

EVALUATING THE TOXICITY OF SILVER NANOPARTICLES ON THE
BEHAVIOUR AND PHYSIOLOGY OF BRISTLENOSE CATFISH (*ANCISTRUS*
CIRRHOISIS)

BY

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List of Abbreviations:

AChE.....	Acetylcholine Esterase
AgNPs.....	Silver Nanoparticles
ANOVA.....	Analysis of variance
ATP.....	Adenosine triphosphate
DNA.....	Deoxyribonucleic acid
DO.....	Dissolved oxygen
MDA.....	Malondialdehyde
MS 222	Tricaine mesylate
N ₂	Nitrogen
O ₂	Oxygen
PVP.....	Polyvinylpyrrolidone
ROS.....	Reactive oxygen species
SEM.....	Scanning Electron Microscopy
WWTP.....	Wastewater Treatment Plant

1. Abstract:

Silver nanoparticles (AgNPs) are a common material found in many household items, but as these items are thrown out, AgNPs can leach into the aquatic environment, and are currently being found in increasingly higher concentrations in our water supply. In the aquatic environment, AgNPs aggregate and fall to the bottom, affecting benthic creatures. In this study, the Bristlenose Catfish (*Ancistrus Cirrhosis*) was used for their benthic nature and their ability to air breathe using their highly vascularized stomachs. By analyzing air breathing frequency (seen only in times of stress), we can better understand what role AgNPs play in a catfish's ability to tolerate environmental stress. Experimentation was done by isolating catfish for seven days with the absence or presence of AgNPs. On the eighth day, these fish were exposed to one of three environmental conditions: control (optimal conditions), hypoxia (20% O₂ saturation), and high temperature (32°C). These fish then had their movement tracked and quantified over 6 hours. In addition to this, fish were placed in a respirometer to measure oxygen consumption, gills were analyzed via scanning electron microscopy, and gut samples were used to determine malondialdehyde (MDA) concentration. It was found that fish regardless of environmental stressor or AgNP presence total distance moved did not change. However, hypoxic fish exposed to AgNPs were found to increase air breathing frequency. This indicates that any movement the catfish is doing is focused on air breathing, which could increase their exposure to aerial predators. Respirometry analysis showed an increase in oxygen consumption only when AgNPs are present, which is in keeping with behavioral data, since an increase in basal oxygen consumption would lead to a higher air breathing frequency. MDA levels varied between environmental stressors in AgNP exposed fish, but qualitative gill analysis indicated that the gills were not damaged from nanoparticles. Together, these results support our hypotheses that AgNPs do interfere with a catfish's ability to tolerate environmental stress. It is expected that with an increase in concentration or exposure times, these effects would increase proportionally.

2. Introduction:

Silver nanoparticle applications and environmental introduction:

Commonly used for their antimicrobial properties and unique physiochemical properties, silver nanoparticles (AgNPs) are found in a variety of items such as cosmetics, clothing, paint, and more². As the average consumer becomes more wasteful, products containing silver nanoparticles are being thrown away at an alarming rate, ending up in landfills³. Rain hitting these landfills can cause nanoparticles to leach into the water, joining the aquatic ecosystem. Back in the home, cosmetics containing AgNPs are washed away, and ultimately end up in wastewater treatment plants (WWTP)⁴. WWTPs reduce the total amount of nanoparticles that end up in the water through filtration. However, AgNPs do not just disappear, but rather they accumulate in sludge (the residue that accumulates in WWTPs). This sludge is commonly repurposed as fertilizer for farmland as it can boost soil quality, and as such, when rainwater hits the fertilized farmland, AgNPs can be washed away, ultimately ending up back in the water².

Due to their role in cosmetics, short and long-term effects of AgNPs have already been investigated, and it has been shown that AgNPs can damage DNA and cause functional impairment in endothelial cells^{3,5,6}. However, this cytotoxicity can also be used to control or reduce angiogenesis (preventing the spread or progression of cancer)⁷, indicating that nanoparticles aren't entirely negative. Even though nanoparticles have been investigated for their effect on humans, much less is known about their impact on a global level, and their impact on other life forms. Currently, AgNPs have been found in concentrations ranging from 0.002 to 10.16mg/L across European surface waters⁸⁻¹², where harm and serious physiological damage to marine life has been seen to occur at concentrations starting within the $\mu\text{g/L}$ range^{13,14}. By understanding the broader ecological impact of AgNPs, we can push companies that currently use AgNPs to find greener alternatives that don't have the same effects on the environment and aquatic wildlife¹⁵.

Characterization of Nanoparticles

While this is a wide concentration range, it's important to recognize that not all nanoparticles are created equally. To understand how biologically available AgNPs are (and how likely they are to interfere with biological systems), it's important to recognize

that there are a multitude of properties that can affect biological availability. First, zeta potentials can affect availability and refer to how likely it is that something will precipitate out. If a nanoparticle has a zeta value close to zero, this means the particle will precipitate out, and directly refers to the possibility of biological uptake in the gills of the fish¹⁶. Nanoparticles that have a more positive zeta potential are taken up more quickly by the gills because the mucus lining of the gills is negatively charged¹⁷. Nanoparticles that are taken up quicker would impair the gills faster, but the overall impact would still vary depending on length of time spent in contact with nanoparticles¹⁸.

Additionally, size and the presence of caps can also play a role in biological availability. Nanoparticle size has a large impact on whether it's taken up by the cell. Depending on the size, the nanoparticle can migrate to different parts of the body or different parts within the cell¹⁹, but all sizes of nanoparticles have demonstrated some sort of cellular toxicity²⁰. One type of cap that is becoming increasingly more common is polyvinylpyrrolidone (PVP). PVP caps can help to maintain the stability of the nanoparticle²¹ and can increase their ability to remain in suspension in various solutions thanks to the amphiphilic nature of PVP²². The capping of the nanoparticles enhances their biological availability by making them more stable in suspension²³, meaning PVP capped particles are more likely to end up in the environment, and more likely to interact with biological systems²³. PVP capped nanoparticles will be used in this research to better mimic the nanoparticles likely encountered in the wild.

To properly investigate how AgNPs affect aquatic life, the nature of the nanoparticle must be taken into consideration as well. One of the reasons why nanoparticles are favoured is because of their high surface-to-volume ratio. This high ratio of available surface sites can accelerate adsorption or reaction²⁴. This high ratio also means that the nanoparticle can disperse evenly and stay suspended in another substance. However, should these nanoparticles collide or come into close contact with each other, they have the tendency to aggregate, creating larger particles. These large particles will then settle out of solution, sinking to the bottom of the suspension²⁵. In the end, most nanoparticles will end up aggregating together, sinking out of solution. In nature, this is also true, and over time, surface waters will become clean with a low concentration of AgNPs. However, the bottom of the same body of water will be much more contaminated²⁶.

Pelagic vs Benthic Creatures:

Due to this nanoparticle separation, the species of fish investigated in a toxicology report must be carefully considered. A pelagic creature such as a perch, would feed primarily in the pelagic layer on other smaller pelagic fish²⁷. In contrast, benthic creatures (bottom dwellers) spend their time on the bottom of lakes or rivers. ocean. These creatures are typically detritus eaters and will consume bioparticles such as algae and other sediments²⁸. As AgNPs enter the water, they will first interact with the pelagic creatures. As AgNPs settle and sink to the bottom, this leaves the pelagic layer clear while contaminating the bottom layer. Benthic creatures directly consume these AgNP aggregates as they feed and breathe²⁹. As such, it's important to consider that benthic creatures are much more likely to interact with NPs in the wild, and this should be reflected when investigating nanoparticle toxicity in aquatic creatures. Previous reports have shown that pelagic fish are minimally affected by nanoparticles, but much less is known about their benthic counterparts³⁰.

Ancistrus cirrhosis:

Knowing how important it is to consider benthic creatures over pelagic creatures, *Ancistrus cirrhosis* (Bristlenose Catfish) was chosen for this experiment. Catfish are benthic creatures, with the bottom rover body type³¹. While there is a plethora of different bottom feeders available, these catfish were chosen for a multitude of reasons. The first reason is that this breed of catfish is quite representative of bottom dwellers. They are bottom feeders, detritus eaters, rarely go into the pelagic layer, and tend to be quite motionless^{32,33}. These qualities tend to be true for most benthic creatures, and so catfish are great representatives of benthic creatures. If catfish were to demonstrate substantial behavioural or physiological changes, that would likely be a good indicator that other animals found in the same environment are also being affected in some way. Additionally, this is a freshwater breed of catfish³³. By specifically investigating if freshwater catfish are impacted by AgNPs, then this would likely indicate that there would be similar effects within humans if our fresh water supply was contaminated. Putting this impact in a human perspective is the key to reducing AgNP water contamination.

Another reason that catfish were chosen was due to their ability to both gill breath in water and air-breathe above water³⁴. Like most fish, catfish can satisfy their oxygen need by using their gills. As the fish moves its mouth, this pushes water past the gills which are capillary rich. These capillaries can then pick up dissolved oxygen, and release carbon dioxide³⁵. However, catfish also have the incredible ability to air breathe thanks to their soft, thin underbellies, and spiralized stomachs¹, which can be seen in Figure 1.



Figure 1. Anatomy of a catfish demonstrating the long stomach in a spiral shape. Image adapted from German *et al.* (2009)¹

As opposed to a human's stomach with folds to help aid digestion and provide surface area, the spiralized stomach is smooth with no folds¹. The internal surface of the epithelial lining contains multiple types of cells, most commonly being respiratory epithelial cells, which also run over capillaries³⁶. While the stomach is mostly used to break down food and begin digestion¹, this interaction between the respiratory epithelial cells and capillaries establishes a very thin air-blood barrier. The highly vascularized stomach is not the primary source of oxygen during hypoxic conditions (assuming gill function is intact), but rather used to help improve the amount of oxygen intake³⁷.

Current literature and aims of this study:

Current literature investigating the effect of AgNPs on aquatic life typically focus on pelagic fish. Literature that investigates benthic creatures typically look at higher nanoparticle concentrations (6.25 to 100mg/L) over shorter periods of time (96 hours)³⁸.

Previous focuses have also been on blood profile (hematological indices), acetylcholine esterase (AChE) activity, and oxidative stress indices^{18,38}. While this helps to understand the changes in physiology when in the presence of nanoparticles, it does not consider how a fish is likely to be affected in the wild.

In this research, AgNP dosage will be much lower than previous literature. This will help to determine the effect of silver nanoparticles at a dose that is more likely to be found in the wild. AgNPs have been found in high concentrations in the wild, but by investigating lower concentrations, the effect when there is minimal nanoparticle presence can be found. By understanding the effects that occur due to lower concentrations, it can be assumed that the effects only increase and worsen as nanoparticle concentration increases.

Additionally, exposure length will be increased to simulate more representative conditions. If the landfill runoff were to go into a fast-flowing river, the concentration would not be as high, and the time of exposure would be shorter. Conversely, if the runoff accumulated in a pond, then the concentration of AgNP would likely be higher, and the time of exposure would be much longer. Catfish that swim through these exposure points can then be exposed to AgNP which can be incorporated into their gills, or even stomach linings³⁸. Additionally, non-migratory animals such as catfish will rarely leave their home area and will only typically move to new area due to population pressure³⁹. By extension, this means that they also feed in these areas which are contaminated by nanoparticles, potentially incorporating nanoparticles into their gut tissue. Longer exposure times are more useful for predicting trends in wild catfish behaviour.

Lastly, this paper aims to investigate how AgNPs affect a catfish's ability to tolerate two different environmental stressors, the first of which is hypoxia. With climate change becoming ever more prevalent and global temperatures rising, it's important to recognize that water temperature increases as well. As water temperature rises, dissolved O₂ levels decrease, which leads to a reduced amount of oxygen being available^{40,41}. It is also important to recognize is that catfish originate from the Amazon⁴². The Amazon is prone to periods of hypoxia due to seasonal variations in rainfall such as the dry season, where rivers no longer flow to pools of water which creates highly hypoxic pond habitats⁴³. As

such, aquatic creatures are forced to rely on alternative methods to gain oxygen or learn to adapt. In catfish, airbreathing is the chosen alternative method. AgNPs are known to integrate into the stomach/intestines and gills³⁷. If the presence of AgNPs is enough to irritate the gut of the catfish, this may lead to a loss in ability to air breathe effectively. As such, this could cause an increase in air breathing frequency, directly going against their benthic nature, and exposing them to predators more frequently. It's thus important to determine if silver nanoparticles are preventing natural adaptation to the environment. This is also true for the other environmental stressor investigated, high temperature. An increase in temperature will lead to a proportional increase in the metabolic rate of a fish at the same time that it decreases oxygen availability. Animals must be able to adapt to this condition in order to survive.

Investigative Methods:

While comprehensive studies have been done demonstrating changes in physiology, one major indicator of stress has been overlooked. One of the most sensitive indicators of stress is often times behaviour⁴⁴. If an animal were to act more aggressive, sporadic, or lethargic, that can often suggest that something is wrong physiologically as an animal will rarely go against its natural state of being. Like most fish, a catfish's gills are the primary site of respiration⁴⁵. Air-breathing is only demonstrated when hypoxic conditions are experienced⁴⁶. O₂ sensors throughout the gills are constantly monitoring the internal environment, and it is only when oxygen levels are critically low that the fish will air-breathe⁴⁷. To quantitatively determine how nanoparticles affect a catfish's ability to tolerate hypoxia, you can count the number of times the catfish air breathes. If nanoparticles irritate the gills or the as expected, the frequency of air breaths taken will increase to get the same amount of O₂. In addition to counting the air breathing episodes, behaviour can be quantified through use of EthoVisionXT technology, where the fish's total distance moved can be tracked. This is a useful analysis as catfish in non-stressful situations are likely to remain stationary (aligning with their benthic nature) but will increase their movement when in a stressful situation. As such, an increase in total movement can be directly linked to how stressed the catfish is.

To further investigate how nanoparticles affect the fish, gills will be analyzed using a scanning electron microscope (SEM) to look for evidence of nanoparticle damage. These deformed gills will no longer be able to exchange oxygen, indicating the fish had a reduced rate of oxygen consumption⁴⁸. To further investigate how gill breathing is impacted by AgNPs, the fish will also be placed in a respirometer overnight, showing the amount of oxygen consumed by the fish over time. It is expected that in the presence of nanoparticles, the fish will consume less oxygen compared to a fish without nanoparticles due to AgNP interference with gills⁴⁸ combined with removing air breathing access.

Lastly, physiological markers will be investigated by sampling stomach tissue. This tissue can then be used to investigate various stress markers. One common indicator of stress is presence of malondialdehyde in tissue. When oxygen levels are low, or temperatures are high, oxidative stress occurs, which is when there is an excess of oxidants present with not enough defense systems. Oxidants include things like reactive oxygen species, as well as reactive nitrogen species⁴⁹. These reactive species will damage a variety of different biomolecules, one of which being lipids within the cell membrane^{49,50}. Lipid oxidation results in numerous products being produced, however one of the most harmful products produced is malondialdehyde (MDA). This aldehyde is highly toxic, and it has the potential to cause DNA mutations⁵⁰. Higher MDA levels indicate oxidative stress and rise in response to an increase in reactive oxygen species⁵¹ due to hypoxia⁵² or AgNPs⁵³. Heat can also cause an increase in oxidative stress due to mitochondrial damage⁵⁴. It would also be expected that as temperatures increase and dissolved oxygen levels decrease, the fish would experience similar hypoxic conditions. As such, it would be expected that a fish experiencing warmer temperatures would have an increase in MDA levels as well⁵⁵.

Summary:

In this research, varying environmental stressors will be used to mimic stressors seen in the wild. This will be done both in the presence and absence of silver nanoparticles at a concentration that is likely to be seen in the wild. By investigating these environmental conditions and increasing the length of exposure, the overall effect that AgNPs have on aquatic wildlife can be evaluated. This effect will be determined by quantifying behavioural changes, finding the change in rate of oxygen consumption (MO₂), determining malondialdehyde levels, and investigating any behavioural changes in the

catfish. The aim is to answer the three following questions: What is the effect of AgNPs at a dose that is more likely found in the wild; what effect does long term exposure have on the physiology and behaviour of catfish; and how do AgNPs affect a catfish's natural ability to tolerate environmental stress? It is hypothesized that when nanoparticles are present, they will irritate the gills, which will increase the frequency of air breathing. Additionally, the presence of nanoparticles may irritate the gut, resulting in less efficient air breathing. If the catfish cannot effectively air breathe in necessary scenarios, the fish may have to air breathe more frequently to get the same amount of oxygen through the gut. It is also hypothesized that the presence of nanoparticles will cause an increase in total distance moved. Lastly, it is expected that stressed catfish will have higher levels of MDA. It is expected that the presence of nanoparticles will further stress the catfish, and the fish subjected to both environmental stressors and nanoparticle presence will have the highest levels of MDA.

3. Methods and Materials:

3.1 Ancistrus dolichopterus (Catfish) Growth and Exposure Conditions

Catfish were sourced from a local supplier and grown in large tanks of 24°C aerated freshwater (sourced from Harold Crabtree Aqualab, Sackville, NB). Catfish were grown for at least 5 months prior to experiments. Catfish were fed a varied diet consisting of fresh zucchini, algae pellets (TopFin and Tetra), and other protein sources like Bug Bites (Fluval) and Catfish Pellets (OmegaOne). Once large enough, one catfish was moved to an isolation tank maintained at 24°C, using water from their original tank system to prevent any chemical differences. Two water treatments were investigated in this experiment: control and nanoparticle. Both control and nanoparticle tanks were maintained at optimal conditions with the nanoparticle tank containing a concentration of 80µg/L AgNPs. Silver nanoparticles were sourced from NanoComposix, using 5mg/mL 5nm Silver Nanospheres – PVP capped (NanoComposix) (Lot No. ECP1598 and MPP0042). Previous characterization was completed by Campbell *et al.* (2019) and Ollerhead *et al.* (2020).^{56,57} Solutions were sonicated for 10 seconds using a Sonic Dismembrator (Fisher Scientific) to disrupt aggregates prior to use.

Every day, 50% water changes were done using water from the original tank system. The nanoparticle concentration of 0.08g/L was maintained with additional doses. To ensure there was no adverse reaction to the dosing regime, each control tank received a sham dose with an equal amount of water. Each fish was kept in the isolation tank for 7 days either in clean water or in the presence of nanoparticles. On the 7th day, the fish was moved to the video tank (with water matching the condition in their isolation tank) and allowed to acclimate overnight.

3.2 Environmental Conditions and Video Analysis

Once acclimated, a 50% water change occurred. The fish was then exposed to one of three environmental conditions: optimal conditions, hypoxic conditions, or high temperatures. Optimal conditions consisted of well oxygenated water (using an air stone and air pump) at 24°C. Hypoxic conditions were created by bubbling nitrogen gas into the tank until an oxygen saturation level of 20% (1.68mg O₂/L) was reached. Oxygen levels were measured using a dissolved oxygen meter and levels were dropped over the course of 90 minutes. Temperature was maintained at 24°C. For the high temperature condition, a heating coil was used to heat the tank to from 24 to 32°C over 90 minutes. Fish were exposed to the environmental stressor for the duration of the video analysis and respirometry.

Recording commenced after the environmental condition was achieved. Videos were filmed by a monochromatic Basler camera, and these videos were directly imported into EthoVision[®] XT (Noldus, Wageningen, Netherlands). Each video was individually analyzed using deep learning to track the total distance moved. In addition to this, the tank was divided, where the top 3 cm constituted the top of the tank, and the rest of the tank was considered the bottom of the tank. EthoVision tracked the amount of time the fish spent in the top portion of the tank and tracked the number of times the fish entered into the zone. Due to video constraints, the tanks were devoid of environmental enrichment, with only the fish being present. To prevent behavior from being affected by human presence, the tank was located behind a curtain. Each fish was videoed for 6 hours before being transferred to the respirometer. Testing for biochemical endpoints, as well as data analysis was the same for all treatment groups.

3.3 Measuring the Rate of Oxygen Consumption (MO_2)

In all trials, mass specific MO_2 activity was tracked by measuring the decrease in water oxygen levels as measured using an intermittent flow respirometry system (Q-box Aqua, Qubit Systems, Kingston ON, Canada). During the recording, the respirometer tank was set up to mimic the same environmental conditions as the recording tank, and maintained with the environmental stressor for the duration of the trial. After the recording was finished, the fish was carefully placed inside the chamber, and a lid was placed over the tank. The program was started on a loop cycle of 150 seconds flush time, 60 seconds of rest, and 350 seconds of circulation. The fish was left in the respirometer overnight for a minimum of 10 hours. The following morning, the fish was euthanized (details in 3.4). The mass of the fish was entered into LoggerPro to determine the mass specific MO_2 . The following equation was used to determine respiration rate.

$$MO_2 = DOSlope * (Vr - Va) * \frac{3600}{m}$$

Where MO_2 = oxygen consumption (mg/kg/h), $DOSlope$ = rate of decrease of DO (mg/L/s), Vr = respirometer volume (L), Va = volume of experimental animal (L), and m = animal weight (kg).

This calculated data was extracted and moved to Excel where it was plotted against time. To find the basal metabolic rate of the fish, 5 representative MO_2 readings were averaged from the lower 5-10% of all readings recorded. This ensured removal of outliers, and that these values were representative of basal metabolic rate.

3.4 Euthanization and Sampling

Catfish were humanely euthanized via anesthetic overdose using 0.5g/L MS-222 (Syncaïne) and death was confirmed if the animal did not respond to repeated tail pinches and inversion. Once euthanized, the catfish was placed on paper towel to pat off excess water. Weight was recorded using an analytical balance, and the fish was placed ventral side up on a surgical block. An incision from the pore (found where the tail and torso meet) to just below the operculum was made. Four horizontal cuts were made to expose the internal cavity. The spiralized stomach was removed, placed in a cryovial, and immediately placed in liquid nitrogen. Gut samples were stored at -80°C until needed. Due to the bony

nature of armoured catfish and the delicate gills, it was difficult to remove the gills from the recently deceased catfish. So, a cut was made just below the operculum moving horizontally, removing the head of the catfish. Heads were fixed in 2.5% (vol:vol) glutaraldehyde in 0.1M phosphate buffer and stored at 4°C until processing for SEM.

3.5 Determining Gill Structure via SEM

Following fixation, gills were dissected out of the heads and placed in microporous specimen capsules (Electron Microscopy Sciences, Hatfield, PA, USA) filled with 2.5% (vol:vol) glutaraldehyde in 0.1M phosphate buffer. Specimens were then rinsed 4 x 10 min in distilled water and dehydrated in a graded series of ethanol (20%, 50%, 70%, 85%, 95%) for 10 min each. This was followed by 4 x 10 min washes in a 100% anhydrous ethanol and then dried using liquid CO₂ in a Denton DCP-1 critical point dryer (Denton Vacuum, Moorestown, NJ, USA). Gills were mounted on 32 mm diameter aluminum stubs using colloidal graphite and coated with ~20 nm gold using a Hummer 6.2 sputtering unit (Anatech Ltd., Union City, CA, USA). Scanning electron microscopy was performed using a Hitachi SU3500 SEM (Hitachi High Technologies, Toronto, Canada) operating at 10 kV accelerating voltage and 20 mm working distance. Three fish from each treatment group were selected at random for gill analysis. Six images were taken of each gill at 100X, 500X, 800X, 2000X, and two images at 5000X.

3.6 Homogenization of Gut Sample and MDA Assay

The gut sample from 3.4 was thawed and placed on ice. A small portion of the gut was weighed and added to phosphate-buffered saline to make a solution with a concentration of 40mg gut/mL, as suggested by the manufacturer's protocol. The gut submerged in buffer was kept on ice and homogenized in 10 second bursts 4 times using a Sonic Dismembrator (Fisher Scientific). This homogenate was stored at -80°C until needed.

MDA was measured through use of a Bioxytech MDA-586 assay (OxisResearch). No deviations from the standard protocol occurred and absorbance was measured at 586nm using a Molecular Devices (Sunnyvale, CA, USA) SpectraMax microplate reader in a Costar Assay Plate, 96 Well (Corning, USA). Using the equation of the standard curve, the

absorbance of the gut homogenate at 586nm could be converted to protein concentration (mg of protein/mL of solution).

3.7 Data + Statistical Analysis

All data was compiled and sorted using Excel. Statistical analysis for comparing the environmental stressors consisted of one-way ANOVAs and post-hoc Tukey HSD tests ($p < 0.05$) to determine if there was any statistical difference between groups. If the variances were found to differ significantly via Bartlett's test, a Kruskal-Wallis test was completed instead of a one-way ANOVA. To determine the effect of nanoparticles on the fish's ability to tolerate environmental stress, unpaired t-tests were used ($P < 0.05$). All statistical analysis was done using Prism5 (GraphPad, USA).

4. Results:

4.1 Video Analysis

Through use of EthoVisionXT (Noldus), the catfish was tracked, and the total distance moved (cm) within the tank could be found. Post-hoc tests indicated that fish exposed to the same environmental condition were not found to have any statistical difference between them. As such, the values presented in Figure 2 are the comparisons of environmental stressors when AgNPs were absent or present.

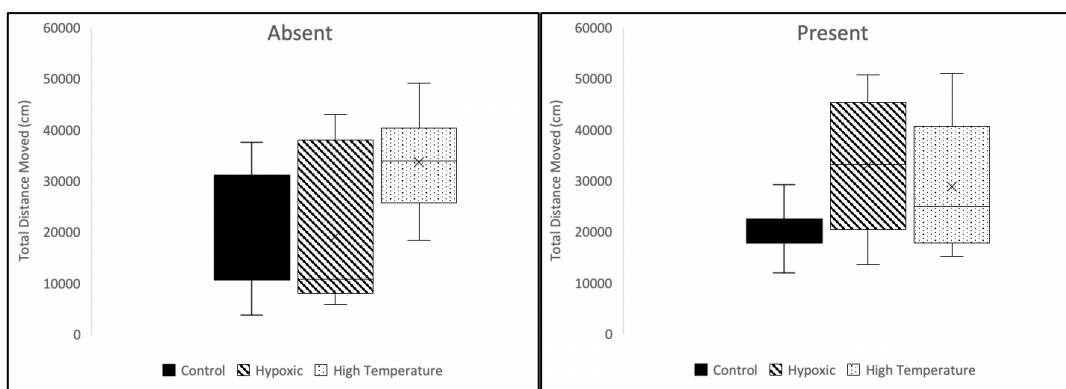


Figure 2. Changes in total distance moved (cm) when exposed to 5nm PVP-AgNPs and environmental stressors. Data was collected and analyzed in EthoVisionXT (Noldus). Control conditions consisted of well oxygenated, 24°C water. Hypoxic conditions consisted of 20% O₂ saturation, and high temperature consisted of 32°C water. ANOVA and post-hoc tests did not show statistical differences between any of the treatment groups within the absent group ($p=0.0639$) and the present group ($p=0.9175$).

When AgNPs were absent, catfish moved a total of 22774.7 ± 11960.7 cm in the control condition, 19229.9 ± 12945.5 cm in the hypoxic condition, and 33685.2 ± 9925.6 cm in the high temperature condition. Testing indicated that variances did not differ significantly ($p=0.5459$) and so a one-way ANOVA was performed and indicated that there was a trend towards significance of environmental stress on the absent fish ($p=0.0639$). A larger sample size or additional experiments are required to confirm or refute this trend.

When AgNPs were present, catfish moved a total of 32577.5 ± 34560.6 cm in the control condition, 33051.2 ± 15222.8 cm in the hypoxic condition, and 28867.5 ± 12697.5 cm in the high temperature condition. Variances were found to differ significantly ($p=0.0099$) and there was no significant effect of environmental stress on the nanoparticle fish ($p=0.4286$). This same process was repeated for frequency of visits to the top, which can be seen in Figure 3.

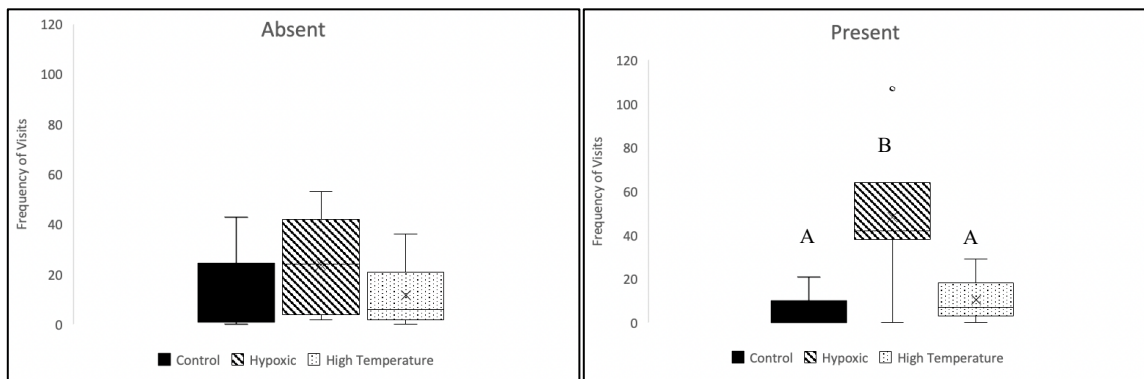


Figure 3. Changes frequency of visits to the top 3cm of the tank when exposed to 5nm PVP-AgNPs and environmental stressors. Data was collected and analyzed in EthoVisionXT (Noldus). Control conditions consisted of well oxygenated, 24°C water. Hypoxic conditions consisted of 20% O₂ saturation, and high temperature consisted of 32°C water. Significant differences are denoted by dissimilar letters.

When AgNPs were absent, catfish visited the top 12.9 ± 15.2 times over the course of 6 hours in the control condition, 23.9 ± 32.2 times in the hypoxic condition, and 11.7 ± 12.1 times in the high temperature condition. A one-way ANOVA was performed and indicated that there was no significance of environmental stress on the absent fish ($p=0.2516$). Bartlett’s test indicated that variances did not differ significantly ($p=0.5467$).

When AgNPs were present, catfish visited the top 6.6 ± 7.3 times over the course of 6 hours in the control condition, 48.3 ± 18.6 times in the hypoxic condition, and $10.6 \pm$

9.9 times in the high temperature condition. Variances did differ significantly ($p=0.0008$), analysis indicated that there was a significant effect of environmental stress on the nanoparticle present fish ($p=0.0191$). Post-hoc tests indicated that there was a significant difference between the present control condition and the present hypoxic condition ($p>0.01$). T-tests also indicated that there was no difference between the absent and present fish within the control, hypoxic, or high temperature conditions.

4.2 Respirometry

It is expected that when the fish is put into the respirometer, MO_2 rates will be high as the fish is acclimating to its new surroundings. Over time, the fish should become more relaxed, and the basal metabolic MO_2 rate should be achieved. A representative curve of the expected shape can be seen in Figure 4.

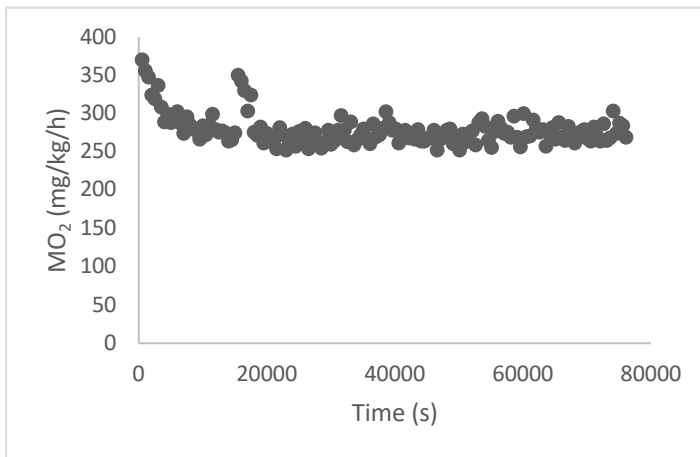


Figure 4. Representative oxygen consumption ($mg\ O_2/kg/h$) curve for control fish over time (s) showing an initial decrease before stabilizing. Oxygen consumption was measured by an intermittent flow respirometry system (Q-box Aqua, Qubit Systems, Kingston ON, Canada).

This was found to be true for fish in control absent, control present, high temperature absent, and high temperature present. However, fish in both hypoxia groups exhibited a low initial MO_2 , which peaked approximately 4 hours into the trial. MO_2 then continued to decrease overnight. An example of this curve can be seen in Figure 5.

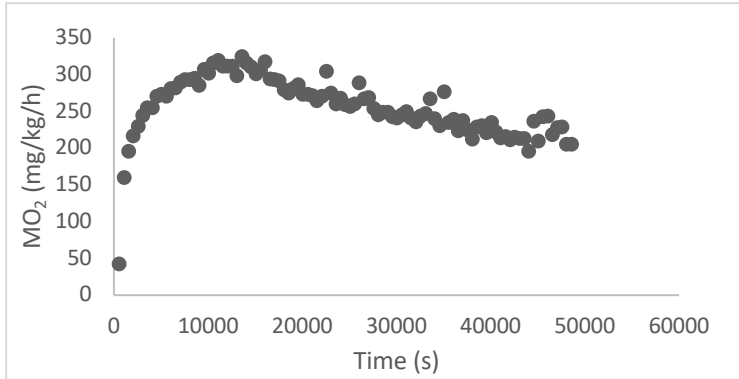


Figure 5. Representative oxygen consumption (mg O₂/kg/h) curve for fish exposed to hypoxia over time (s) showing an initial decrease before stabilizing. Oxygen consumption was measured by an intermittent flow respirometry system (Q-box Aqua, Qubit Systems, Kingston ON, Canada).

Ultimately, the goal of this procedure was to determine the basal metabolic rate for the fish. This basal rate would be expected after the fish has calmed down, usually later in the night. To collect data points to analyze, five points were selected at random within the lowest 5-10% range. These values when then averaged and are presented in Figure 6. However, with the fish that endured the hypoxic trials, demonstrated two instances where they had a lower MO₂. As such, for these fish only, two sets of data points were examined: one set from the start of the respirometry trial and one from the end. These values can be seen in Figure 6.

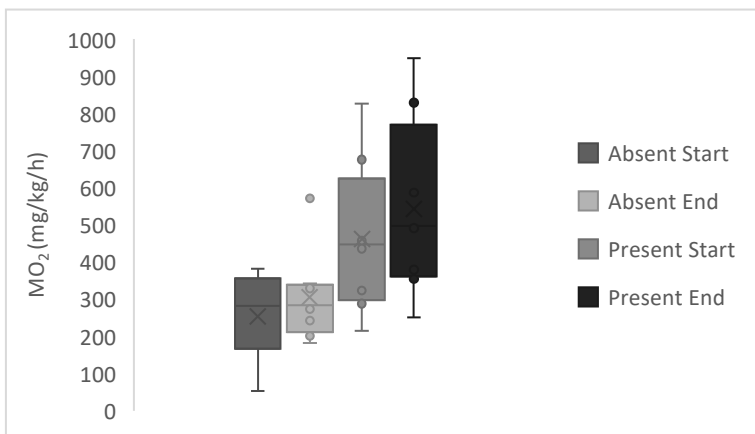


Figure 6. Comparison of average starting metabolic rates (MO₂) (mg O₂/kg/h) and ending metabolic rates in *Ancistrus cirrhosis* exposed to hypoxic conditions, with and without the presence of 5nm AgNPs. Oxygen consumption was measured by an intermittent flow respirometry system (Q-box Aqua, Qubit Systems, Kingston ON, Canada). ANOVA and post-hoc tests did not show statistical differences between any of the treatment groups within the absent group (p=0.4124) and the present group (p=0.4774).

The MO_2 values of the absent hypoxic fish at the start of respirometry trial was found to be 254.4 ± 40.8 mg O_2 /kg/h and 304.4 ± 43.0 mg O_2 /kg/h at the end of the respirometry trial. Through an unpaired, two-tailed t-test, no significant difference in MO_2 was seen between the start of the absent hypoxic trials and the end of the trial ($p=0.4124$). The MO_2 values of the present hypoxic fish at the start of respirometry trial was found to be 543.0 ± 84.6 mg O_2 /kg/h and 462.0 ± 71.8 mg O_2 /kg/h at the end of the respirometry trial. Through an unpaired, two-tailed t-test, no significant difference in MO_2 was seen between the start of the present hypoxic trials and the end of the trial ($p=0.4774$).

To determine if the observed increase in respiration after the fish was placed in the respirometer was statistically different from the basal metabolic rate, unpaired t-tests were completed comparing the end of the respirometry trial with the peak within both nanoparticle conditions. The absent hypoxic basal metabolic did not differ from the absent hypoxic peak in respiration ($p=0.3668$) and the present hypoxic basal metabolic rate did not differ from the present hypoxic metabolic rate peak ($p=0.3110$). For comparison and consistency, all further analysis of MO_2 data from the hypoxia fish refer to the data taken from the end of the trial. Post-hoc tests indicated that fish exposed to the same environmental condition were not found to have any statistical difference between them, regardless of nanoparticle exposure. As such, the values presented in Figure 7 are the comparisons of environmental stressors when AgNPs were absent or present.

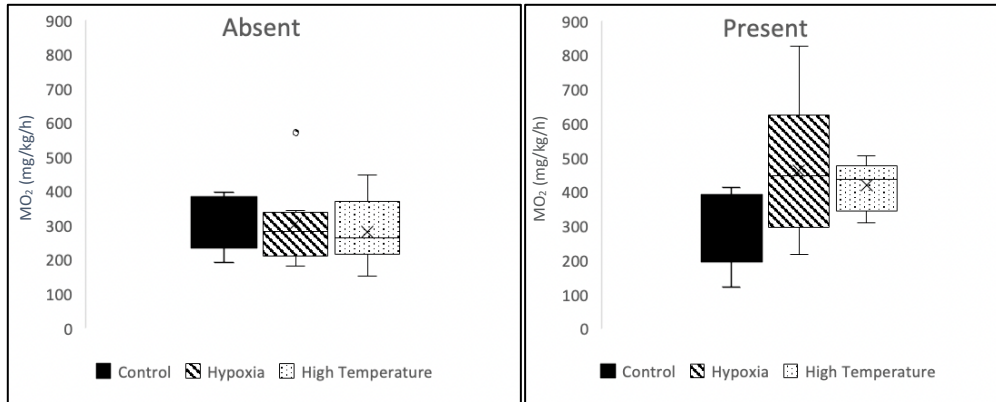


Figure 7. Effect of environmental stressors with and without 8-day exposure to 5nm AgNP on average *Ancistrus cirrhosis* oxygen consumption rates (MO_2) ($mg\ O_2/kg/h$). Control conditions consisted of well oxygenated, 24°C water. Hypoxic conditions consisted of 20% O_2 saturation, and high temperature consisted of 32°C water. ANOVA and post-hoc tests did not show statistical differences between any of the treatment groups within the absent group ($p=0.8926$) and the present group ($p=0.0669$). Oxygen consumption was measured by an intermittent flow respirometry system (Q-box Aqua, Qubit Systems, Kingston ON, Canada).

When AgNPs were absent, catfish reached basal metabolic rate of $299.1 \pm 78.0\ mg\ O_2/kg/h$ in the control condition, $304.4 \pm 121.7\ mg\ O_2/kg/h$ in the hypoxic condition, and $281.1 \pm 98.3\ mg\ O_2/kg/h$ in the high temperature condition. A one-way ANOVA was performed and indicated that there was no significance of environmental stress on the absent fish ($p=0.8926$). Bartlett's test indicated that variances did not differ significantly ($p=0.5607$).

When AgNPs were present, catfish reached a basal metabolic rate of $286.2.0 \pm 110.4\ mg\ O_2/kg/h$ in the control condition, $462.0 \pm 203.2\ mg\ O_2/kg/h$ in the hypoxic condition, and $419.3 \pm 71.1\ mg\ O_2/kg/h$ in the high temperature condition. A one-way ANOVA was performed and indicated that there was a trend towards significance of environmental stress on the nanoparticle present fish ($p=0.0669$). Bartlett's test indicated that variances did differ significantly ($p=0.0311$), so a Kruskal-Wallis test was completed which again indicated that there was a trend towards significance of environmental stress on the nanoparticle fish ($p=0.0649$). Post-hoc tests also indicated that there was no difference between the absent and present fish within the control, hypoxic, or high temperature conditions.

4.3 Gill Structures

Using a scanning electron microscope, the gills from three fish from each experimental group were imaged and analyzed. Representative images from each group at 800x are presented in Figure 8.

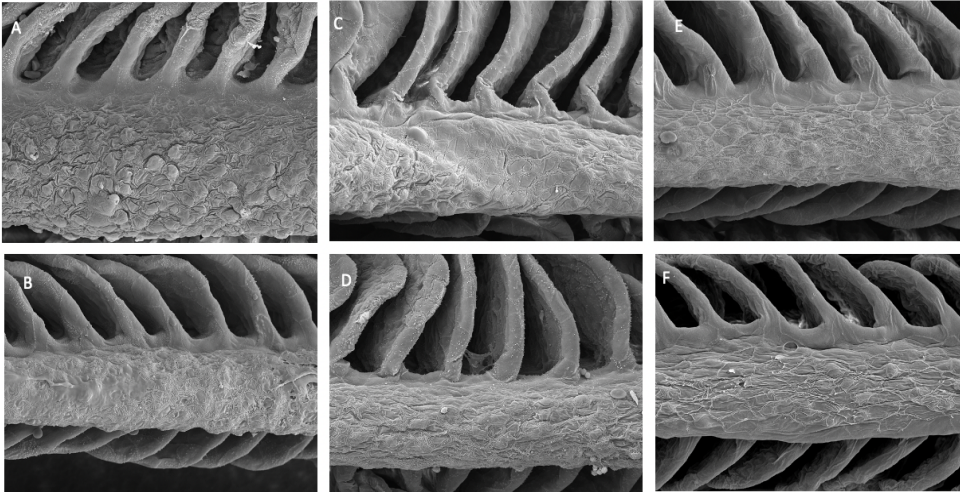


Figure 8. 800X SEM view of gill filaments and portions of primary and secondary lamellae in *Ancistrus cirrhosis*. Images show no signs of damage with or without exposure to 5nm AgNPs and regardless of environmental condition. A: Absent Control, B: Present Control, C: Absent Hypoxia, D: Present Hypoxia, E: Absent Heat, F: Present Heat. Images courtesy of J. Ehrman (Mount Allison University).

Qualitative analysis of the primary and secondary lamellar indicates that there was no clear physical damage to the gills within any of the treatment groups. No fused lamellae were seen, nor an increase in debris. This was true for all images derived from SEM analysis. Any abnormalities may be explained by any remaining mucus or artifacts from the preparatory methods or a result of drying out during the gill isolation process. Quantitative analysis with this set of data is not possible as common gill analysis includes measuring gill filament length and width of different gill arches. Due to the bony nature of catfish, it was difficult to isolate gill filaments without damaging them, so photos were taken of the best gill filament, regardless of gill arch position.

4.4 MDA assay

To determine oxidative stress at the cellular level, an MDA assay was performed to determine if there was a higher concentration of oxidative damage. Post-hoc tests indicated that fish exposed to the same environmental condition were not found to have

any statistical difference between them, regardless of nanoparticle exposure. As such, the values presented in Figure 9 are the comparisons of environmental stressors when AgNPs were either absent or present.

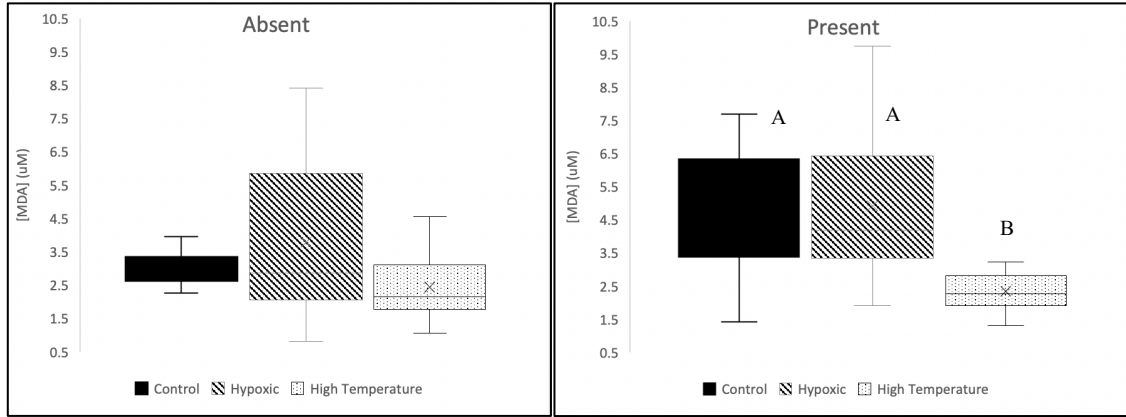


Figure 9. Effect of 5nm silver nanoparticles on malondialdehyde (MDA) concentration (μM) in *Ancistrus cirrhosis* in combination with 3 different environmental stressors. MDA content was determined through use of Bioxytech MDA-586 assay (OxisResearch), a commercially available colorimetric assay. Significant differences are denoted by dissimilar letters.

When AgNPs were absent, catfish gut samples were found to contain 2.93 ± 0.56 μg MDA in the control condition, 3.79 ± 2.44 μg MDA in the hypoxic condition, and 2.44 ± 0.91 μg MDA in the high temperature condition. A one-way ANOVA was performed and indicated that there was a trend towards significance of environmental stress on the nanoparticle present fish ($p=0.0630$). Bartlett's test indicated that variances did differ significantly ($p<0.0001$), so a Kruskal-Wallis test was completed which indicated that there no difference of environmental stress on the absent fish ($p=0.1967$).

When AgNPs were present, catfish gut samples were found to contain 4.55 ± 1.85 μg MDA in the control condition, 4.92 ± 2.24 μg MDA in the hypoxic condition, and 2.35 ± 0.55 μg MDA in the high temperature condition. A one-way ANOVA was performed and indicated that there was a significant effect of environmental stress on the nanoparticle present fish ($p<0.0001$). Bartlett's test indicated that variances did differ significantly ($p<0.0001$), so a Kruskal-Wallis test was completed which indicated that there was a significant effect of environmental stress on the nanoparticle present fish ($p<0.0001$). Post-hoc tests (Dunn's multiple comparison) indicated that the present heat condition was

significantly difference from the control present group ($p < 0.0001$) and the hypoxic present group ($p < 0.0001$). Post-hoc tests also indicated that there was no difference between the absent and present fish within the control and hypoxic groups.

5. Discussion:

5.1 AgNPs have the Potential to Influence Behaviour when Combined with Environmental Stressors

AgNP presence resulted in an increase in frequency of air breathing in the hypoxic fish, however this was not seen for high temperatures or control conditions. This indicates that AgNPs interfered with a catfishes ability to respond to hypoxic conditions, but not high temperatures. The increase in air-breathing means that in the wild, a fish in an AgNP polluted environment would be more susceptible to aerial predation as they would have to visit the top more frequently. MO_2 data within the present group was found to have a p-value of 0.0669, indicated a trend towards significance of the environmental stressor on the present fish. It thus seems likely that an increase in either AgNP concentration or an increase in exposure time could lead to a statistically significant result. If AgNP treatment was found to increase MO_2 , this could explain the increase in air-breathing frequency observed. If AgNPs damaged or irritated the gills or gut of the catfish, that catfish would need to visit the top more frequently as gut damage could lead to gas exchange efficiency impairment.

5.2 AgNPs Increase the Variability of Fish Response to Environmental Conditions

In all four quantitative analyses (total distance moved, frequency of air-breathing, MO_2 , and MDA), variance in groups was found to differ significantly from one another when nanoparticles were present. When nanoparticles were absent, homogenous variances were seen in all analyses except for MDA. Increased variance between individuals was seen primarily in nanoparticle present conditions indicating that AgNPs have some effect on the catfish. With an increase in variability, extreme values are more likely. This could mean that individual catfish may be responding to a much greater extent than others, making it difficult to discern a general response. In the wild, this means that catfish could

respond in a variety of different ways to environmental stressors when exposed to AgNPs but have the potential to act against their natural benthic tendencies.

*5.3 Potential Novel Mechanism for Anaerobic Metabolism in *Ancistrus cirrhosis**

Two different respirometry curves were observed; one that steadily decreased over time, and one that started low, peaked, and then returned to a basal metabolic rate. Considering that the first peak was observed in both control and high temperature fish, whereas the second curve was only seen in hypoxic fish regardless of AgNP presence, it is likely the hypoxic stressor was the cause. Prior to entering the respirometer, the catfish in the video tank had the ability to air-breathe. Around 30-70% (depending on catfish species) of arterial oxygen content in hypoxic scenarios can be attributed to air gulping³⁷. This can account for the low initial rate of oxygen consumption as the catfish may have had sufficient red blood cell and/or myoglobin oxygen stores. The longer the catfish goes without surface access, however, the more hypoxemic the fish becomes. As blood O₂ levels become insufficient and stress levels rise, respiration rates increase. After this peak, it appears that the catfish reacclimatizes as respiration rates decrease again. It is likely that the later decrease in MO₂ is associated with an activation of anaerobic metabolism, which is characteristic of armored catfish⁵⁸. With the length of this experiment, no significant difference was seen between the initial basal metabolic rate and the final basal metabolic rate. This was also true comparing the final basal metabolic rate with the peak in metabolic rate. If the experiment had been continued for longer, respiration may have decreased until mortalities occurred, as anaerobic metabolism cannot be relied on indefinitely due to the buildup of waste products like H⁺ and lactate. For fish that cannot air breathe, a significant increase in mortality is expected for those in hypoxic conditions for periods longer than 24 hours³⁷.

Between all three environmental conditions, no difference was observed when AgNPs were absent. This is indicative that it was the presence of nanoparticles that causes the greatest change in basal metabolic rate. While the control group did not differ when nanoparticles were present, both hypoxia trials and high temperature trials had a roughly 150% increase in respiratory rate. This increase is a maladaptive adjustment, and with no access to the surface, the fish would likely not survive long. In a broader context, if a catfish

was found in a warm polluted river, and could not escape the environment, it would be forced to breathe air more frequently and increase its risk of predation. If it was unable to breathe air, mortalities may occur as an indirect result of AgNP exposure.

The results of the hypoxia trials are above the expected range when compared to previously reported literature values. *Hypostomus aff. Pyreneusi* is a closely related freshwater facultative air-breather.³⁴ It was found that mass specific MO_2 can go as low as 120.8 mg O_2 /kg/h for *Hypostomus aff. Pyreneusi*. In this experiment, catfish reached a minimal rate of 304.4 ± 121.7 mg O_2 /kg/h.

Fish exposed to higher temperatures were seen to have no change in respiration when compared to other environmental stressors both in the presence and absence of nanoparticles. This was surprising as it is expected that an increase of 8 degrees would approximately double metabolic rate⁵⁹. This result in combination with the significantly lower MDA levels in the fish exposed to high temperatures has the potential to be explained by one of three reasons; the first is that these high temperature experiments were done after the control and hypoxia groups had been completed. The fish within the control and hypoxia groups were 3.5 ± 1.6 g while fish in the high temperature trials were 5.9 ± 1.4 g. The second is that catfish are tropical creatures that originate from the Amazon River basin. While these fish were grown in 24°C, in the wild these fish are often found in warmer climates where water temperatures can reach higher than 32°C⁶⁰. As such, 32°C may not have been enough of a stressor to elicit a change in behaviour or physiology, or not enough to induce significant mitochondrial damage⁶¹. Lastly, it is possible that the high temperatures may cause uncoupling of mitochondria, which decreases ROS production⁶².

5.4 Eight Day Exposure at 0.08ug/L Does Not Lead to Overt Gill Damage

Gill analysis was inconclusive due to the inability to conduct quantitative analysis, but there were no obvious indicators of damage in the images collected. Nanoparticles are expected to integrate into a variety of different systems within fish, and silver is by far the most likely metal to enter into the gills⁶³, and is recognized to cause inhibition of $Na^+/K^+ATPase$ ⁶⁴. Bioaccumulation has been reported primarily in the liver, followed by the intestines, and the gills. This accumulation has been shown to induce damage to gill tissues at concentrations as low as 0.03mg/L, but bioaccumulation increases proportionally

to the concentration of nanoparticles⁶⁵. Kakakhel *et al.* (2021) investigated bioaccumulation of AgNPs in carp and reported that the highest rate of mortalities only occurred at concentrations higher than 0.09mg/L after 12 days⁶⁵. The current experiment only exposed fish to nanoparticles for a maximum of 8 days at 0.08mg/L. As such, it is reasonable to assume that if exposure length was increased, or concentration was increased, damage may be more likely to occur. While this paper did not report when damage to the gills was initially observed, it did report that the most damage was seen at higher concentrations over longer durations⁶⁵, indicating that the assumption made above is reasonable. As mentioned in 4.3, quantitative analysis of SEM images was not possible with this gill set. If exposure length was longer, it is possible that blood vessels will be congested which would lead to clubbing of the tips of secondary lamellae⁶⁶. This clubbing is also known as loss of structure, and loss of structure ultimately results in loss of function⁶⁷.

6. Conclusion:

Results indicated that AgNPs did impact hypoxic stress tolerance. Fish exposed to both nanoparticles and the hypoxic conditioned increased air breathing frequency. AgNPs may have also increased respiratory rate in the hypoxic condition, indicating that frequency of air-breathing must increase to meet the fishes metabolic needs, but further analyses are required to confirm this hypothesis. AgNPs were also seen to greatly increase the variability of fish response to their environment, further indicating that AgNPs did impact stress tolerance in catfish. Ultimately, the video analysis results together (no change in distance moved but an increase in air breathing frequency for the present hypoxic fish) indicates that any movement that the catfish is doing is focused on moving to the top in times of stress. Decreased levels of MDA in high temperature fish could indicate that high temperatures cause uncoupling of mitochondria, which decreases ROS production. These results indicate that AgNPs are damaging to the benthic creatures of our aquatic ecosystems. Armed with this knowledge, we can encourage and demand that companies using AgNPs find greener alternatives.

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