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Silver nanoparticles are lethal to the ciliate model *Tetrahymena* and safe to the pike silverside *Chirostoma estor*

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ABSTRACT

Ciliate ectoparasites are one of the most important groups of pathogens in fish culture, and the traditional treatments are sometimes harmful to the fish and the environment. Thus, the search for novel compounds that are effective at low concentrations and safe for fish are necessary to optimise treatments in aquaculture. The antiprotozoal capacity of silver nanoparticles (AgNPs) against the ciliate *Tetrahymena* has been documented; however, their toxicity may vary with the synthesis methodology and nanoparticle size. The objectives of this study were a) to evaluate the acute toxicity *in vitro* of two AgNPs (Argovit™ and UTSA) on *Tetrahymena* sp., a biological model for ciliated ectoparasites of fish and b) to test the safety of lethal and higher doses of UTSA AgNPs for ciliates on the fish *C. estor*. Light microscopy and scanning electron microscopy (SEM) were used to determine whether AgNPs affected the structure of the cell surface of *Tetrahymena*. The mortality, histopathological alterations and metagenomics of the fish were used to determine the major effects of UTSA AgNPs. In *Tetrahymena*, the median lethal concentration (LC_{50}) for Argovit™ was 2501 ± 1717 ng/L at 15 min and 796 ± 510 ng/L at 60 min, while the LC_{50} for UTSA AgNPs was 4 ± 2 and 1 ± 0.6 ng/L at 15 min and 60 min, respectively. A concentration of 3300 ng/L Argovit™ and 10.6 ng/L UTSA AgNPs for 15 and 60 min, respectively, was 100% effective against *Tetrahymena*. After 60 min of exposure to 0.25 and 0.50 ng/L UTSA AgNPs, the number of cilia significantly reduced, there were small holes on the cell surface, and the cellular membrane was ruptured. In fish exposed to lethal (10.6 ng/L) and higher (31.8 and 95.4 ng/L) doses of UTSA, the AgNPs did not affect fish survival after 96 h, and there were no signs of histopathological damage or gut microbial changes. This study is the first report on microscopic and ultrastructural changes in *Tetrahymena* after exposure to significantly low concentrations of UTSA AgNPs with antiprotozoal efficacy without evidence of harmful effects on fish. These results provide the basis for further studies of both pet aquarium and commercial fish that may validate these findings at a larger experimental scale, taking into account AgNPs bioaccumulation, safety for human consumption and environmental impact.

1. Introduction

Protozoan parasites are one of the most important groups of pathogens negatively influencing the welfare of both cultured and wild fish (Abdel-Baki and Al-Quraishy, 2014). Among these protozoans, the ciliates *Chilodonella hexasticha*, *C. piscicola*, *Tetrahymena pyriformis*, *T. corlissi* and *Ichthyophthirius multifiliis* are common parasites causing severe mortality and important economic losses in both ornamental fish and freshwater aquaculture worldwide (Bruno et al., 2006; Picon-Camacho et al., 2012; Shinn et al., 2015). *Ichthyophthirius multifiliis* is the most pathogenic protozoan parasite of freshwater fish and is a major problem in aquaculture (Xu et al., 2016); thus, removing the

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small migratory infective stage of this parasite is essential to break the cycle of infection.

Chemical treatments remain the principal method for controlling protozoan infections in aquaculture, despite numerous attempts to develop and implement physical and farm management-based alternatives (Picon-Camacho et al., 2012). However, the use of chemicals to control protozoan parasites can sometimes be difficult to administer, costly, not completely efficient and even environmentally hazardous (Tieman and Goodwin, 2001). For these reasons, the search for new effective compounds is a necessity to optimise antiparasitic treatments in aquaculture to minimise the amount of drug released in the environment and to ensure best performance.

Nanotechnology is a new discipline that promises the development of new "smart" target-specific drugs, with a broad spectrum and without developing multi-resistance (Prakash et al., 2013). Silver nanoparticles (AgNPs) are a main commercialised nanomaterial (Valerio et al., 2017) since they possess unique properties, such as their large surface area and great capacity to produce reactive oxygen species (ROS), which allow them to have effective antimicrobial properties (Elechiguerra et al., 2005; Gherbawy et al., 2013; Juganson et al., 2017). Protists have highly developed systems for the internalisation of microscale and nanoscale particle sizes and thus are good models of organisms for nanotoxicology (Holbrook et al., 2008). The antiprotozoal capacity of AgNPs against the flagellates *Leishmania tropica* (Allahverdiyev et al., 2011) and *L. amazonensis* (Bergmann et al., 2012), and the ciliates *Tetrahymena pyriformis* (Shi et al., 2012), *Tetrahymena thermophila* (Juganson et al., 2017), and trophonts and free-living stages of *I. multifiliis* have been documented (Kiruba et al., 2016; Saleh et al., 2017). However, the toxicity of AgNPs may vary with the size, structure and composition of engineered nanoparticles (Griffitt et al., 2008). The precise mechanism by which silver nanoparticles exert their action in protists is unknown but likely involves rupture of the outer membrane, as reported in *Cryptosporidium parvum* and *I. multifiliis* (Cameron et al., 2016; Saleh et al., 2017). Lara et al. (2015) showed ultrastructural differences under SEM in the fungus *Candida albicans* after being subjected to silver nanoparticle treatment, indicating damage to the outer cell wall.

Pike silverside (*Chirostoma estor)* is an interesting emerging aquaculture fish species and freshwater model organism (Martínez-Chávez et al., 2018), which would greatly benefit from novel strategies to control ectoparasitic ciliates that have been known to cause high mortalities in culture (Martínez-Chávez, pers. comm.). Importantly, *C. estor* is an agastric species with a short intestine (Ross et al., 2006) that could be sensitive to AgNPs exposure.

The objective of this study was two-fold: a) to evaluate the acute *in vitro* toxic effects of two AgNPs (Argovit™ and UTSA) on *Tetrahymena* sp. as a biological model for ciliated ectoparasites of fish and b) to test the safety of lethal and higher doses of UTSA AgNPs for ciliates on the fish *C. estor*.

2. Materials and methods

2.1. Characteristics of AgNPs and solutions

Two different types of AgNPs were evaluated.

2.1.1. Argovit™ is an AgNP chemically synthetised solution (Juárez-Moreno et al., 2017) and was donated by Professor Dr. Vasily Burmistrov from the Scientific and Production Center Vector-Vita (Russia). Argovit™ is a preparation of highly dispersed AgNP silver nanoparticles with an overall concentration of 200 mg/mL (20%) of Polyvinylpyrrolidone (PVP)-coated AgNPs in water. The content of metallic silver in the Argovit™ preparation is 12 mg/mL, and the dispersion was stabilised with 188 mg/mL of PVP.

The particle size is 35 ± 15 nm, the shape is spherical, and the hydrodynamic diameter of metallic silver with PVP is 70 nm. The zeta potential (mV) is −15; the surface plasmon resonance was observed at 420 nm; and the structure of the PVP was confirmed by Fourier transform infrared spectroscopy. The AgNP dilutions were calculated according to metallic silver content in the Argovit™ preparation. The solutions were prepared in deionized water and kept at 4 °C in darkness. The Argovit™ AgNPs have multiple certificates for their usage in veterinary and human applications (Borrego et al., 2015).

2.1.2. The UTSA AgNPs were donated by Dr. Miguel José Yacamán from the University of Texas at San Antonio. These AgNPs were prepared in the Department of Physics and Astronomy, San Antonio, Texas University, by microwave-assisted techniques that have the advantage of achieving fast heating and reaction completion to produce large-scale metal nanoparticles.

The particle size is 1–3 nm, the shape is spherical, and the zeta potential (mV) is −2.9 to 13.4 (Romero- Urbina et al., 2015). The solutions were prepared in deionized water and kept at 4 °C in darkness. The content of metallic silver in the UTSA preparation is 0.032 mg/mL (Lara et al., 2015).

2.2. Ciliate culture

An axenic culture of free-living ciliates was obtained from a water-based organic culture with *Lactuca sativa* after 5 days at 20 °C. The ciliates were maintained in Elbp culture medium at 30 °C according to Arany et al. (2014). These ciliates were routinely observed and photographed using an inverted microscope (Zeiss AX10 Vert. A1). For the experiments, the culture in logarithmic phase was used, and the cells were counted under an Olympus BX3 microscope with a haemocytometer after they were immobilised with one drop of Bouin's solution (Miyoshi et al., 2003).

The morphology of the ciliates was first identified *in vivo*, and the ciliates were later immobilised. They were observed using a high-power oil immersion objective and interference contrast. The ciliates were fixed and stained by the wet Chatton-Lwoff silver nitrate technique (Foissner, 1992) and Klein's dry silver impregnation method to identify their genus according to Lom and Dykova (1992).

2.3. Preparation of AgNPs concentrations and controls

The concentrations of the Argovit™ and UTSA AgNPs were calculated based on the metallic silver content. The solutions were prepared in deionized water immediately before being used and kept in the dark at 4 °C, following the methodology of Juárez -Moreno et al. (2017).

For the experiments, fresh stock solutions (Argovit™: 2,000 μg Ag/ml; UTSA: 269.67 μg Ag/ml) were prepared in deionized water.

Sodium chloride 30 g/L and formalin 1 ml/L (FDA, 2018), and AgNO3 0.4 ml/L solutions were used as positive controls and prepared using deionized water. All chemicals used in the study were from J.T. Baker ® analytical grade.

2.4. In vitro toxicity test of AgNPs against Tetrahymena sp.

For the *in vitro* tests, the 96-well microplate counting method was used. A volume of 100 μL of different concentrations of AgNPs was placed in each well of the microplate (Xu et al., 2016). Subsequently, 20 ciliates were added to each well (in triplicate) containing different concentrations of nanoparticles and controls (Abe et al., 2014 modified). Six Argovit™ AgNPs concentrations (300, 900, 1500, 2100, 2700, and 3300 ng/L) were selected to determine the 1-h LC_{50}. For the UTSA AgNPs, five concentrations (1.0, 3.4, 5.8, 8.2 and 10.6 ng/L) were selected to determine the 1-h LC_{50}. For both AgNPs types, positive controls of formalin and sodium chloride were used according to the FDA (2018). To compare the toxicity of AgNPs (Argovit ™ and UTSA) against ionic silver in the ciliated, a solution of silver nitrate (Baker ® analytical grade) was used as a reference for representing the most toxic form of silver at a concentration of 0.4 mg/L (Kvitek et al., 2009). The cumulative mortality of the protozoan was recorded every 15 min post-

exposure for 1 h using a Zeiss (model AX 10) inverted microscope. The criterion of cell death was the absence of ciliary movement (Xu et al., 2016).

2.5. Effect of AgNPs against Tetrahymena under light microscopy and scanning electron microscopy (SEM)

To observe microscopic changes in trophozoite morphology, *Tetrahymena* sp. (n = 20) was exposed to 300 and 3300 ng/L of Argovit™ and 1 and 10.6 ng/L UTSA AgNPs for 60 min. The control in deionized water and the treated trophozoites of *Tetrahymena* sp. were observed every 15 min under the Zeiss inverted microscope (model AX).

To document ultrastructural changes in *Tetrahymena* sp., the trophozoites (n = 200) were exposed for 60 min to the UTSA AgNPs (0.25 and 0.5 ng/L). The control and treated trophozoites of *Tetrahymena* were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) for 24 h at 4 °C, followed by secondary fixation in 1% osmium tetroxide (OsO_4) in the same buffer for 1 h at 4 °C. The samples were then dehydrated through a graded ethanol series and critical-point dried with carbon dioxide. The specimens were mounted on metal stubs, coated with gold, and examined in a Hitachi Stereoscan Model SU1510 SEM (Hitachi Ltd., Tokyo, Japan).

2.6. In vivo safety test of UTSA AgNPs in Chirostoma estor

Pike silverside (*Chirostoma estor)* juveniles were obtained from the Laboratory of Aquaculture and Aquaculture Biotechnology of the Institute of Agricultural and Forestry Research (IIAF), Michoacán University. A total of 248 three-month-old (3.4–4.4 cm post-hatch length) pike silverside were acclimated in an experimental tank and were observed over 7 days to document any mortalities. Then, 15 individuals were randomly selected and placed in each of 16 9 L containers at 21 °C. The water was changed every 24 h in a static experimental system. The fish were fed flakes to satiety at a rate of 8 feedings a day.

Due to the commercial availability of UTSA and their *in vitro* efficacy vs that of Argovit™, only UTSA AgNP safety was tested in the fish. Treatment concentrations used in pike silverside were the ones causing 100% mortality in *Tetrahymena* 10.6 ng/L, and 3 and 6 times this value being: 31.8 and 95.4 ng/L. Additional freshwater was used as a negative control. Freshly made UTSA AgNPs concentrations were added daily to the water of each treatment for 4 days of exposure, ensuring a constant concentration (Afifi et al., 2016). The experimental period was divided into three phases: phase 1, the acclimation period of 7 days (A1-A7); phase 2, the exposure period of four days (D1-D4); and phase 3, a 6-day follow-up without treatment (D5-D10). Mortality, behaviour change (*i.e.* isolated fish, erratic swimming and/or fish not feeding) and any possible clinical observations were assessed during the 8 times that fish were daily fed as well as during the change of fresh water with AgNPs added.

The mortality rate of the fish was registered daily throughout the experiment.

2.7. Histology analysis

At the beginning of the trial, 8 *C. estor* juveniles were sampled at random for histological analysis to assess their initial health status. During the experiment, 8 specimens from each treatment and controls were collected on day D4. In the sampling of day D10, the remaining fish were collected at the end of the experiment: control (n = 11) and those exposed to UTSA AgNPs concentrations (ng/L) of 10.6 (n = 13), 31.8 (n = 12) and 95.4 (n = 12). An incision was made in the abdomen of each specimen for fixation. Davidson's solution for fish was used as a fixative, and at 24 h post-fixation, the fish were transferred to 70% ethanol until further processing. The whole fish (except the tail) was processed for histological analysis following the methods of Lee and Luna (1968), Drury and Wallington (1980), and Kamonporn et al. (1999): dehydration in an automatic processor (Histokinette) and embedding in paraffin. Afterwards, 5-μm transverse sections were obtained and stained with Harris haematoxylin (Sigma) and eosin (Sigma-Aldrich) (H&E) to determine if there was cytotoxic damage on gills, skin and digestive tract because these tissues and organs were directly exposed to water containing the three treatments. For morphological examination, we used an Olympus BX50 compound microscope, and photographs were obtained with a QImagine digital camera.

2.8. Gut microbiota analysis

Six fish intestines per treatment (negative control and highest UTSA AgNPs concentration treatment 95.4 ng/L) were sampled for microbiome analysis on days 0, 4 and 10. For this, each individual was sedated and rendered insentient with ice water and culled by decapitation. Then, a precise ventral incision was done to access the abdominal cavity and excise the intestine by cutting both ends of the intestine which was immediately placed on individual vials containing 96% ethanol and placed at 4 °C until extraction.

Metagenomic DNA of fish intestines was extracted with a standard CTAB extraction protocol (Doyle and Doyle, 1987), and the V3 variable region of the 16S rRNA gene was PCR-amplified with the primer pair V3-338f and V3-533r and sequenced in an Illumina Miniseq at CIAD-Mazatlán. Samples that did not pass the quality standards were discarded. The reads were assembled with Flash v.1.2.7 software (Magoc and Salzberg, 2011), and VSEARCH (Rognes et al., 2016) was used for further processing and finally obtaining an abundance matrix. The matrix was normalised using the metagenomeSeq method (Mc Murdie and Holmes, 2014).

2.9. Statistical analyses

Significant (α0.05) differences in *Tetrahymena* mortality between the treatments were detected with ANOVA and an a posteriori Newman–Keuls test in Statistica version 7. The mean lethal concentration (LC_{50}) of the AgNPs and their confidence intervals were calculated using the Probit model (version 15.0) of the SPSS® program. For metagenomic data, a non-parametric multidimensional scaling plot (NMDS) was generated with vegan R library v2.4-6 (Dixon, 2003), and statistical analysis with the ANOSIM function from the vegan R package was used to evaluate groups using the Bray-Curtis distance and 999 permutations.

3. Results

3.1. Ciliate morphology

The ciliates cultivated were pyriform and measured 30.44 ± 0.41 × 13.87 ± 0.37 μm in size. The buccal cavity is anteroventrally located on the left side. The oral ciliary apparatus is distinct from the somatic ciliature and consists of a paroral membrane and three membranelles localised on the left side of the buccal cavity. The cytostome at the bottom of the buccal cavity opens into an inconspicuous cytopharynx. The ciliates had 13 kineties running meridionally from the posterior pole of the body to the buccal cavity and the pre-oral suture. Two post-oral kineties ended at the posterior margin of the buccal cavity. A spherical macronucleus of approximately 11.29 ± 3.52 × 9.62 ± 2.48 μm near of the centre of the cell. A single spherical micronucleus, about 3 μm in diameter, slightly posterior to the macronucleus.

3.2. In vitro test of Argovit™ and UTSA AgNPs

The percentage of cumulative mortality of *Tetrahymena* trophozoite post-exposure to Argovit™ (0–3300 ng/L) and UTSA (0–10.6 ng/L)

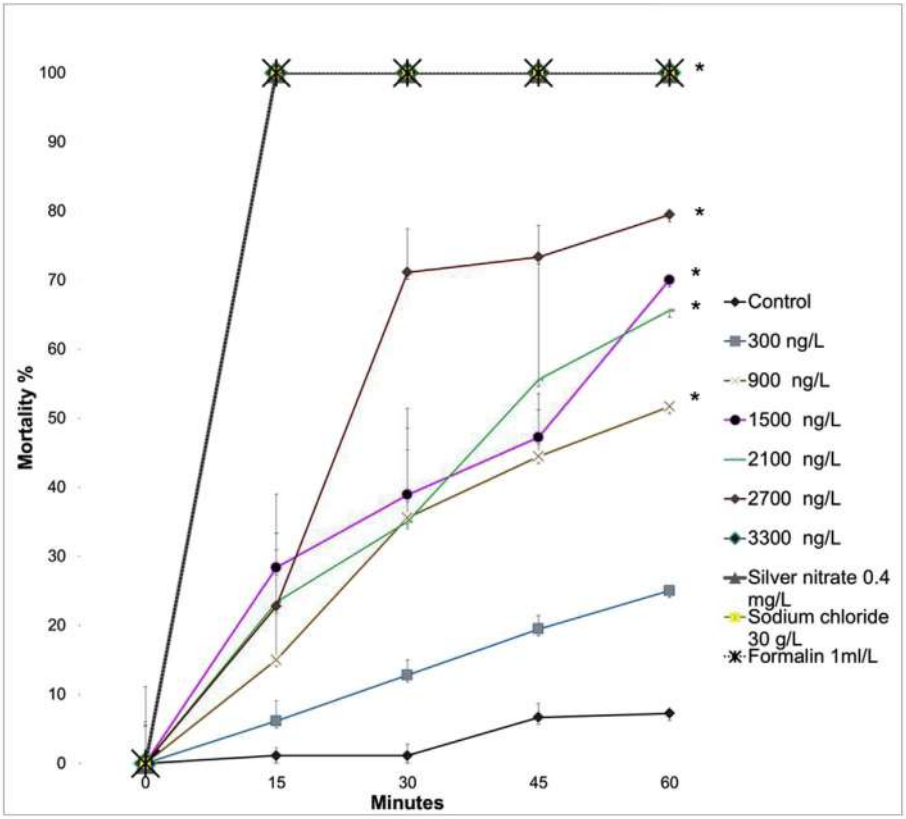

Fig. 1. Cumulative mortality of *Tetrahymena* sp. exposed to different concentrations of Argovit™ AgNPs. Error bars indicate SE. * indicate significant difference against the control at 60min.

AgNPs was measured every 15 min for 1 h and is shown in Fig. 1 and Fig. 2. Both AgNPs had a concentration-dependent effect.

In Fig. 1 shows three groups, Argovit™ AgNPs concentrations (0 and 300 ng/L) that did not cause 50% mortality, concentrations (900–2100 ng/L) that caused 50–70% mortality, and concentrations (2400–3300 ng/L) that caused mortalities of more than 70–100%.

The negative control, Argovit™ AgNPs, showed a mortality of 7% at 60 min. The positive controls silver nitrate, formalin and sodium chloride caused 100% mortality at 15 min.

In Fig. 2 shows three groups, UTSA AgNPs concentrations (0 and 1 ng/L) that did not cause 70% mortality, concentrations (3.4 and 5.8 ng/L) that caused 70–90% mortality, and concentrations (8.2–10.6 ng/L) that caused mortalities of more than 90–100%. The negative control, UTSA AgNPs, showed a mortality of 9% at 60 min. The positive controls silver nitrate, formalin and sodium chloride caused 100% mortality at 15 min.

For both the Argovit™ and UTSA AgNPs, the percentage of mortality associated with the concentrations tested was significantly different ($P < 0.05$) at 60 min of exposure in relation to that of the control. Argovit™ at 3300 ng/L and UTSA at 10.6 ng/L showed 100% mortality in *Tetrahymena* trophozoites at 15 and 60 min, respectively (Figs. 1 and 2).

Table 1 shows the mean lethal concentration (LC_{50}) and confidence intervals (95%) for each of the concentrations of Argovit™ and UTSA AgNPs in *Tetrahymena* trophozoites during the 1 h treatment. The mean lethal concentration for both nanoparticles decreased as the exposure time increased and indicated that the UTSA AgNPs ($LC_{50} = 4 \pm 2$ and 1 ± 0.6 ng/L at 15 min and 60 min, respectively) were more effective against *Tetrahymena* than were the Argovit™ AgNPs ($LC_{50} = 2501 \pm 1717$ and 796 ± 510 ng/L at 15 min and 60 min, respectively).

3.3. Microscopical and ultrastructural changes of AgNPs in Tetrahymena sp.

The light microscopy data revealed that compared to the control (Fig. 3a) *Tetrahymena* treated for an hour with Argovit™ and UTSA AgNPs underwent important morphological changes at the minimum concentration of Argovit™ (300 ng/L), the ciliate only changed their oval morphology to circular (Fig. 3b); while the highest concentration (3300 ng/L) caused the plasma membrane to rupture and the cellular content to leak (Fig. 3c). The lowest concentration of UTSA AgNPs (1 ng/L) caused a slight rupture of the cellular membrane of *Tetrahymena* (Fig. 3d), while the maximum concentration (10.6 ng/L) showed plasma membrane rupture and nucleus fragmentation (apoptosis) (Fig. 3 e).

The SEM visualisation of *Tetrahymena* exposed to UTSA AgNPs revealed serious damage after 60 min of treatment. Without the UTSA AgNPs, cilia covered the entirety of the control *Tetrahymena* cells, and the kinetids were running regularly (Fig. 4a). However, after 60 min of treatment with 0.25 and 0.50 ng/L UTSA AgNPs, a significant reduction in the number of cilia, damaged kinetids, swelling areas and small holes on the surface of the cells was observed (Fig. 4 b, c and d).

3.4. Safety of UTSA AgNPs in Chirostoma estor: survival, histopathological and gut microbiome alterations

All *C. estor* treated with UTSA AgNPs survived without showing clinical signals of toxicity or evident abnormal behaviour, including feeding. Histological analysis did not show any relevant pathological alterations in fish from the control or under treatment, except for light or medium hydropic degeneration of epithelial and chloride cells of the secondary lamellae (Fig. 5 a, b). Skin and digestive tract did not show any pathological alterations neither in the control and those exposed to UTSA AgNP concentrations (ng/L) of 10.6, 31.8 or 95.4 of the samples

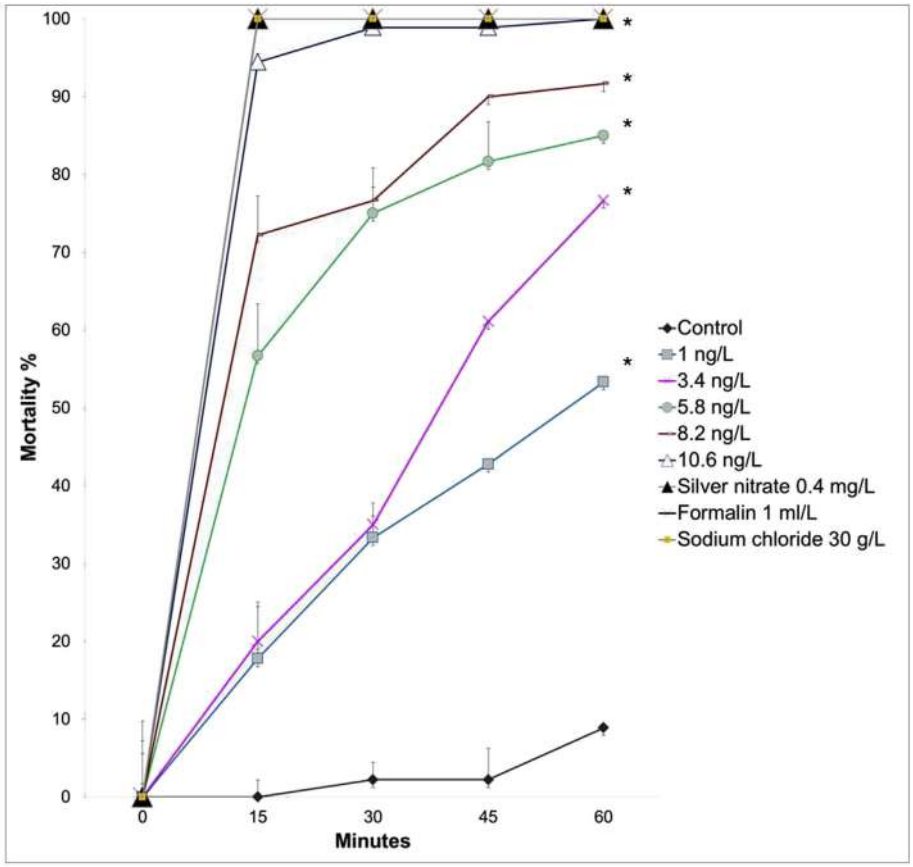

Fig. 2. Cumulative mortality of *Tetrahymena* sp. exposed to different concentrations of UTSA AgNPs. Error bars indicate SE. * indicate significant difference against the control at 60min.

Table 1
Mean lethal concentration (LC_{50}) and confidence intervals (95%) for each of the concentrations of Argovit™ and UTSA AgNPs in *Tetrahymena* sp. for 60 min of treatment.

Argovit™ AgNPs		95% Confidence Intervals	
Time (min)	**LC_{50} (ng/L)**	Lower limit	Upper limit
15	2501	1717	6920
30	1554	1007	2337
45	1160	791	1527
60	796	510	1047
UTSA AgNPs			
15	4	2	6
30	3	1	4
45	2	0.50	3
60	1	0.6	2

from day eight and day ten.

With respect to the gut microbiota, we observed no effects. The cumulative microbiota was evaluated for each sampled intestine, and no differences were shown between control and UTSA treatment (95.4 ng/L) after ANOSIM analysis (R = 0.037, significance = 0.205). NMDS also shows how the grouping of each treatment is undifferentiated (Fig. 6).

4. Discussion

The use of *Tetrahymena thermophila* has been an important model system for biological research for many years (Cassidy, 2012). However, Xu et al. (2016) reported that *Tetrahymena thermophila*, *T. pyriformis* and *Tetrahymena* sp. respond similarly to tested parasiticides in terms of lethal concentration. Thus, in this study, we evaluated the toxicity of two different AgNPs using cultivated ciliates identified as *Tetrahymena*.

The cytotoxicity of AgNPs tested caused an abnormal swimming behaviour in *Tetrahymena*, but also a change in cell morphology, and ultimately cell death. Both the Argovit™ and UTSA AgNPs showed dose-dependent antiprotozoal activity (as the concentration increased, the toxicity increased), coinciding with the response of *T. pyriformis* to other types of AgNPs (Shi et al., 2012). However, the concentrations of Argovit™ (3300 ng/L) and UTSA (10.6 ng/L) AgNPs, which caused 100% of mortality in *Tetrahymena,* were much lower than the concentrations of other types of AgNPs tested on *Tetrahymena pyriformis* (2 913 000 ng/L) previously mentioned by the authors for *Paramecium caudatum* (30 000 000 and 50 000 000 ng/L), which was reported by Abe et al. (2014), and for the theronts and tomonts of *Ichthyophthirius multifiliis* (10 000 ng/L) (Saleh et al., 2017). One or multiple physical, chemical and biological known factors could explain these important differences. For example, the toxicity studies by the aforementioned authors were carried out on specific culture medium and not deionized water as was the case in this study. This is relevant as deionized water has shown to be a more suitable exposure medium in comparable assays with similar results in the degree of magnitude found with the current study which are possibly due to the fact that potential interactions between culture media and AgNPs are avoided (Juganson et al., 2013; Suppi et al., 2015; Lee et al., 2018). In fact, deionized water could have facilitated the dissolution of Ag^+ ions decreasing the LC_{50} of AgNPs by two orders of magnitude less than in the *Tetrahymena thermophila* tests performed in the culture medium (JemecKahruPotthoff et al., 2016; Juganson et al., 2017). A biological aspect to be considered is the differential toxic sensitiveness of ciliate strains as has been previously reported in *Tetrahymena* (Juganson et al., 2017) and which could me more relevant in our study by the use of wild *Tetrahymena* strains,

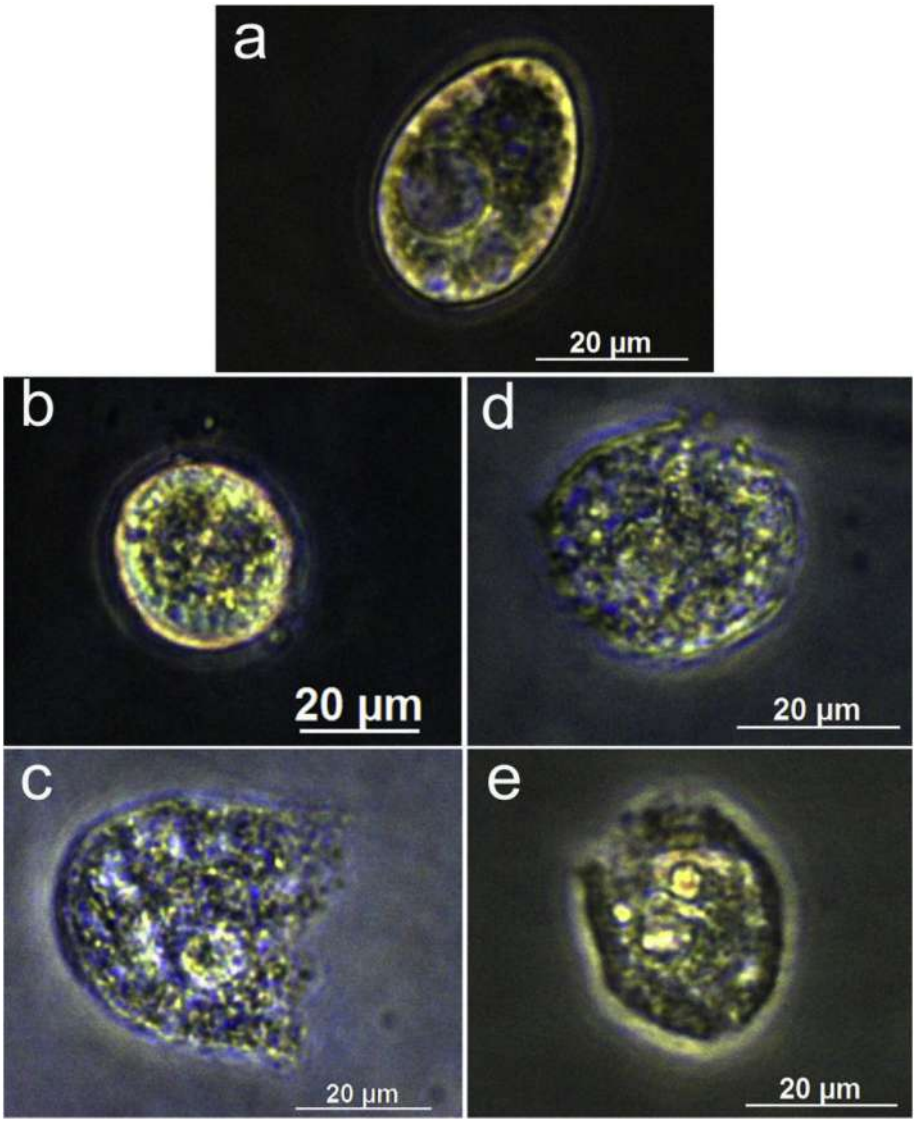

Fig. 3. Microscopic observations of Argovit™ and UTSA AgNPs treatments on *Tetrahymena* sp. after 60 min exposure. a) Control (deionized water), b) 300 ng/L Argovit™: showing changed their oval morphology to circular, c) 3300 ng/L Argovit™: rupture of plasma membrane and leakage of cellular content, d) 1 ng/L UTSA: a little rupture of cellular membrane of *Tetrahymena*, d) 10.6 ng/L UTSA: plasma membrane rupture and nucleus fragmented.

however this should be further pursued as the identification level of *Tetrahymena* in this study was done only at genus level.

Importantly, the concentration of *Tetrahymena* cells used in this study (≥100 cell/mL) allowed to accurately observe changes under the microscope caused by AgNPs while recording mortality every 15 min. These concentrations have previously shown to cause infection and mortality in some freshwater fish species (Ponpornpisit et al., 2000; Leibowitz et al., 2009; Sharon et al., 2015). All the above described strategies were aimed to provide some degree of external validity to this study and may explain in part the observed augmented toxicity of both AgNPs tested.

The comparison of the toxicity of 3300 ng/L Argovit™ and 10.6 ng/L UTSA AgNPs with 0.4 mg/L $AgNO_3$ used as an ionic silver control on *Tetrahymena* showed comparable effects, which could indicate a similar mode of action for these compounds, which is in accordance with previous findings suggesting that Ag^+ are important in the toxicity of AgNPs against microbes and protozoans (Duran et al., 2016; Cameron et al., 2016; Hedayati et al., 2019). Both AgNPs evaluated in this study against *Tetrahymena,* exhibited the lower LC_{50} reported for protozoans (10–100 mg Ag/L of AgNPs and 1–10 mg Ag/L of Ag salt (Bondarenko et al., 2013), suggesting that the ions of Ag + released were dissolved in the aqueous medium becoming very toxic to the organism tested although other interactions such as the ratio of Ag+/cells compared to other studies could also account for some of the differences in toxicity magnitude.

However, this difference is also, likely due to the different nanoparticle sizes, as has been previously reported (Shi et al., 2012, 2013; Ivask et al., 2014). The UTSA AgNP size was 1–3 nm, and that of the Argovit™ AgNP was 35 nm. A negative correlation was found between nanoparticle size and toxicity: small nanoparticles (5–10 nm) are more toxic to *Tetrahymena pyriformis* than are large nanoparticles (15–25 nm) (Shi et al., 2013). Similarly, the LC_{50} at 60 min of *Tetrahymena* sp. to Argovit™ and UTSA AgNPs were 796 and 1 ng/L, respectively, values much lower than those (39 000 000 ng/L) reported by Kvitek et al. (2009) for *Paramecium caudatum*.

Another factor influencing the efficacy of UTSA AgNPs against *Tetrahymena* when compared to that of Argovit™ is the method of the AgNPs synthesis. Argovit™ AgNPs are obtained by chemical methods and are covered with low-molecular-weight PVP to avoid agglomeration and increase stability. These AgNPs have shown better performance in several biological assays than that of non-capped AgNPs or AgNPs capped with other agents (Borrego et al., 2015; Durán et al.,

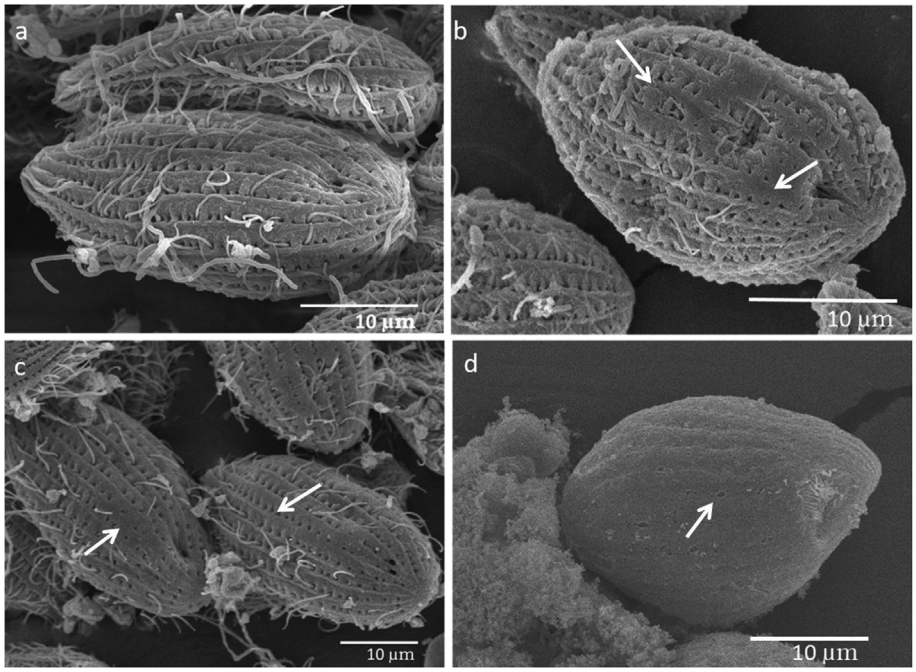

Fig. 4. Scanning electron micrographs of *Tetrahymena* sp. trophozoites after 60 min exposure to UTSA AgNPs. a: control without AgNPs, b: 0.25 ng/L: significant reduction in the number of cilia and kinetids damaged, c: 0.50 ng/L: swelling and d: small holes on surface of the cells.

2016). However, microwave technique used to produce UTSA AgNPs is a good alternate methodology to synthesize pure metallic nanoparticles, as efficient control of nanoparticle size distribution is obtained, and handling of potential contaminants or cytotoxic reducing agents is avoided. This technique reduces the formation of agglomerates in nanomaterial with narrow size distributions (Gutiérrez et al., 2012; Zhu and Chen, 2014; Lara et al., 2015). Although the precise mechanisms of nanoparticle toxicity are largely unknown (Griffitt et al., 2008), factors such as the technology by which nanoparticles are prepared, the type of stabilising additives used, and the shape and size of the nanoparticles greatly influence the efficiency of silver nanoparticles (Podkopaev et al., 2014). However, is important to remark that UTSA AgNPs have no additives (coating or protective agents) and are positively charged (Bhattarai et al., 2011) making them more soluble and therefore highly active against pathogens (Lara et al., 2015; Romero-Urbina et al., 2015). The latter may ultimately explain the differences between both AgNPs tested *in vitro* against *Tetrahymena* sp in this study.

The application of AgNPs (Argovit™ and UTSA) in *Tetrahymena* damaged and ruptured the cell membrane, resulting in the gradual disappearance of its functionality (Liu et al., 2004). These results coincide with those of Dash et al. (2012), Razack et al. (2016), Lara et al. (2015) and Cameron et al. (2016), who applied AgNPs on algae, the fungus *Candida albicans* and the protozoan *Cryptosporidium parvum*, respectively.

Like other ciliates, *Tetrahymena* cells have a striking variety of highly complex and specialised cell structures. The surface structure of untreated *Tetrahymena* was intact with regular lengthwise ciliary rows and abundant cilia. However, cells exposed to 0.25 and 0.50 ng/L UTSA AgNPs for 60 min showed swollen and disrupted tegument (Fig. 4 b and c). The ciliary rows were damaged, and small holes appeared on the surface of the cells (Fig. 4 d). The damage of the outer membrane by UTSA AgNPs coincides with the effect of the experimental exposure of *Tetrahymena* to 0.232 mg/L metal cadmium (Zhang et al., 2013). To the best of our knowledge, no previous studies had shown evidence of ultrastructural changes in ciliates after AgNPs exposure. However, future research is needed to observe internal damage in the ultrastructure of ciliates and molecular changes leading to their death to account for other non-physical mechanisms leading to their death such as reactive oxygen species as have been reported by Juganson et al. (2017).

Some previous studies have reported mortality in different fish species exposed to AgNPs. Wu and Zhou (2013) mentioned that in medaka (*Oryzias latipes*), in the 96-h acute toxicity test, no death was observed. However, the mortality in the 4.8 mg/L AgNP group was 70% after 6 h of treatment and reached 100% after 12 h of exposure. The

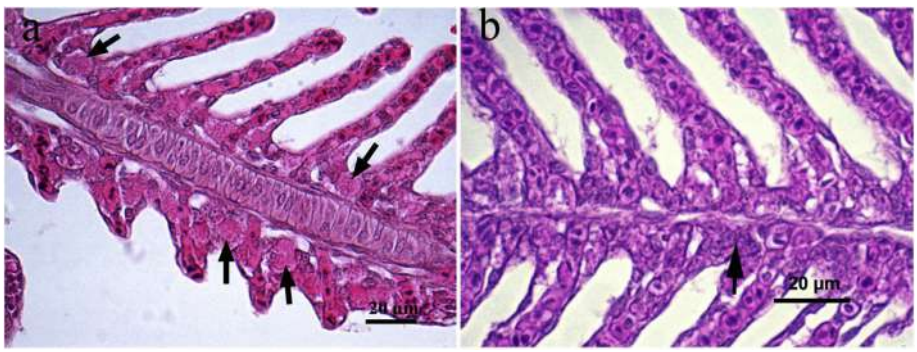

Fig. 5. Transversal cut of the gills of: a) control fish showing chloride cells of secondary lamellae (arrows). b) gills of the fish with the highest doses of AgNp's (95.4 ng/L) also with some chloride cells but no pathological alterations (H & E).

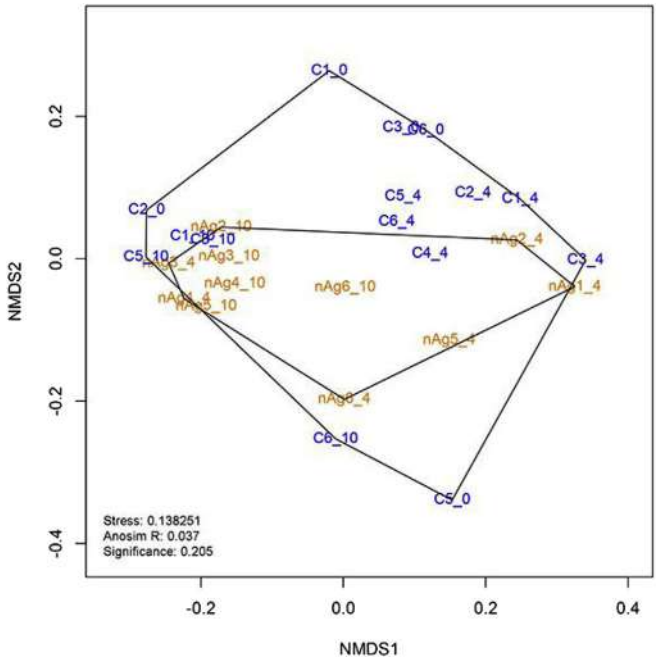

Fig. 6. Microbiota profile analysed in a NMDS plot. Each microbial profile was given a 2D value after the NMDS algorithm. Control sampling is in blue and UTSA treatment in yellow, each are framed by a polygon. Not real separation of groups is observed and stress value shows a good level of representation (i.e. < 0.2). ANOSIM R value also indicates groups are not significantly different (i.e. R < 0.25). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

time and dose are related with increase in mortality. Afifi et al. (2016) showed that as in the control group of tilapia, no mortality was observed in fish exposed to Ag-NPs at a concentration of 5 mg/L. However, 40 mg/L Ag-NP suspension caused 100% mortality with a calculated 96 h LC_{90} of 27.3 ± 3.2 and 28.2 ± 2.9 mg/L to *O. niloticus* and *T. zillii*. In the present study, no mortality was observed in pike silverside *C. estor* at 96 h post-treatment, allegedly because the concentrations used in previous studies are much higher than the concentrations used presently of 0.0000106 mg/L; 0.0000318 mg/L and 0.0000954 mg/L. Future studies analysing chronic exposure should be done to determine if negative effects arise in such case.

Interestingly this high concentration - high mortality rates previously reported in the literature correlate well with histological damage observations in different tissues of several freshwater fish after AgNPs exposure. These included the intestine of tilapia (*Oreochromis niloticus*) (Sarkar et al., 2015); gills of zebra fish (*Danio rerio*) (Mansouri and Johari, 2016) and in rohu (*Labeo rohita*) Khan et al. (2017); in the gills and liver of medaka (*Oryzias latipes*) (Wu and Zhou, 2013) and in the golden fish (*Carassius auratus*) Abarghouei et al. (2016); skin, liver and gills (Ostaszewska et al., 2016) in sturgeon (*Acipenser baerii*); and pathological alterations in gills, intestine and muscle of catfish (*Mystus gulio*) (Abirami et al., 2017). Again, all the previous studies applied AgNps in higher concentration or doses above the concentration used in this study, being the smaller of them 0.0015 mg/L applied in zebra fish. In contrast, no pathological effects were found in pike silverside (*C. estor*) in the current study which is also consistent with the absence of mortality at 96h and the lower concentration of AgNPs used in this trial being at least two orders of magnitude less. As previously stated for *Tetrahymena in vitro,* that the small size and low concentrations (10.6, 31.8 and 95.4 ng/L) of UTSA AgNPs used may explain the lack of detrimental effects observed in *C. estor*. Ivask et al. (2014) suggested that AgNPs 10 nm and smaller seem to interact with cells and become bioavailable by dissolving in the close vicinity, on the outer cell surface or inside of the cells. However, difference in the toxicity threshold (TT) is hardly due to a single factor, such as the particle size and would requires good evidence to be accepted (Akter et al., 2018).

Another important aspect to consider is that in teleosts, the gut, skin and gills are the main mucosal surfaces and immunological barriers (Fröhlich and Roblegg, 2012). The intestinal epithelium contains absorptive cells and mucus-secreting cells (goblet cells), among others (Ferguson, 2006). Georgantzopoulou et al. (2016) evaluated the effects of AgNPs (20–200 nm) on intestinal mucus secretion and found that the mucus layer presents a mechanical barrier mostly towards large particles, reducing their interaction with the cellular membrane and subsequently leading to minor toxicity compared to that of 20 nm Ag particles. In our study, no abnormal mucus secretion was observed in the fish during the whole experimental period. No mucus was observed in the cells, gills, intestine or skin in the histological analysis of the treated fish compared with that of the control fish. Because different AgNPs produce different shapes and particle sizes further research should elucidate the uptake mechanisms of different sized particles and ions, the role of mucus on the transit rate of different sized AgNPs and the mechanism leading to increased inflammatory response (Georgantzopoulou et al., 2016). Also, concentration, coating materials and aggregation are likely to interact differently on the biological system and therefore should be taken into consideration to determine the toxicity of AgNPs. The only pathology observed irrespective of the treatment in our study was the hydropic degeneration of epithelial and chloride cells of secondary lamellae. This phenomenon is commonly observed and poorly understood (Ferguson, 2006) but at least in this case not associated to AgNPs exposure.

As previously stated, the gut is one of the main mucosal surfaces and immunological barriers that also contain a highly dynamic and sensitive microbiota population. Importantly, gut microbiota profiles can reflect the healthy state of a host and microbiota has been implicated in differentiation, morphology, immunity, and nutrient absorption in fish (Merrifield and Rodiles, 2015). Studies suggested that AgNPs could affect digestive microbiota when ingested with food (Merrifield et al., 2013; Fröhlich and Fröhlich , 2016; and recent evidence of AgNPs in water have shown sex dependent effects in fish gut microbiota (Yambo et al., 2018). In this study, we further test the hypothesis that AgNP treatments would modify the gut microbiota in an agastric short intestine model. Our results show no evidence of gut microbiota alterations by UTSA AgNPs as observed in Fig. 6. This phenomenon is possibly related to the low concentrations of AgNPs used but may also be due to the fact that the AgNPs were not delivered directly through food. In addition, in contrast to gut alterations shown in zebrafish (Yambo et al., 2018) our study was evaluated during a shorter period and with a different digestive model which should be taken in consideration when comparing effects across species. Thus, taken together, the results of the present study indicate that UTSA AgNPs administered in water at low concentrations are non-toxic to the pike silverside *C. estor*.

Importantly however, despite the observed potential of AgNPs as control of ciliated ectoparasites in fish, these nanoparticles may still pose a risk to other organisms and the environment (Bondarenko et al., 2013; Zhang et al., 2019). Therefore, further experiments should be directed at elucidating the precise mechanisms of action of these types of AgNPs which at the same time would help evaluate the potential effects on the surrounding environment if future aquaculture applications are to be undertaken.

5. Conclusions

To the best of our knowledge, this study is the first report on microscopic observations of ultrastructural changes in ciliates (*Tetrahymena sp.*) after AgNPs treatments, demonstrating high antiprotozoal efficacy and nontoxicity to pike silverside *C. estor*. UTSA AgNPs have the potential to be a good candidate as a ciliated ectoparasite control, especially in fish not destined for human consumption, such as ornamental fish, where the AgNPs could be readily applied.

Additional research is necessary to evaluate the *in vivo* efficacy of these AgNPs in fish infected by ciliate ectoparasites on a larger experimental scale, taking into account AgNPs bioaccumulation, safety for human consumption and environmental impact.

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