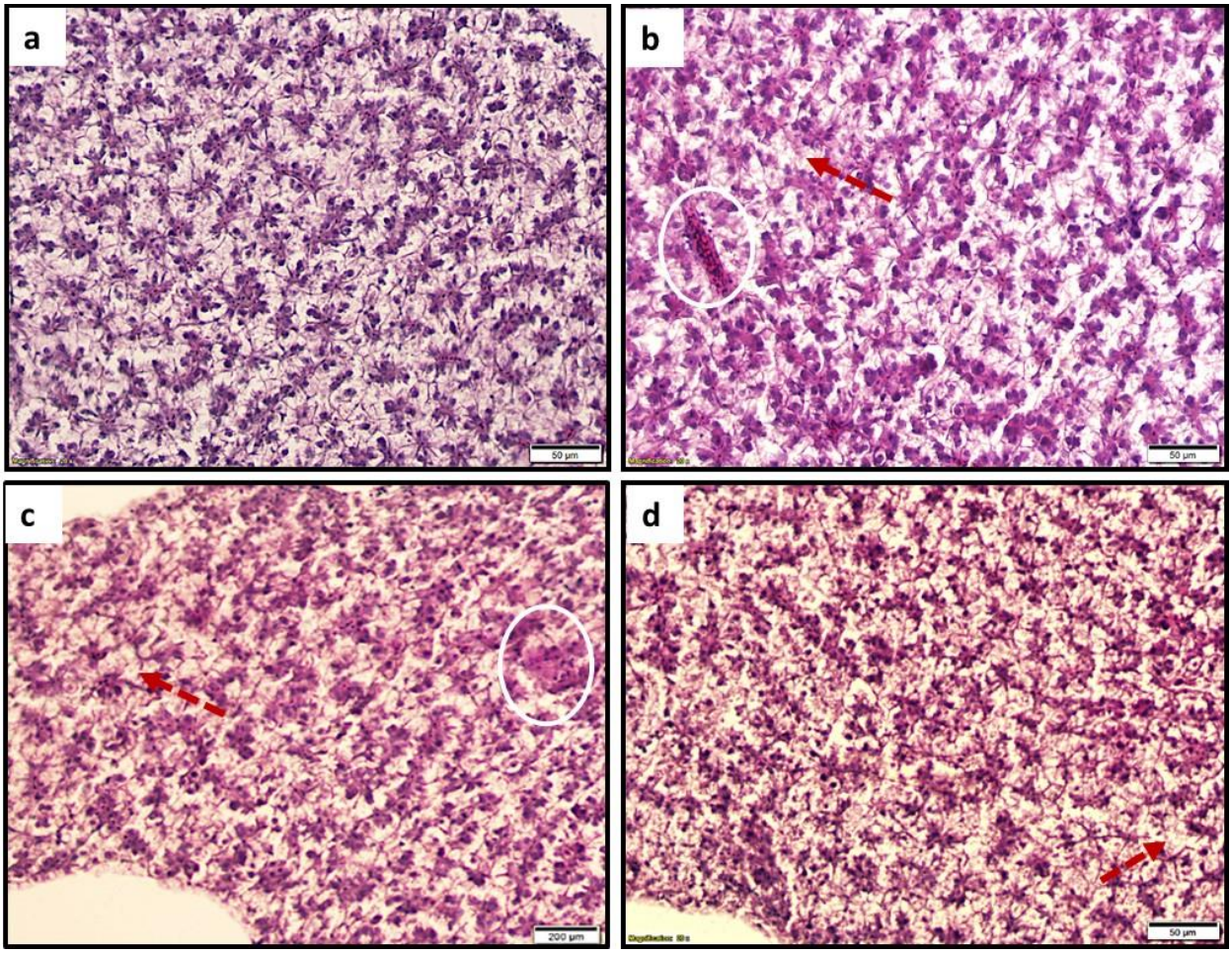

Figure 10. Liver light microscopy of histological sections of *Gambusia holbrooki* exposed to three different types of nanoclays at 0.1 mgL^{-1} (**a:** control (0 mgL^{-1}); **b:** natural nanoclay; **c:** Cloisite® 30B; **d:** Novaclay^{TM}). Blood cell aggregation with increased number of Kupffer cells (*white marked circularly*), cell death (*red dashed arrow*); x 20 magnification and scale bars = 50µm.

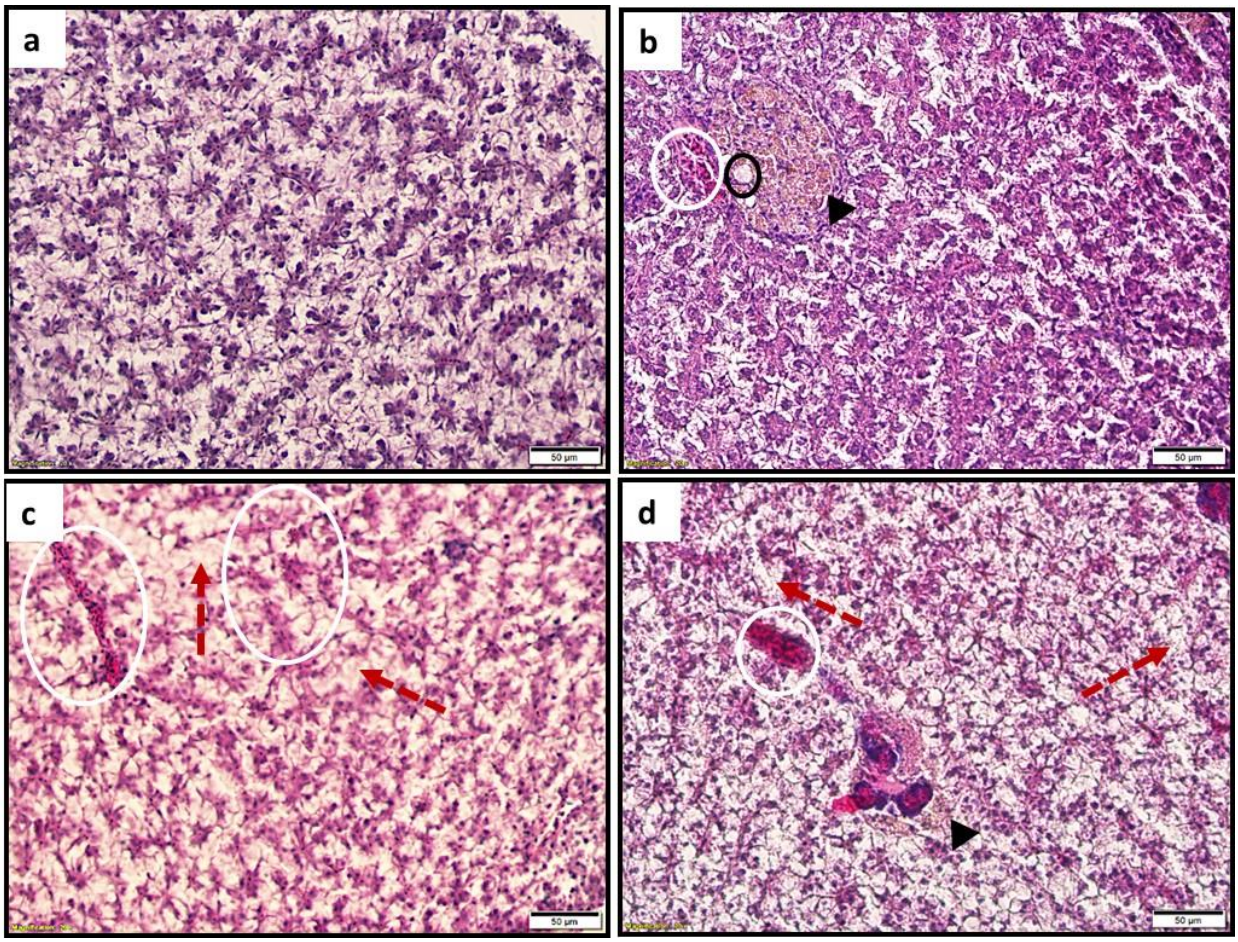

Figure 11. Liver light microscopy of histological sections of *Gambusia holbrooki* exposed to three different types of nanoclays at 1 mgL^{-1} (**a:** control (0 mgL^{-1}); **b:** natural nanoclay; **c:** Cloisite® 30B; **d:** Novaclay™). Blood cell aggregation with increased number of Kupffer cells (*white marked circularly*), hemosiderin (*black arrowhead*), cell death (*red dashed arrow*), hepatocyte vacuolization (*black marked circularly*); x 20 magnification and scale bars = 50µm.

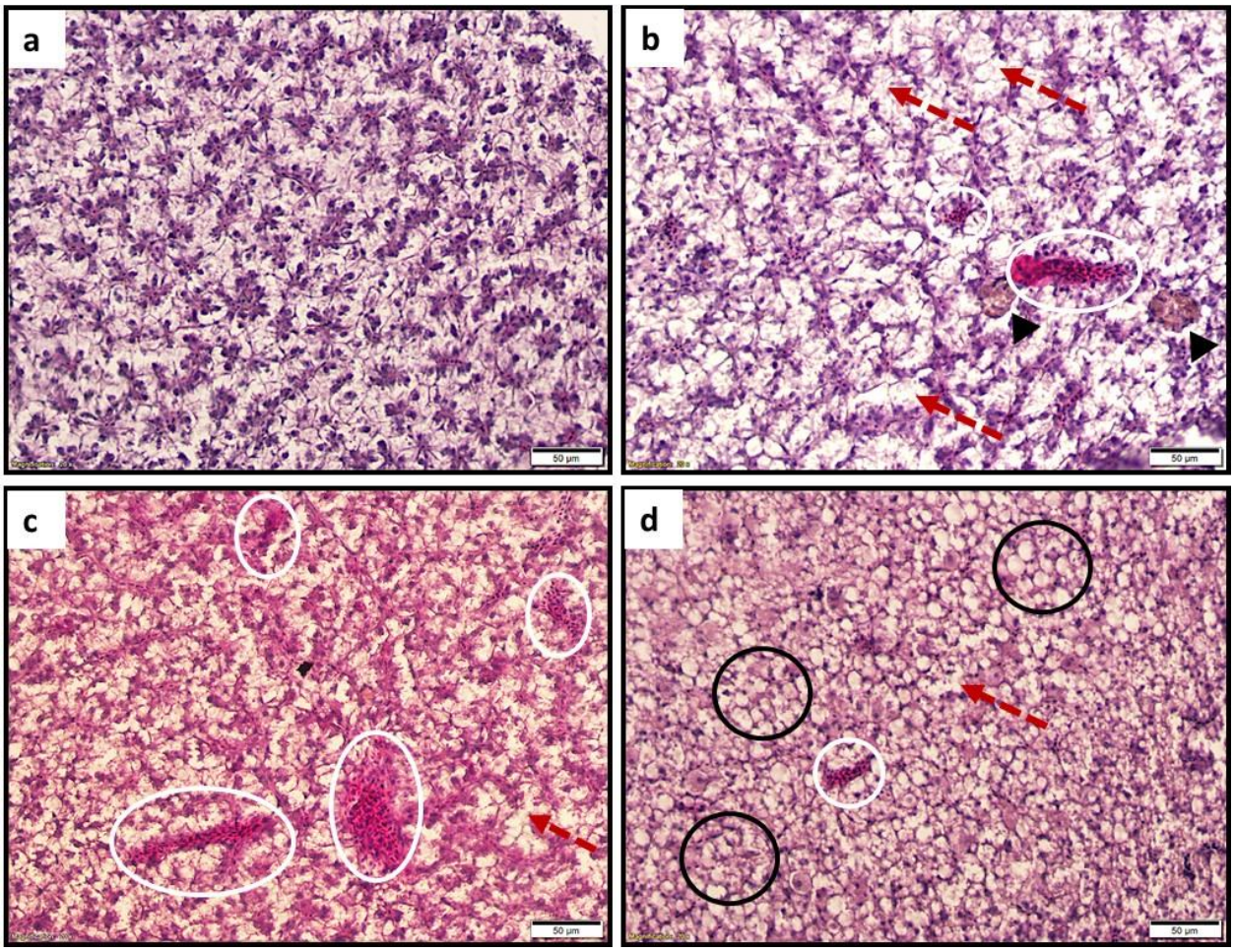

Figure 12. Liver light microscopy of histological sections of *Gambusia holbrooki* exposed to three different types of nanoclays at 10 mgL^{-1} (**a:** control (0 mgL^{-1}); **b:** natural nanoclay; **c:** Cloisite® 30B; **d:** Novaclay™). Blood cell aggregation with increased number of Kupffer cells (*white marked circularly*), hemosiderin (*black arrowhead*), cell death (*red dashed arrow*), hepatocyte vacuolization (*black marked circularly*); x 20 magnification and scale bars = 50µm.

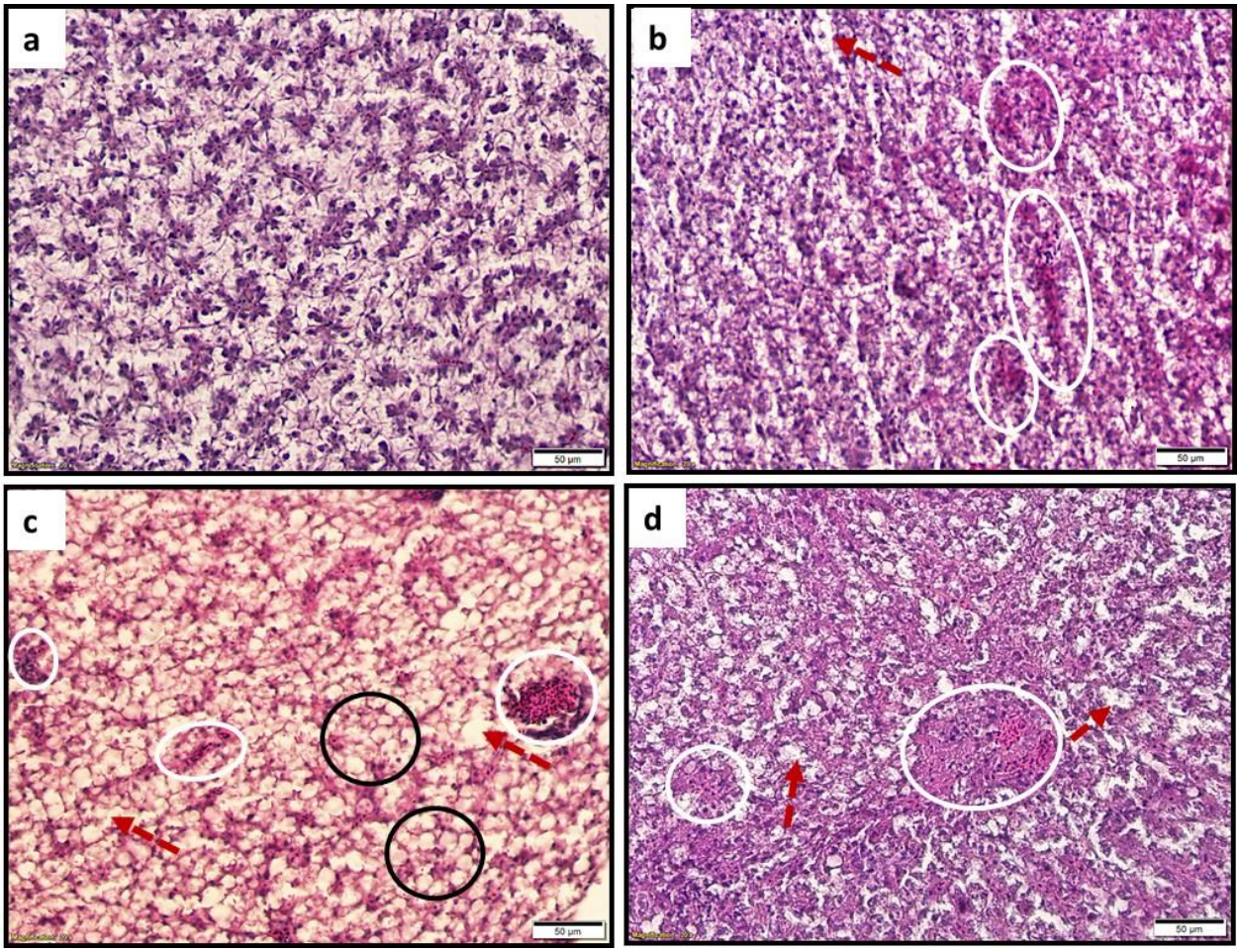

Figure 13. Liver light microscopy of histological sections of *Gambusia holbrooki* exposed to three different types of nanoclays at 100 mgL^{-1} (**a:** control (0 mgL^{-1}); **b:** natural nanoclay; **c:** Cloisite® 30B; **d:** Novaclay™). Blood cell aggregation with increased number of Kupffer cells (*white marked circularly*), cell death (*red dashed arrow*), hepatocyte vacuolization (*black marked circularly*); x 20 magnification and scale bars = 50µm.

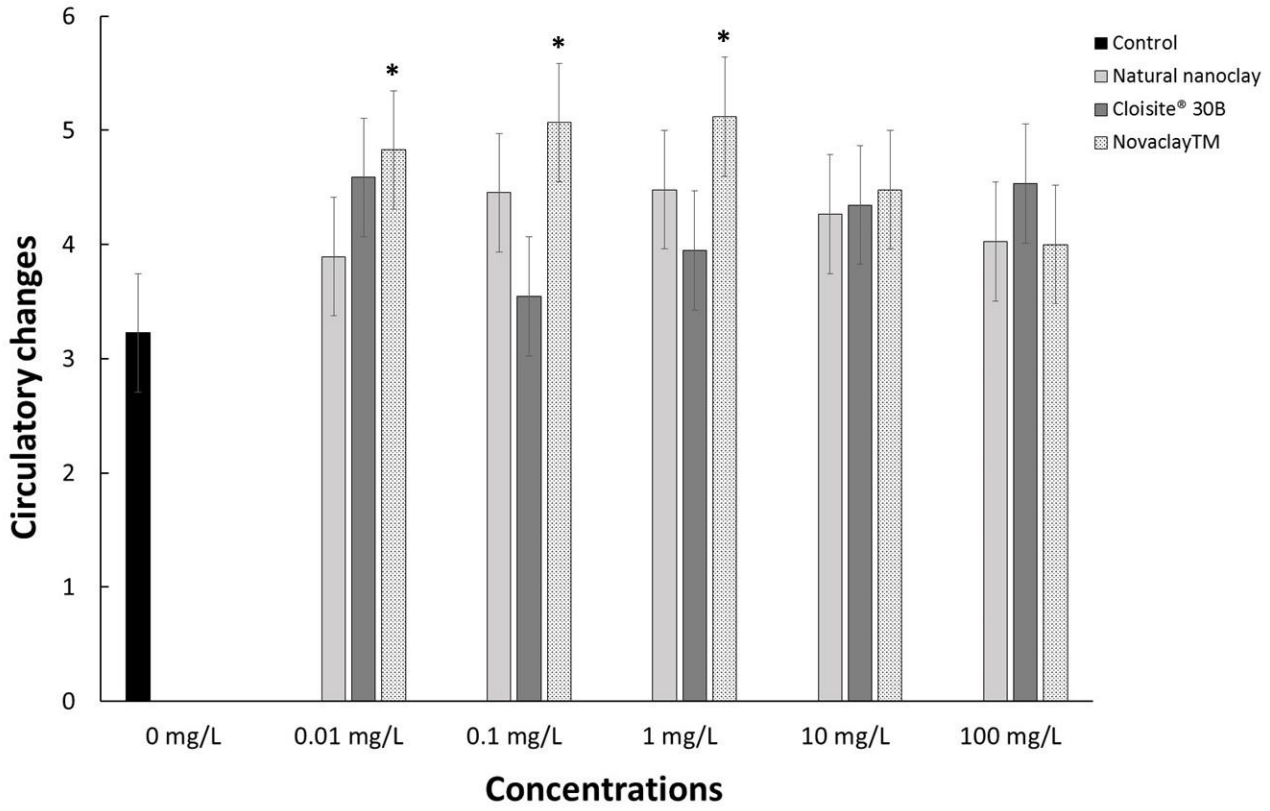

Figure 14. Least square mean estimates ± standard error (SE) of the scores of circulatory disturbances of *Gambusia holbrooki* when exposed to three types of nanoclays at six different concentrations (0, 0.01, 0.1, 1, 10, and 100 mgL^{-1}) using a general linear mixed model (PROC Mixed). (*****) Treated groups statistically different than control by using Dunnet's procedure ($p<0.05$).

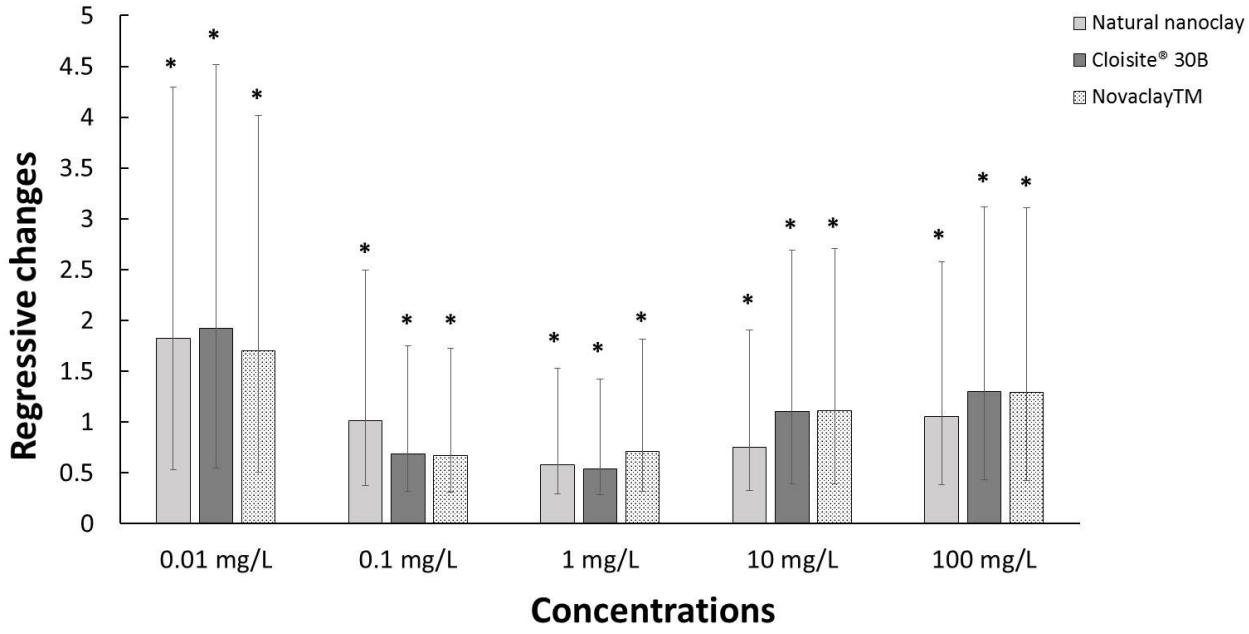

Figure 15. Least square mean estimates ± standard error (SE) of the scores of regressive changes of *Gambusia holbrooki* when exposed to three types of nanoclays at five different concentrations (0.01, 0.1, 1, 10, and 100 mgL^{-1}) using a general linear mixed model (PROC Mixed). (*****) Treated groups statistically different than control by using Dunnet's procedure ($p<0.05$). Results from control treatments (0 mgL^{-1}) were not showed in Figure 15, because there were no signs of regressive changes in mosquito fish in the control groups.

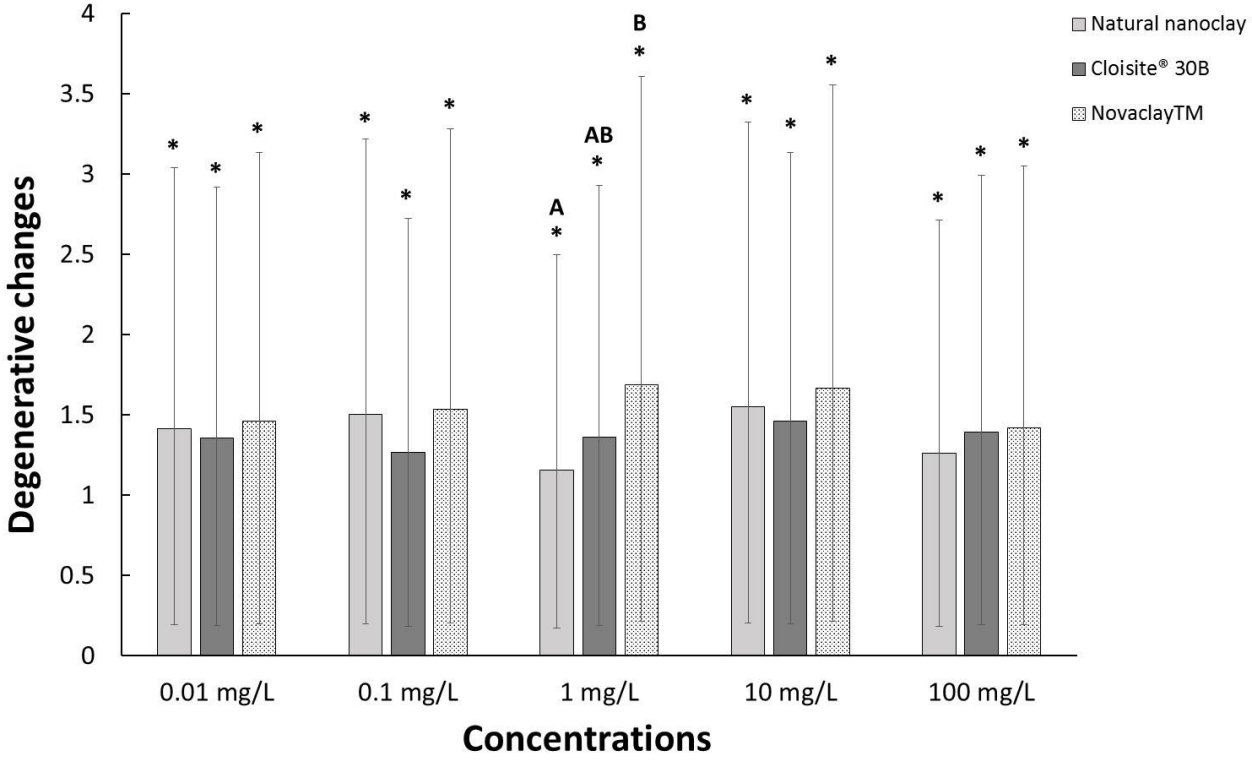

Figure 16. Least square mean estimates ± standard error (SE) of the scores of degenerative changes of *Gambusia holbrooki* when exposed to three types of nanoclays at five different concentrations (0.01, 0.1, 1, 10, and 100 mgL^{-1}) using a general linear mixed model (PROC Mixed). (*****) Treated groups statistically different than control by using Dunnet's procedure ($p<0.05$). Different letters (**A, B**) above bars within each concentration identify which nanoclay types differ from each other in their effects at that particular concentration, while (**AB**) above bars within each concentration show which nanoclay types do not differ from each other in their effects on the degenerative changes of mosquito fish at that particular concentration. Adjusted p-values for each group comparison were generated using the False Discovery Rate (FDR). Results from control treatments (0 mgL^{-1}) were not showed in Figure 16, because there were no signs of degenerative changes in mosquito fish in the control groups.

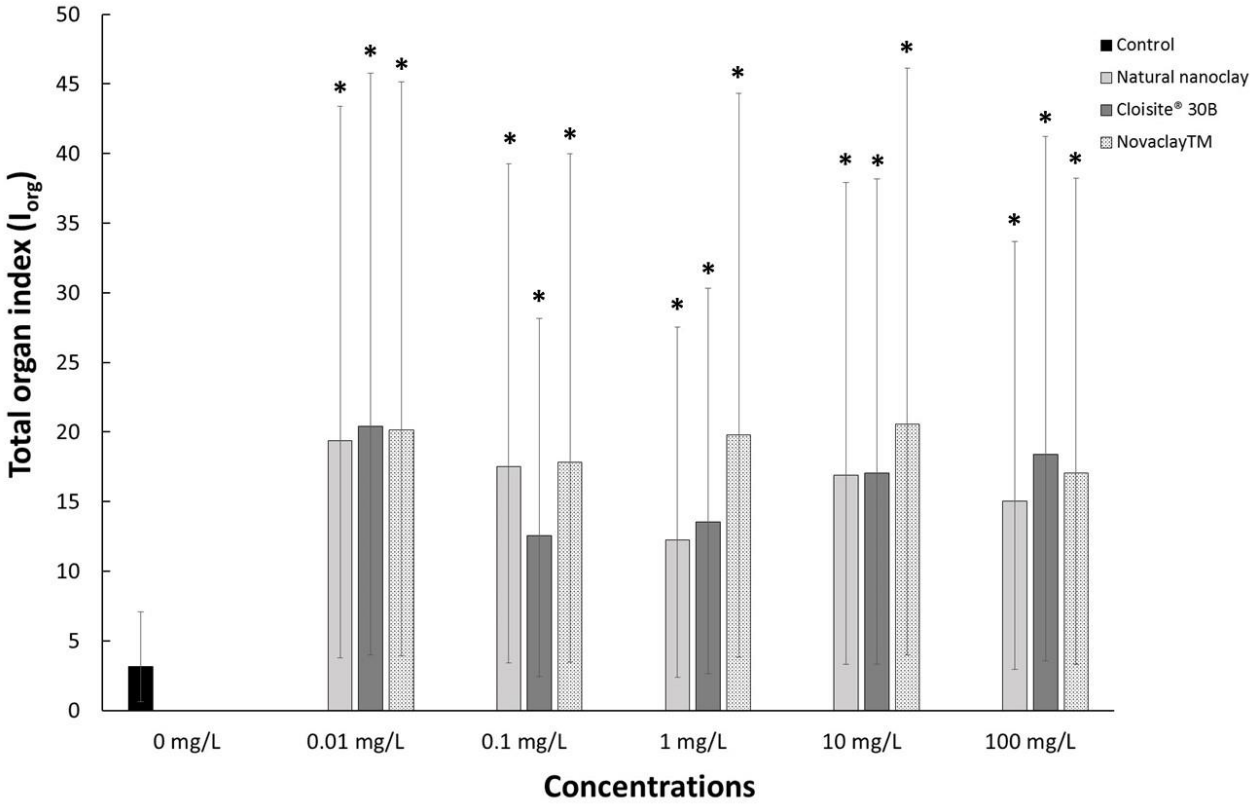

Figure 17. Least square mean estimates ± standard error (SE) of the total organ index ($I_{org.}$) of *Gambusia holbrooki* when exposed to three types of nanoclays at six different concentrations (0, 0.01, 0.1, 1, 10, and 100 mgL^{-1}) using a general linear mixed model (PROC Mixed). (*****) Treated groups statistically different than control by using Dunnet's procedure ($p<0.05$).

CHAPTER 4: Summary of findings and recommendations for future research in nanoecotoxicology

This study indicated that natural and modified nanoclays have adverse toxic effects on multiple species that differ in their trophic position within aquatic food webs. We did observe that nanoclays differed in their toxicity due to their physicochemical properties and species-specific sensitivity to nanoclay exposure. Cloisite® 30B adversely affected all four aquatic species (*Chlamydomonas reinhardtii*, *Daphnia magna*, *Chironomus dilutus*, and *Gambusia holbrooki*), while natural nanoclay and Novaclay™ only affected *Daphnia magna* and *Gambusia holbrooki* (Table 3). Cloisite® 30B likely has higher toxic effects on aquatic species due to the following factors: i) presence of quaternary ammonium compounds in its composition that may cause oxidative stress, and ii) lower stability in solution (zeta potential: -11 mV), which can make Cloisite® 30B particles more likely to agglomerate, fall out of solution, and deposit on sediments. In fact, the lower stability of Cloisite® 30B particles may favor their agglomeration to the cell wall/membranes of aquatic biota, inhibiting the photosynthetic activity, body growth, and survivorship of pelagic species. Furthermore, benthic species may be vulnerable to Cloisite® 30B particles that are deposited and adsorbed on sediment particles that settle to the bottoms of aquatic environments. In contrast, natural nanoclay (zeta potential: -18.7 mV) and Novaclay™ (zeta potential: -21.4 mV) have higher stability in solution, making them more bioavailable and potentially dangerous to highly mobile species such as daphnids and mosquito fish. Although, both natural nanoclay and Novaclay™ affected similar aquatic species, Novaclay™ seems to have higher toxic effects than natural nanoclay at lower concentrations.

This study took an important first step in assessing and comparing how a natural nanoclay and two modified nanoclays affect aquatic species. Indeed, our results can help regulatory agencies to make decisions regarding risk assessment of modified nanoclays, because we were able to identify that, in general, modified nanoclays have increased toxicity relative to natural nanoclays on aquatic organisms. However, some key issues still need particular consideration in testing the toxicity of nanoclays. Those include:

1. Identify the concentration and toxicity of materials added to the modified nanoclays (e.g., quaternary ammonium compounds (MT2EtOH), and stearic acid of calcium (AMS-32^{TM})) in solution, since they may become dissociated, inducing additional toxic effects on aquatic species. Modified nanoclays are synthetized with the addition of either MT2EtOH (methyl, tallow, bis-2-hydroxyethyl, quaternary ammonium chloride) (Cloisite®30B) or stearic acid of calcium (AMS-32TM) within the interfacial lamella of the nanoclays (Novaclay^{TM}), which favor their application in different areas. However, there is lack of knowledge about how those particular modifiers dissociate from clay minerals, causing isolated toxic effects on aquatic species. With one exception (Sharma et al. 2010), there is no study quantifying and investigating the dose-response relationship of materials added to some kinds of modified nanoclays.

2. Evaluate the mechanisms through which nanoclays can induce the formation of reactive oxygen species (ROS), causing oxidative stress in aquatic species. Although, previous studies have already suggested that the interaction between nanomaterials and cellular components can cause the generation of reactive oxygen species (ROS), resulting in the subsequent formation of oxidative stress in biological systems, there is still no information about the mechanisms through which the nanomaterials, including the three types of nanoclays in this study, can induce the oxidative stress on aquatic life (Nel et al. 2009; Oukarroum et al. 2012). Thus, further studies

evaluating the molecular mechanisms of nanoclays toxicity are required to better elucidate their mode of action on humans and aquatic species.

3. Study different types of natural nanoclays and modified nanoclays as well as different aquatic species. Though this study showed that natural nanoclay and modified nanoclays adversely affected different aquatic species, we only assessed the potential toxicity of a particular natural nanoclay (Na^+ montmorillonite) and two modified nanoclays (Cloisite® 30B and Novaclay^{TM}). In fact, there is a need in investigating the potential toxic effects of other types of naturally occurring nanoclays and modified nanoclays, because we need to understand if different forms of nanoclays similarly affect aquatic life. Furthermore, our study only indicated differences in sensitivity of four sentinel aquatic species (*D. magna = G. holbrooki > C. reinhardtii > C. dilutus*) to nanoclay exposure, making it necessary to investigate if natural and modified nanoclays also induce negative effects on others pelagic and benthic species.

4. Characterize the physicochemical properties of nanoclays in real environmental media. Characterizing the physicochemical properties of nanoclays (e.g., surface charge, shape, state of dispersion, particle size and size distribution) is an important initial step to better understand the potential effects of nanoclays on aquatic ecosystems (Powers et al. 2006). However, it is very important to characterize nanoclays within environmental media found in nature, because potential physicochemical changes can occur in solution (e.g., surface chemistry, and agglomeration state) due to the interaction between nanoclays and a range of molecules naturally present in surface waters and sediments (Bernhardt et al. 2010). Indeed, different water chemistries and the presence of organic matter likely influence the surface coating and agglomeration state of nanoclays, affecting their fate, bioavailability, and toxicity (Christian et al. 2008). Thus, we suggest that future investigations consider performing the characterization of

nanoclays as well as the assessment of organismal responses in environmental media, which may provide more realistic scenarios of nanoclay exposures.

5. Investigate the potential trophic transfer of natural nanoclay and modified nanoclays in aquatic food webs. While modified nanoclays will soon deposited in aquatic ecosystems, there is no information about the propensity of modified nanoclays to be transfer through trophic transfer relative to natural nanoclays already present in the environment. Though the assessment of the trophic transfer of nanoclays in aquatic food webs can be the most important mechanism of transport and fate of nanoclays, only few studies have addressed this issue (Holbrook et al. 2008; Zhu et al. 2010). The aquatic species selected in this study are well suited to assess the trophic transfer of different types of nanoclays, since they either belong to different trophic levels or differ in their microhabitat usage (i.e. water column versus sediment).

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Table 3. *NOAEL* (No Observed Adverse Effect Level) and *LOAEL* (Low Observed Adverse Effect Level) for four sentinel aquatic species (*C. reinhardtii, D. magna, C. dilutus, and G. holbrooki*) when exposed to a natural nanoclay (Na^+ montmorillonite) and two manufactured nanoclays (Cloisite® 30B and Novaclay™). (–) indicates that the nanoclay had no adverse effect on the particular organism for any of the concentrations considered in this study.

	C. reinhardtii		*D. magna*		*C. dilutus*		*G. holbrooki*	
	NOAEL	**LOAEL**	**NOAEL**	**LOAEL**	**NOAEL**	**LOAEL**	**NOAEL**	**LOAEL**
Natural nanoclay	-	-	10 mg/L	100 mg/L	-	-	-	0.01 mg/L
Cloisite® 30B	1 mg/L	10 mg/L	0.1 mg/L	1 mg/L	10 mg/L	100 mg/L	-	0.01 mg/L
Novaclay™	-	-	0.1 mg/L	1 mg/L	-	-	-	0.01 mg/L

APPENDIX A: ANIMAL USE PROTOCOL (AUP)

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

August 2, 2016

David Chalcraft, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. Chalcraft:

Your Animal Use Protocol entitled, "Does Nanoclay Technology Adversely Affect Freshwater Food Webs Relative to Natural Nanoclays?" (AUP #D342) was reviewed by this institution's Animal Care and Use Committee on August 2, 2016. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Eddie Johnson/jd

Eddie Johnson
Vice-Chair, Animal Care and Use Committee

EJ/jd

Enclosure