



Boron Oxide Nanoparticles Exhibit Minor, Species-Specific Acute Toxicity to North-Temperate and Amazonian Freshwater Fishes

Tyson J. MacCormack^{1*}, Patrick T. Gormley¹, B. Ninh Khuong¹, Olivia A. Adams¹, Susana Braz-Mota², Rafael M. Duarte³, Christopher M. Vogels¹, Luc Tremblay⁴, Adalberto L. Val², Vera M. F. Almeida-Val² and Stephen A. Westcott¹

¹ Department of Chemistry and Biochemistry, Mount Allison University, Sackville, NB, Canada, ² Laboratory of Ecophysiology and Molecular Evolution, Brazilian National Institute for Research of the Amazon, Manaus, Brazil, ³ Institute of Biosciences, São Paulo State University (UNESP), São Vicente, Brazil, ⁴ Department of Chemistry and Biochemistry, Université de Moncton, Moncton, NB, Canada

OPEN ACCESS

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*Correspondence:

Tyson J. MacCormack
tmaccormack@mta.ca

Specialty section:

This article was submitted to
Biosafety and Biosecurity,
a section of the journal
Frontiers in Bioengineering and
Biotechnology

Received: 01 April 2021

Accepted: 05 May 2021

Published: 28 May 2021

Citation:

MacCormack TJ, Gormley PT,
Khuong BN, Adams OA,
Braz-Mota S, Duarte RM, Vogels CM,
Tremblay L, Val AL, Almeida-Val VMF
and Westcott SA (2021) Boron Oxide
Nanoparticles Exhibit Minor,
Species-Specific Acute Toxicity
to North-Temperate and Amazonian
Freshwater Fishes.
Front. Bioeng. Biotechnol. 9:689933.
doi: 10.3389/fbioe.2021.689933

Boron oxide nanoparticles (nB_2O_3) are manufactured for structural, propellant, and clinical applications and also form spontaneously through the degradation of bulk boron compounds. Bulk boron is not toxic to vertebrates but the distinctive properties of its nanostructured equivalent may alter its biocompatibility. Few studies have addressed this possibility, thus our goal was to gain an initial understanding of the potential acute toxicity of nB_2O_3 to freshwater fish and we used a variety of model systems to achieve this. Bioactivity was investigated in rainbow trout (*Oncorhynchus mykiss*) hepatocytes and at the whole animal level in three other North and South American fish species using indicators of aerobic metabolism, behavior, oxidative stress, neurotoxicity, and ionoregulation. nB_2O_3 reduced *O. mykiss* hepatocyte oxygen consumption ($\dot{M}O_2$) by 35% at high doses but whole animal $\dot{M}O_2$ was not affected in any species. Spontaneous activity was assessed using $\dot{M}O_2$ frequency distribution plots from live fish. nB_2O_3 increased the frequency of high $\dot{M}O_2$ events in the Amazonian fish *Paracheirodon axelrodi*, suggesting exposure enhanced spontaneous aerobic activity. $\dot{M}O_2$ frequency distributions were not affected in the other species examined. Liver lactate accumulation and significant changes in cardiac acetylcholinesterase and gill Na^+/K^+ -ATPase activity were noted in the north-temperate *Fundulus diaphanus* exposed to nB_2O_3 , but not in the Amazonian *Apistogramma agassizii* or *P. axelrodi*. nB_2O_3 did not induce oxidative stress in any of the species studied. Overall, nB_2O_3 exhibited modest, species-specific bioactivity but only at doses exceeding predicted environmental relevance. Chronic, low dose exposure studies are required for confirmation, but our data suggest that, like bulk boron, nB_2O_3 is relatively non-toxic to aquatic vertebrates and thus represents a promising formulation for further development.

Keywords: nanotoxicology, engineered nanomaterials, acetylcholinesterase, ionoregulation, energy metabolism, oxidative stress, aquatic toxicology

INTRODUCTION

When released into the environment, bulk boron compounds and boric acid (BA) can decompose into nanoparticles (NPs) (Zhou and Bai, 2015). Boron NPs are also manufactured as engineered nanomaterials (ENMs) and boron ENMs in the form of boron oxide ($n\text{B}_2\text{O}_3$) are used in fuel and propellant applications for their high heats of combustion and rapid energy release rates (Liu et al., 2010). As $n\text{B}_2\text{O}_3$ use increases, more will inevitably end up in the environment and could pose risks to human and ecosystem health. The toxicity of bulk boron in the form of BA has been well established in a number of terrestrial and aquatic organisms (Weir and Fisher, 1972; Butterwick et al., 1989; Cochran, 1995), however little is known about the toxicity of $n\text{B}_2\text{O}_3$. The only available study suggested that $n\text{B}_2\text{O}_3$ was more toxic to a model invertebrate than titanium dioxide and aluminum oxide ENMs (Strigul et al., 2009). $n\text{B}_2\text{O}_3$ is unlikely to find its way into the environment in the same quantities as more common ENM formulations (e.g., silver ENMs), but understanding its potential ecotoxicity remains important, especially if its use increases.

Boric acid is a natural form of boron found in minerals such as boracite, ulexite, and colemanite, as well as in seawater and plants (Lodge, 1994; Wolska and Bryjak, 2013), and is used in a variety of applications including clinical products, antibacterial and antifungal agents, and insecticides (Siegel and Wason, 1986; Sander et al., 1991; Xue and Barnard, 2003). Boron deficiency limits growth in many plants and the agricultural application of boron is common, particularly in some areas of Brazil where soil boron levels are sub-optimal (Fageria and Nascente, 2014). Boron is also an effective pesticide since it is essentially inert to mammals (Weir and Fisher, 1972) but is highly toxic to invertebrates (Cochran, 1995). For example, in German cockroaches, BA exhibits neurotoxicity by inhibiting acetylcholinesterase (AChE) activity and disrupting reactive oxygen species metabolism, as evidenced by increased glutathione S-transferase activity (Habes et al., 2006). In fish, boron does not bioaccumulate and toxicity depends on differences in osmoregulatory function, water uptake rates, membrane permeability, and environmental salinity (Thompson and Davis, 1976). Boric acid is relatively non-toxic to chinook and coho salmon, with 96 h LC_{50} s $> 100 \text{ mg L}^{-1}$ (Hamilton and Buhl, 1990). At low concentrations, boron can actually be beneficial to fish; at $< 925 \mu\text{mol L}^{-1}$, BA stimulated embryonic growth in rainbow trout (Eckert, 1998) and similar effects have been observed in zebrafish (Rowe and Eckert, 1999).

Numerous ENM formulations exhibit some degree of acute toxicity to freshwater fish, although usually at exposure concentrations far in excess of those expected in the environment (Callaghan and MacCormack, 2017). Some commonly observed effects of acute ENM exposure are disruptions to ionoregulatory homeostasis (Schultz et al., 2012), oxidative stress (Federici et al., 2007), inhibition of AChE (Callaghan et al., 2016), and damage to the gill epithelium (Griffitt et al., 2009; Bessemer et al., 2015). Direct and/or indirect effects on energy metabolism at the tissue and whole animal level have also been observed (Dieni et al., 2014; Callaghan et al., 2016), in some cases at environmentally relevant ENM concentrations (Black et al., 2017). Under aerobic conditions, these effects can manifest as changes in rates of

oxygen consumption ($\dot{M}\text{O}_2$) at either the cell or whole animal levels. At the whole animal level, routine and maximum $\dot{M}\text{O}_2$ ($\dot{M}\text{O}_{2\text{min}}$ and $\dot{M}\text{O}_{2\text{max}}$, respectively) and aerobic scope, the difference between them, are ecologically relevant indicators of health and fitness (Brown et al., 2004). Contaminant-induced reductions in aerobic scope can have negative consequences on fish populations (Johansen and Esbaugh, 2017). ENM exposure influences whole animal $\dot{M}\text{O}_2$ in fish under certain conditions (Callaghan et al., 2016), sometimes in the absence of clear biochemical indicators of toxicity (Black et al., 2017; Campbell et al., 2019). Understanding how $n\text{B}_2\text{O}_3$ exposure affects aerobic metabolism in a variety of fish species will contribute valuable information on its overall environmental safety.

Thus, the present study had two objectives: the first was to characterize the impact of BA and $n\text{B}_2\text{O}_3$ on aerobic metabolism in isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes as a proxy for cellular health. Lowest observable effects concentrations (LOECs) from these experiments, or 48 h LC_{50} trials, were then used to characterize the *in vivo* impacts of acute $n\text{B}_2\text{O}_3$ exposures on a common north-temperate fish, *Fundulus diaphanus*, and two Amazonian species, *Apistogramma agassizii* and *Paracheirodon axelrodi*. We have previously illustrated that these Amazonian species exhibit different sensitivities to ENM exposure as a result of their differing ionoregulatory and metabolic strategies (Braz-Mota et al., 2018), so they represent a powerful model system for studying the potential toxicity of $n\text{B}_2\text{O}_3$. Whole animal aerobic metabolic rates were determined, along with various biochemical indicators of toxicity to gain insight into potential mechanisms of $n\text{B}_2\text{O}_3$ bioactivity.

MATERIALS AND METHODS

Nanoparticles and Characterization

Spherical B_2O_3 nanopowder (#1303862) was purchased from American Elements (Los Angeles, CA, United States) with advertised diameters of 20–80 nm and a specific surface area of $10\text{--}50 \text{ m}^2 \text{ g}^{-1}$. Supplemental scanning electron microscopy (SEM) analysis was carried out to confirm primary particle size and shape. Dry $n\text{B}_2\text{O}_3$ was dusted onto carbon adhesive tabs (Cat. #77825-09, Electron Microscopy Sciences, Hatfield, PA, United States) affixed to aluminum stubs and sputter coated with ca. 5 nm gold using a Hummer 6.2 sputtering system (Anatech United States, Union City, CA, United States). Preparations were examined using a Hitachi SU3500 scanning electron microscope (Hitachi High Technologies, Toronto, Canada) operating at 5 kV and 5 mm working distance. The non-conductive and magnetic nature of the material prevented the use of lower energy beams for improved surface resolution. Morphological measurements of particles were manually acquired from SEM images with dmfMeasure, an image analysis program developed at Mount Allison University's Digital Microscopy Facility.

Hydrodynamic diameter and zeta (ζ) potential were assessed via dynamic light scattering analysis using a Zetasizer Nano ZS (Malvern Panalytical, Malvern, United Kingdom) according to the recommendations of the instrument manufacturer. Fresh $n\text{B}_2\text{O}_3$ stock suspensions (100 mg L^{-1}) were prepared in

0.2 μm -filtered well water from Mount Allison University's Harold Crabtree Aqualab (Sackville, New Brunswick, Canada) or the Instituto Nacional Pesquisas de Amazônia (INPA; Manaus, Amazonas, Brazil). The composition of individual well waters has been described elsewhere (Bessemmer et al., 2015; Braz-Mota et al., 2018) but additional characterization of the dissolved organic carbon (DOC) concentration in Mount Allison University's well water was obtained. Measurements were carried out on filtered and acidified (pH 2 with HCl) samples with an Elementar isoTOC high-temperature catalytic oxidation analyzer (Elementar Americas Inc., NY, United States). Stock nB_2O_3 suspensions were sonicated for 30 s using a wand-type sonicator (F60 Sonic Dismembrator, Fisher Scientific, Ottawa, Canada) immediately prior to analysis.

Animal Collection and Housing

Rainbow trout (*Oncorhynchus mykiss*; body mass 600 ± 25 g) were obtained from Fraser's Mills Fish Hatchery (Nova Scotia, Canada) and banded killifish (*Fundulus diaphanus*; body mass 8.29 ± 0.28 g) were captured by hand netting from a local pond in Sackville, New Brunswick, Canada. Both species were housed in Mount Allison University's Harold Crabtree Aqualab at $16 \pm 1^\circ\text{C}$ in separate 750 L tanks supplied with aerated, flow-through well water. The dwarf cichlid (*Apistogramma agassizii*; body mass 0.320 ± 0.018 g) and cardinal tetra (*Paracheirodon axelrodi*; body mass 0.161 ± 0.010 g) were purchased from an ornamental fish supplier in Manaus and housed at INPA, where they were maintained at $28 \pm 1^\circ\text{C}$ in 450 L tanks of aerated, flow-through well water. All animals were adults. Throughout the acclimation period, all fish were fed daily with dry commercial trout pellets and were kept on a 12 h light, 12 h dark photoperiod. All animals were allowed to acclimate for at least 2 weeks before the start of experiments and food was withheld 24 h before and during experiments. Procedures were approved by the Mount Allison University Animal Care Committee (protocols 15–15 and 101877). For Amazonian fish, procedures followed the CONCEA (National Council of Animal use in Research and Education) animal care guidelines and were approved by INPA's animal care committee (protocol number: 026/2015).

Hepatocyte Isolation and Cellular $\dot{M}\text{O}_2$ Measurements

Initial bioactivity testing was carried out on *O. mykiss* hepatocytes extracted following established protocols (Moon et al., 1985) with minor modifications. Fish were anesthetized with tricaine methanesulfonate (MS222; 300 mg L^{-1}) buffered with NaHCO_3 (600 mg L^{-1}) and euthanized by severing the spinal cord. The hepatic vein was perfused with medium containing (in mmol L^{-1}) NaCl (176), KCl (54), MgSO_4 (0.81), KH_2PO_4 (0.44), Na_2HPO_4 (0.35), NaHCO_3 (5.0), HEPES (10.0) and EGTA (1.0), pH 7.63. After 10 min, the liver was excised and transferred onto a watch glass containing the above medium but with added 0.3 mg mL^{-1} type II collagenase (#9001121, Sigma-Aldrich, United States) and no EGTA. After 30 min, the liver was gently teased apart and the suspension was repeatedly pipetted to break up cell aggregates. Cells were then filtered through a

100 μm nylon mesh, washed three times with perfusion medium containing 2% BSA and 1.5 mmol L^{-1} CaCl_2 , and centrifuged at $1,000 \times g$ for 10 min. Isolated cells were resuspended in Ca^{2+} -free perfusion medium and counted with a Neubauer hemocytometer. Cell viability was assessed by Trypan blue exclusion and preparations with viabilities $<85\%$ were rejected. Cell suspensions were supplied with 99.5% O_2 and 0.5% CO_2 and kept on ice prior to measuring $\dot{M}\text{O}_2$.

Hepatocyte $\dot{M}\text{O}_2$ was measured using OX1LP Dissolved O_2 Cuvette Electrodes (Qubit Systems Inc., Kingston, ON) interfaced to Logger Pro Software via a LabQuest Mini data acquisition system (Vernier Software and Technology, Beaverton, OR, United States). Stock solutions of BA and nB_2O_3 were prepared in isolation medium and the nB_2O_3 stock solution was sonicated using a wand-type F60 sonic dismembrator (Fisher Scientific, Waltham, MA, United States) for 30 s prior to use. Temperature was maintained at $16 \pm 0.1^\circ\text{C}$ using a recirculating water bath and cell suspensions were stirred throughout the experiment. 0.5 mL of cell suspension was transferred into each of the two cuvettes, one with additional isolation medium (control) and one with nB_2O_3 to final concentrations 0.1, 1.0, and 10.0 mg L^{-1} . A triplicate $\dot{M}\text{O}_2$ measurement using aliquots of cell suspension from multiple independent cell isolations was done for each concentration. The average number of cells (16.13×10^4 cells) in the cuvette (0.6 mL) was used to normalize $\dot{M}\text{O}_2$ to $\text{nmol O}_2 \text{ min}^{-1} 10^4 \text{ cells}^{-1}$.

In vivo Exposures

For *F. diaphanus*, nB_2O_3 exposures were carried out in a 30 L static reservoir of continuously aerated well water maintained at $16 \pm 1^\circ\text{C}$. This species was exposed to a final concentration of 1.0 mg L^{-1} nB_2O_3 based on the results of the hepatocyte studies described above. Seven fish were placed in the tank and allowed to acclimate overnight prior to the addition of nB_2O_3 in the form of a freshly prepared and sonicated stock suspension. For control fish, an equivalent volume of ddH_2O was added to the tank as a sham treatment. Animals were held for 48 h following nB_2O_3 or sham treatment before tissue sampling (see below). The relatively large volume of the experimental tank relative to the biomass of fish made a water change unnecessary, as ammonia did not increase above background levels during the exposure (data not shown).

Initial studies on *A. agassizii* and *P. axelrodi* focused on establishing a 48 h LC_{50} concentration for nB_2O_3 . Static renewal exposures were carried out in glass aquaria containing 2 L of continuously aerated INPA well water at $28 \pm 1^\circ\text{C}$, with 10 fish per aquarium. Fish were allowed to acclimate overnight, after which 50% of the water was changed and nB_2O_3 was added to final concentrations of 0, 0.1, 1.0, 10, and 100 mg L^{-1} . A second 50% water change including the appropriate concentration of nB_2O_3 was carried out 24 h later and animals were monitored up to 48 h exposure.

At the end of the exposure period, the fish were anesthetized with MS222 buffered with Na_2CO_3 . The fish were euthanized by severing the spinal cord and tissue samples were collected, flash frozen in liquid nitrogen, and stored at -80°C until use. For *F. diaphanus*, brain, heart, liver, and gill samples were collected,

and for *A. agassizii* and *P. axelrodi*, gill and whole body samples were collected. For *A. agassizii* and *P. axelrodi*, only fish from the control and 10 mg L⁻¹ nB₂O₃ exposure groups were used for biochemical analyses.

Whole Animal Oxygen Consumption Measurements

The effect of nB₂O₃ exposure on aerobic metabolism *in vivo* was assessed by measuring $\dot{M}O_2$ in whole animals. For *F. diaphanus*, exposures were carried out on individual fish housed in a 140 mL intermittent flow respirometry chamber (Q-box Aqua, Qubit Systems, Kingston, Ontario, Canada) within the same 30 L static reservoir of continuously aerated well water maintained at 16 ± 1°C described above. Animals were placed in the respirometer in the afternoon and allowed to recover overnight before being exposed to 1.0 mg L⁻¹ nB₂O₃ or a sham control. Routine $\dot{M}O_2$ ($\dot{M}O_{2min}$) was then determined in undisturbed fish up to 46 h post-exposure, after which fish were moved to a 1 L beaker of exposure water and manually chased to exhaustion (Healy et al., 2017). When *F. diaphanus* no longer responded to stimulation, they were quickly returned to the respirometer to measure maximum $\dot{M}O_2$ ($\dot{M}O_{2max}$) as described elsewhere (Black et al., 2017).

Based on the results of the LC₅₀ testing, *A. agassizii* and *P. axelrodi* were exposed to 10 mg L⁻¹ nB₂O₃ or a sham control. Exposures were carried out on 6 fish simultaneously using the same static renewal protocol described above for LC₅₀ testing. Immediately following the 48 h exposure, animals were transferred to a multi-channel intermittent flow respirometry system (Oxy-4; Loligo Systems, Vidborg, Denmark) and left undisturbed for a minimum of 12 h in clean well water. $\dot{M}O_{2min}$ was then quantified as previously described for these species (Braz-Mota et al., 2018). The layout of the multi-channel respirometry system precluded accurate measurements of $\dot{M}O_{2max}$. The individual *A. agassizii* and *P. axelrodi* used for $\dot{M}O_{2min}$ measurements were not sampled for biochemical analysis.

Biochemical Procedures

Lactate levels were measured in all species as an indicator of anaerobic stress. For *F. diaphanus*, lactate was measured in liver, while in *A. agassizii* and *P. axelrodi*, it was assessed in whole body homogenates prepared as previously described (Braz-Mota et al., 2018). *Fundulus diaphanus* liver samples were homogenized in 2% perchloric acid, centrifuged at 10,000 × *g* for 10 min, and the supernatant collected for analysis. In all instances, lactate was quantified by following the reduction of NAD⁺ to NADH by lactate dehydrogenase (LDH) at 340 nm in a glycine-hydrazine buffer and referenced to an L-lactic acid standard curve.

For *F. diaphanus*, lipid peroxidation and glucose-6-phosphate dehydrogenase (G6PDH) and glutathione reductase (GR) activities were used as indicators of oxidative stress in liver. Hepatic lipid peroxidation in *F. diaphanus* was quantified as malondialdehyde (MDA) using a commercially available kit (Bioxytech® MDA-586, Oxisresearch, Berlingame, CA, United States) according to the manufacturer's instructions.

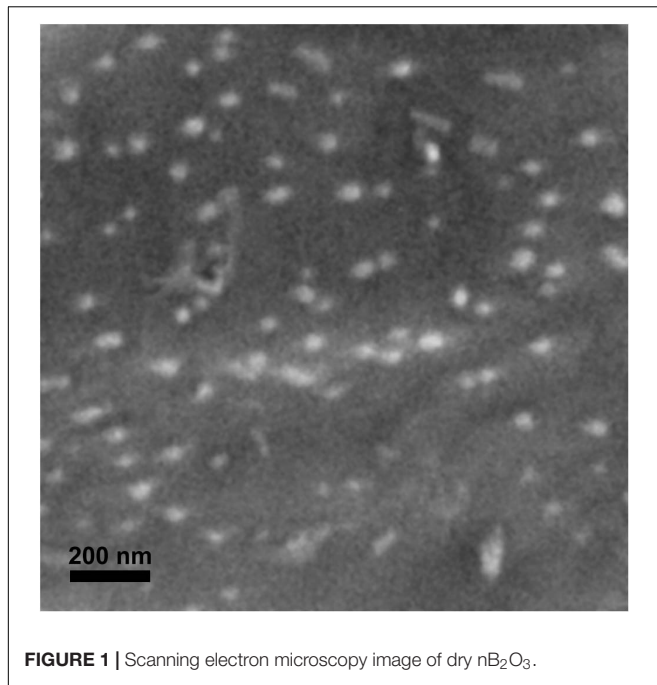
The activities of liver G6PDH and GR were measured using colorimetric assays that followed changes in NADPH absorbance at 340 nm (Dieni et al., 2014) and an extinction coefficient of 6.22 mM⁻¹ cm⁻¹. Oxidative stress assessments in *A. agassizii* and *P. axelrodi*, were carried out on whole body homogenates as previously described (Braz-Mota et al., 2018). In these species, lipid peroxidation was quantified using the colorimetric assay described by Jiang et al. (1991) and glutathione S-transferase (GST) activity was measured according to Keen et al. (1976). For GST assays, changes in 1-chloro-2,4-dinitrobenzene absorbance at 340 nm were converted to activities using an extinction coefficient of 9.6 mM⁻¹ cm⁻¹ and normalized to protein content using a Bradford assay (Bio-Rad Laboratories, Hercules, CA, United States).

Na⁺/K⁺-ATPase (NKA) activity is a sensitive indicator of ionoregulatory disruptions in fish gills following exposure to a variety of ENM formulations (Federici et al., 2007; Farnen et al., 2012; Schultz et al., 2012; Katuli et al., 2014; Bessemer et al., 2015; Black et al., 2017). NKA activity was measured in gill tissue from *F. diaphanus*, *A. agassizii*, and *P. axelrodi* by following the oxidation of NADH at 340 nm using a coupled colorimetric assay protocol (McCormick, 1993) and assuming an extinction coefficient of 6.22 mM⁻¹ cm⁻¹. Activities were normalized to total protein content using a DC protein assay (Bio-Rad Laboratories). Acetylcholinesterase (AChE) activity, which is a common target of xenobiotics and ENMs in fish (Katuli et al., 2014; Callaghan et al., 2016), was assayed in brain, gill, and heart tissue of *F. diaphanus* and in whole body homogenates of *A. agassizii* and *P. axelrodi* using the Ellman method (Ellman et al., 1961). Activity was calculated using an extinction coefficient of 13.6 mM⁻¹ cm⁻¹.

In all cases, absorbance was followed using either a Spectramax 190 or M2 microplate spectrophotometer (Molecular Devices, CA, United States).

Data Analysis and Statistics

For whole animal studies, mass specific $\dot{M}O_2$ was automatically calculated from decreases in dissolved O₂ using the software associated with each respirometry system. In all species, $\dot{M}O_{2min}$ was determined from a minimum of 3 representative $\dot{M}O_2$ values taken from undisturbed fish in the final 2 h of each trial. In *F. diaphanus*, $\dot{M}O_{2max}$ represented the highest $\dot{M}O_2$ value recorded following the exhaustive chase protocol and aerobic scope was calculated as the difference between $\dot{M}O_{2min}$ and $\dot{M}O_{2max}$. Variability in $\dot{M}O_2$ recordings from undisturbed fish can be used as a proxy for spontaneous aerobic activity in fish (Campbell et al., 2019). Frequency distributions of $\dot{M}O_2$ recordings from the final 5 h of each respirometry trial were generated using Prism 7 (GraphPad Software, Inc., CA, United States). Plots illustrate the number of $\dot{M}O_2$ recordings from each species that fell within each 30 mg kg⁻¹ h⁻¹ bin. The frequency distributions thus provide a qualitative means to visualize variability in the aerobic activity of the animals over the selected recording period (Campbell et al., 2019). In all trials, $\dot{M}O_2$ returned to baseline levels within the first 4 h after insertion into the respirometry system and fish were



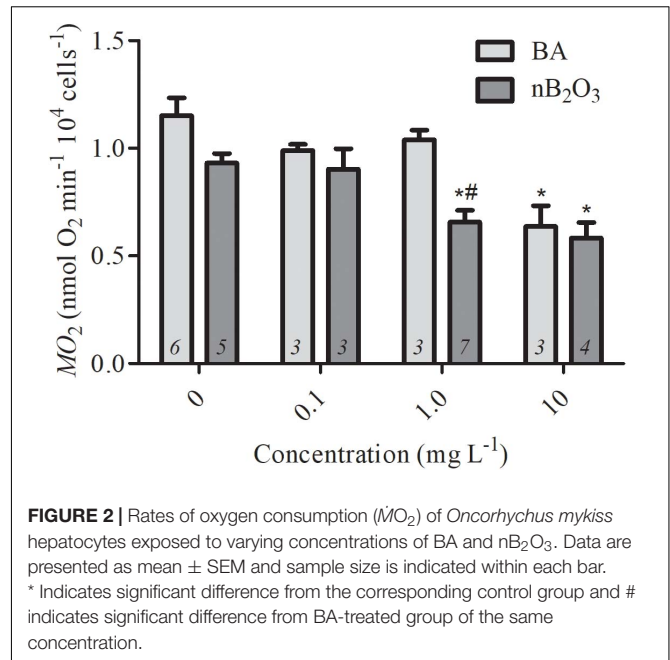
not disturbed during the period over which the frequency distribution analysis was examined.

Oncorhynchus mykiss hepatocyte $\dot{M}O_2$ data were analyzed using a two-way ANOVA followed by a Bonferroni *post hoc* test. Data from *F. diaphanus* studies were analyzed by one-way ANOVA to assess differences between control, BA, and nB₂O₃ treatment groups. When significance was detected, Tukey *post hoc* tests were used for multiple comparisons. Data from the two Amazonian species were analyzed with two-tailed Student's *t*-tests. $P < 0.05$ was used to indicate statistical significance. Box and whisker plots display the range and median values of the data set while all other data is presented as the mean \pm standard error of the mean (SEM).

RESULTS

nB₂O₃ Characterization

The low atomic density of B₂O₃ prevented acquisition of high resolution images with the available instrument but images were sufficient for basic characterization purposes. Dry nB₂O₃ were spherical or oblong in shape with a mean primary particle diameter of 36.2 ± 1.4 nm ($n = 20$; **Figure 1**). Spherical particles ranged from 17 to 60 nm, while several higher aspect ratio particles were $\sim 125 \times 50$ nm. Dynamic light scattering illustrated hydrodynamic diameters of 189 ± 9 nm in *F. diaphanus* exposure water and 368 ± 43 nm in *P. axelrodi* and *A. agassizii* exposure water. Some degree of agglomeration thus occurred in all exposure scenarios and it was more evident in Amazonian water. This interpretation is supported by polydispersity indices of 0.230 for nB₂O₃ in *F. diaphanus* exposure water and 0.348 in Amazonian water (*P. axelrodi* and *A. agassizii* exposures). The DOC content of *F. diaphanus* exposure water determined



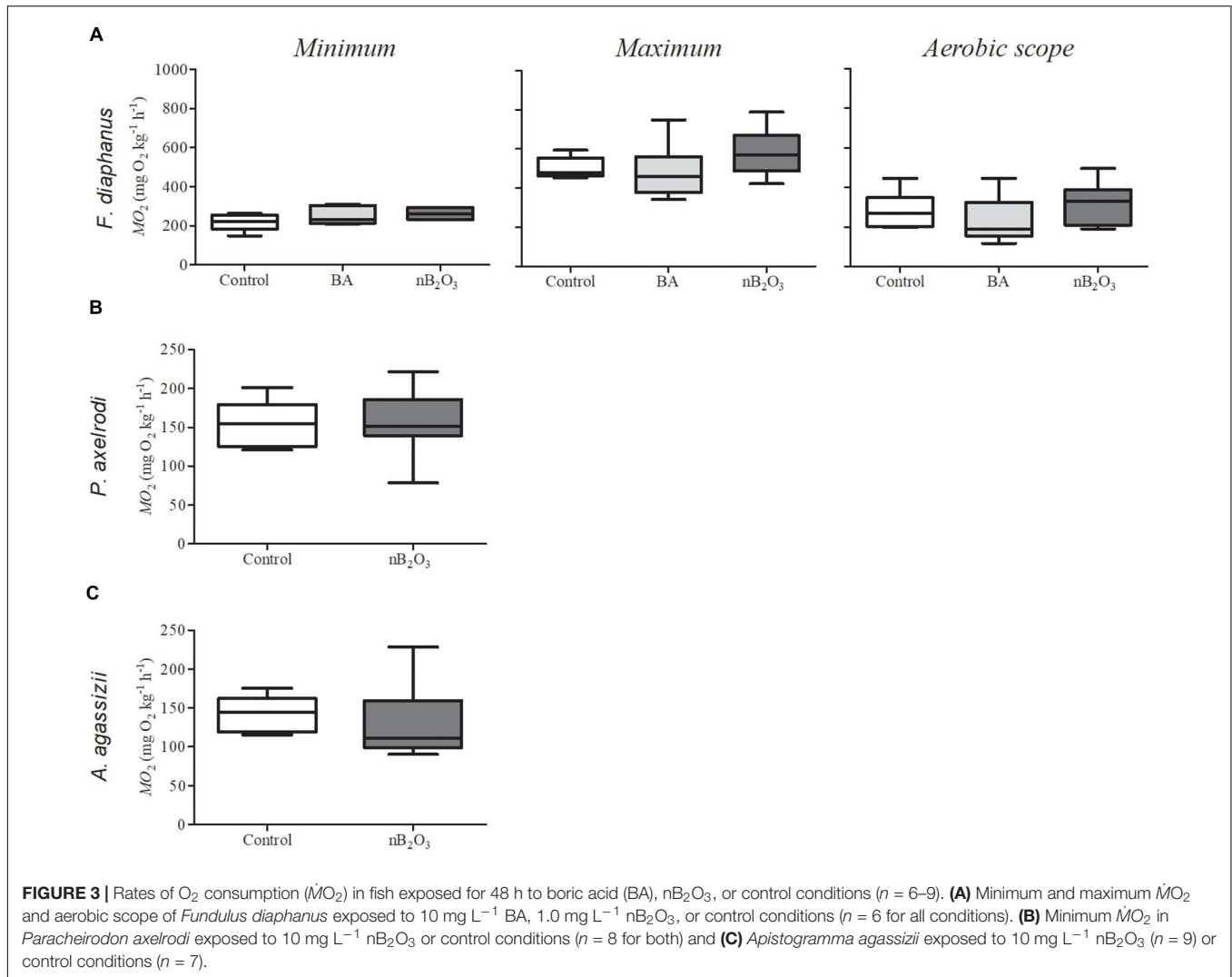
here was 2.14 ± 0.55 and 0.8 mg L⁻¹ in the Amazonian water (Sadauskas-Henrique et al., 2016). The higher DOC content of *F. diaphanus* exposure water may act to stabilize nB₂O₃ suspensions and limit agglomeration. ζ -potential was -17.2 ± 2.0 and -20.3 ± 1.2 mV for *F. diaphanus* exposure water and *P. axelrodi* and *A. agassizii* exposure water, respectively. The pH of the Amazonian water was 5.5 and nB₂O₃ had no effect on pH at concentrations up to 100 mg L⁻¹. The pH of *F. diaphanus* exposure water was 7.5 and 100 mg L⁻¹ nB₂O₃ increased it by 0.5 units.

$\dot{M}O_2$ of Rainbow Trout Hepatocytes Exposed to BA and nB₂O₃

The bioactivity of nB₂O₃ has not been well-characterized so a lowest observable effect concentration was first established in isolated *O. mykiss* hepatocytes and compared to BA to assess potential nano-specific effects (**Figure 2**). Both treatment ($p = 0.0025$) and concentration ($p < 0.0001$) significantly impacted hepatocyte $\dot{M}O_2$ and no interaction was noted (**Figure 2**). nB₂O₃ significantly reduced cellular $\dot{M}O_2$ at 1.0 mg L⁻¹ relative to both the BA and control groups ($p < 0.01$). The $\dot{M}O_2$ of both treatment groups at 10 mg L⁻¹ were lower than those of the corresponding control groups (both $p < 0.01$) but were not significantly different from one another. Based on these data, exposure concentrations of 1.0 and 10 mg L⁻¹ were assigned for nB₂O₃ and BA, respectively, and used for subsequent whole animal studies on *F. diaphanus*.

Paracheirodon axelrodi and *Apistogramma agassizii* nB₂O₃ 48 h LC₅₀ Tests

During 48 h LC₅₀ testing with nB₂O₃, two *P. axelrodi* died at the highest exposure concentration of 100 mg L⁻¹ (data not shown).



No other mortalities were noted in either species so an LC₅₀ for nB₂O₃ could not be calculated. Sub-lethal effects were obvious at the highest doses; at 100 mg L⁻¹ nB₂O₃, almost all fish (both species) lost equilibrium ~30 min after the initial addition of nB₂O₃ and subsequently recovered several hours later. A mass loss of equilibrium was again observed after the 50% water change 24 h into the exposure and the animals again recovered several hours later. At the next highest dose of 10 mg L⁻¹, fish of both species were clearly lethargic relative to untreated controls. As our focus was on potential sub-lethal effects of nB₂O₃, follow-up studies on *P. axelrodi* and *A. agassizii* employed an exposure dose of 10 mg L⁻¹.

Effects on Whole Animal and Tissue Energy Metabolism

Loss of equilibrium and/or lethargy was not noted in *F. diaphanus* immediately following nB₂O₃ addition but, objectively, this species is less active than the Amazonian species and studies were carried out in opaque holding tanks which precluded

accurate behavioral observations. In *F. diaphanus*, whole animal $\dot{M}O_{2min}$, $\dot{M}O_{2max}$, and aerobic scope were not affected by a 48 h BA (10 mg L⁻¹) or nB₂O₃ (1.0 mg L⁻¹) exposure (**Figure 3A**). $\dot{M}O_{2min}$ was similarly unaffected in *P. axelrodi* and *A. agassizii* exposed to nB₂O₃ (10 mg L⁻¹) for 48 h (**Figures 3B,C**, respectively).

Qualitatively, spontaneous aerobic activity in undisturbed fish appeared similar between control and nB₂O₃ treatment groups in all species, with the possible exception of *P. axelrodi* (**Figure 4**). $\dot{M}O_2$ was very consistent and stable in *F. diaphanus*, highly variable in *P. axelrodi*, and intermediate in *A. agassizii*. nB₂O₃ treated *P. axelrodi* exhibited periods of elevated aerobic activity not evident in control fish, reaching 5–6-fold above $\dot{M}O_{2min}$.

In *F. diaphanus*, there were no significant differences in liver glucose levels among treatment groups (**Table 1**). Both BA and nB₂O₃ treated *F. diaphanus* exhibited significant hepatic lactate accumulation relative to controls ($p = 0.001$) and nB₂O₃ treated fish accumulated more lactate than BA treated animals (**Table 1**). Whole body lactate levels were low and unaffected by nB₂O₃ treatment in the two Amazonian species.

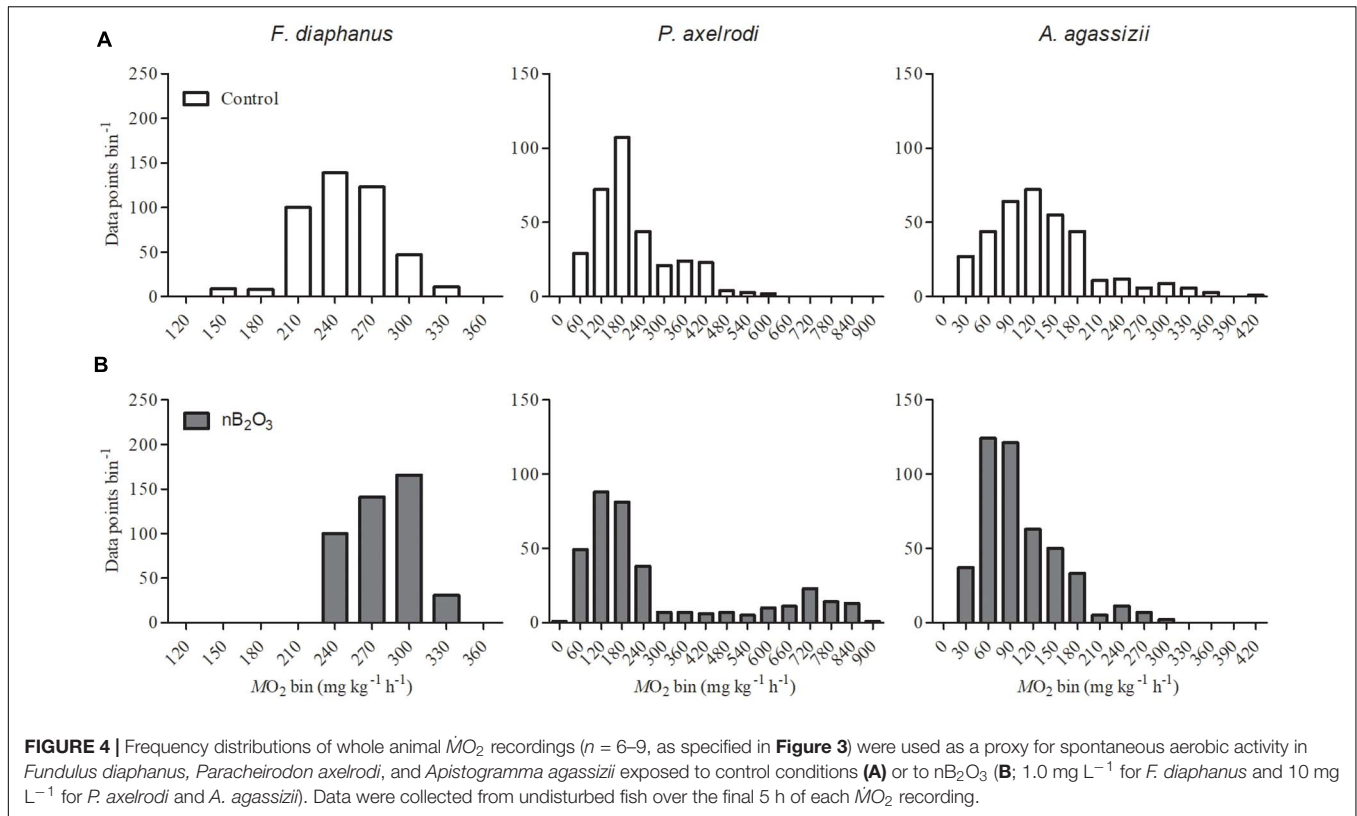


FIGURE 4 | Frequency distributions of whole animal $\dot{M}O_2$ recordings ($n = 6-9$, as specified in **Figure 3**) were used as a proxy for spontaneous aerobic activity in *Fundulus diaphanus*, *Paracheirodon axelrodi*, and *Apistogramma agassizii* exposed to control conditions (**A**) or to nB_2O_3 (**B**: 1.0 mg L^{-1} for *F. diaphanus* and 10 mg L^{-1} for *P. axelrodi* and *A. agassizii*). Data were collected from undisturbed fish over the final 5 h of each $\dot{M}O_2$ recording.

TABLE 1 | Effects of BA (10 mg L^{-1}) and nB_2O_3 (1.0 mg L^{-1} for *Fundulus diaphanus*, 10.0 mg L^{-1} for *Paracheirodon axelrodi*, and *Apistogramma agassizii*) exposure on biomarkers of energy metabolism and oxidative stress.

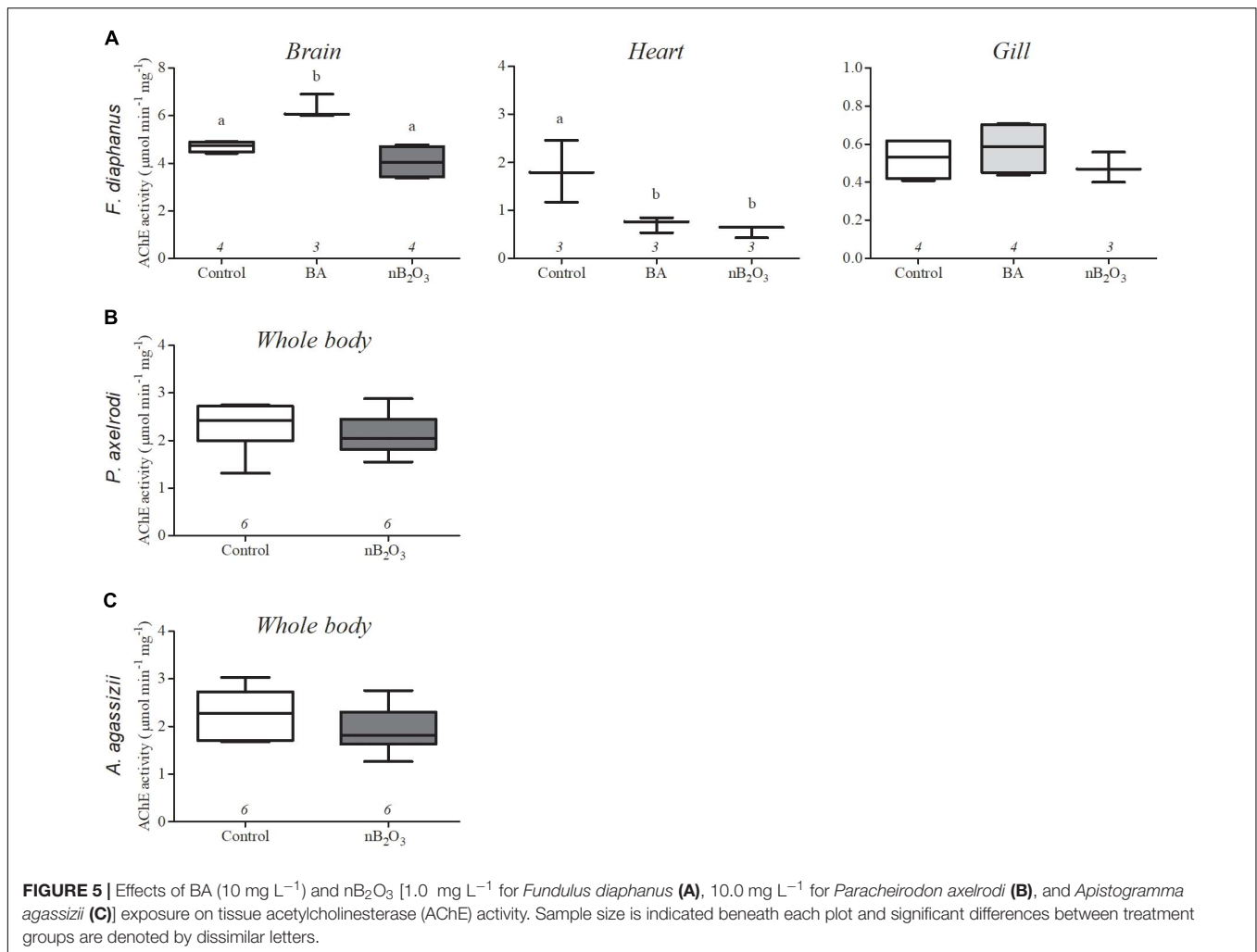
Biomarker	Species	Tissue	Treatment		
			Control	BA	nB_2O_3
Energy metabolism					
Glucose ($\mu\text{mol g tissue}^{-1}$)	<i>F. diaphanus</i>	Liver	24.56 ± 1.87 (6)	19.83 ± 4.11 (6)	21.55 ± 2.01 (6)
Lactate ($\mu\text{mol g tissue}^{-1}$)	<i>F. diaphanus</i>	Liver	11.18 ± 1.22 ^a (9)	16.36 ± 0.80 ^b (9)	20.38 ± 2.17 ^c (9)
	<i>P. axelrodi</i>	W.B.	0.67 ± 0.09 (6)		0.57 ± 0.07 (6)
	<i>A. agassizii</i>	W.B.	0.96 ± 0.22 (6)		1.51 ± 0.22 (6)
Oxidative stress					
G6PDH ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$)	<i>F. diaphanus</i>	Liver	0.19 ± 0.02 (3)	0.18 ± 0.03 (3)	0.14 ± 0.05 (3)
GR ($\text{nmol min}^{-1} \text{mg protein}^{-1}$)		Liver	35.98 ± 7.90 (3)	38.28 ± 5.37 (3)	24.98 ± 7.51 (3)
MDA ($\mu\text{mol mg protein}^{-1}$)		Liver	1.53 ± 0.42 (3)	1.59 ± 0.32 (3)	1.77 ± 0.38 (3)
LPO ($\mu\text{mol L}^{-1} \text{cumene H}_2\text{O}_2 \text{mg protein}^{-1}$)	<i>P. axelrodi</i>	W.B.	46.87 ± 5.70 (6)		55.65 ± 8.84 (6)
	<i>A. agassizii</i>	W.B.	59.21 ± 7.61 (6)		57.36 ± 6.37 (6)
GST ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$)	<i>P. axelrodi</i>	W.B.	1.46 ± 0.20 (6)		1.43 ± 0.21 (6)
	<i>A. agassizii</i>	W.B.	1.35 ± 0.68 (6)		0.78 ± 0.10 (6)

WB, whole body. Sample size is indicated in parentheses and statistically significant differences between treatment groups are indicated by dissimilar letters.

Indicators of Oxidative Stress and Toxicity

A number of oxidative stress indicators were assessed in the 3 study species used for *in vivo* exposures (**Table 1**). Activities of anti-oxidant defense enzymes in *F. diaphanus* were unchanged following exposure to BA or nB_2O_3 and a similar lack of response was noted for *P. axelrodi*, and *A. agassizii*. Lipid peroxidation products, which are indicative of oxidative damage, did not vary significantly in any of the species examined.

In *F. diaphanus*, brain AChE activity was significantly impacted by treatment ($p = 0.0012$) and increased in response to BA but not to nB_2O_3 (**Figure 5**). Heart AChE activity was also significantly affected by treatment ($p = 0.0159$), with inhibition by both BA and nB_2O_3 , while activity was unaffected in gill. Whole body AChE activities were not altered by nB_2O_3 exposure in either *P. axelrodi* or *A. agassizii*.



Gill NKA activity in *F. diaphanus* was significantly altered by treatment ($p = 0.0234$), with exposure to either BA or nB₂O₃ modestly increasing activity relative to controls (Figure 6). *Paracheirodon axelrodi* exhibited the highest gill NKA activity of the 3 species assessed but activity was not affected by nB₂O₃ exposure in either it or *A. agassizii*.

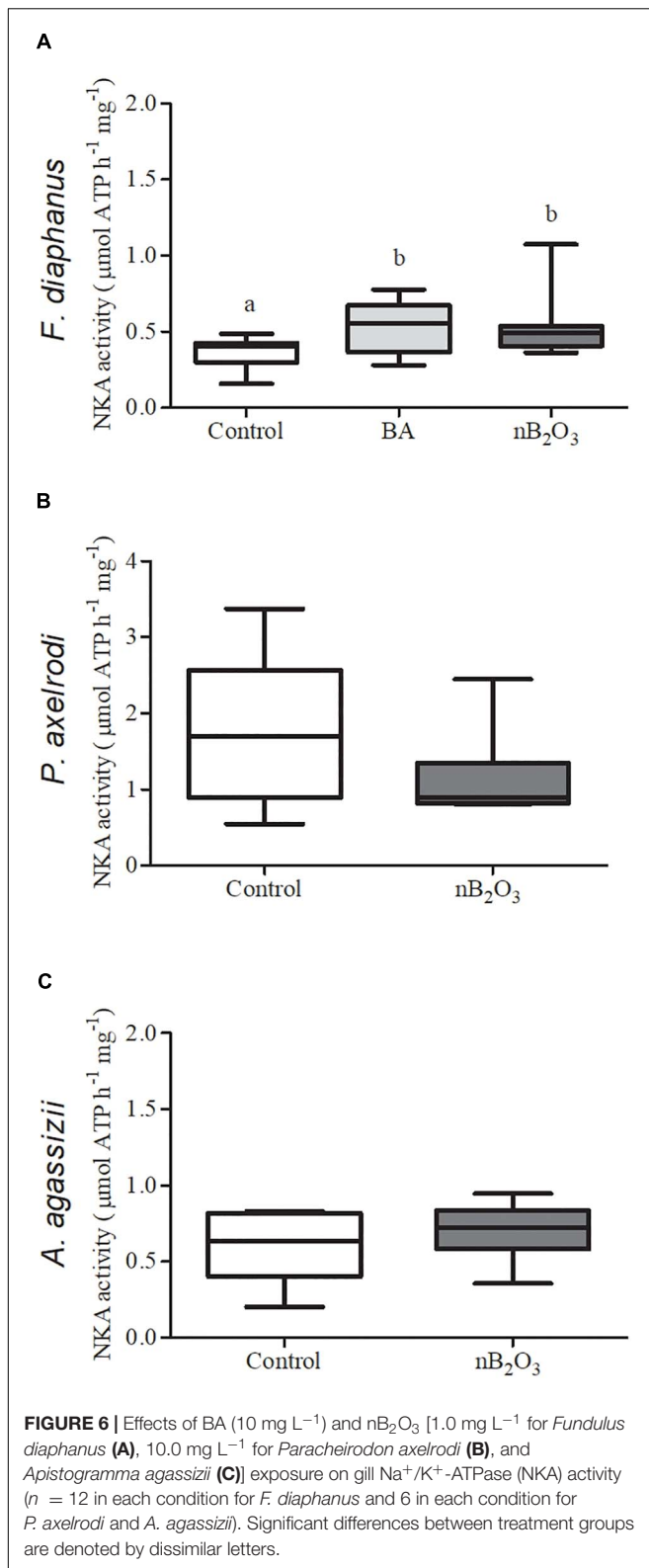
DISCUSSION

Increased industrial use of nB₂O₃ will inevitably lead to their release into the environment but their potential impacts on non-target organisms are almost entirely unknown. The natural form of boron (BA) is considered essentially non-toxic to vertebrates (Woods, 1994), but it is difficult to predict if bioactivity will also be low in nanoparticulate boron formulations. We addressed this gap in knowledge by examining cellular, whole animal, and biochemical indicators of nB₂O₃ toxicity in 3 fish species known to exhibit differing sensitivities to contaminants. Bioactivity was evident at all levels of biological organization examined and species-specific responses were observed, but only at relatively high exposure doses.

nB₂O₃ Effects on Metabolism, Ionoregulation, and Neurotransmission Are Species-Specific

In rainbow trout hepatocytes, nB₂O₃ was bioactive at a lower concentration than BA, with $\dot{M}O_2$ decreased at exposure doses of 1.0 vs. 10 mg L⁻¹, respectively. The mechanism(s) underlying $\dot{M}O_2$ inhibition were not assessed but boron can interact with a variety of enzymes and cofactors (Woods, 1994), which could subsequently reduce aerobic metabolic demand or trigger cell death. The high exposure doses required to elicit hepatocyte responses suggest that neither nB₂O₃ nor BA are particularly toxic, at least for this cell type.

Some ENM formulations specifically alter $\dot{M}O_{2min}$ or $\dot{M}O_{2max}$ in fish (Black et al., 2017; Campbell et al., 2019), while in other species, subtle effects on both contribute to reductions in aerobic scope (Callaghan et al., 2016). The aerobic metabolic depression induced by nB₂O₃ in isolated hepatocytes was not replicated in whole animal exposures with any species tested. However, hepatocyte $\dot{M}O_2$ was measured immediately following nB₂O₃ addition, while whole animal respirometry trials were only carried out following 48 h of nB₂O₃ exposure. The initial



loss of equilibrium and lethargy we witnessed immediately after nB₂O₃ exposure in the 2 Amazonian species could be associated with a decrease in $\dot{M}O_2$, but additional studies are necessary to

confirm this short-term effect of nB₂O₃ on aerobic metabolism of both species. In white sucker, *Catostomus commersonii*, zinc oxide ENM exposure (1.0 mg L⁻¹) triggers a temporary decrease in heart rate (Bessemmer et al., 2015) and reduces aerobic scope (Callaghan et al., 2016). We speculated that this related to a disruption of parasympathetic control of the cardiorespiratory system, either via ENM interactions with gill chemoreceptors or through direct inhibition of cardiac AChE activity, which led to a pseudo-hypoxic response. In white sucker, zinc oxide ENMs inhibited cardiac AChE activity and enhanced gill NKA activity (Bessemmer et al., 2015; Callaghan et al., 2016), the latter likely resulting from an increase in gill blood flow. Inhibition of cardiac AChE and increased gill NKA were also observed here in *F. diaphanus* exposed to nB₂O₃. It is possible that acute nB₂O₃ exposure may trigger a similar, temporary pseudo-hypoxic response in some fish species, although more data is needed to support this supposition.

The $\dot{M}O_2$ variability analysis indicated that after 48 h exposure, spontaneous activity might increase in nB₂O₃ treated *P. axelrodi*, but not in the other species tested. The highly variable $\dot{M}O_2$ data from *P. axelrodi* may be related to its biology; *P. axelrodi* is an active species that is highly dependent on aerobic metabolism, and the large increases in $\dot{M}O_2$ may be associated with a stress response. This species is likely to exploit aerobic activity in an attempt to escape the nB₂O₃ contaminated environment. In contrast, *A. agassizii* and *F. diaphanus* are less active species and may be more resilient to stressors. This same stress pattern was seen for *P. axelrodi* whereby $\dot{M}O_{2min}$ increased almost threefold when fish were acutely exposed to 45 μg L⁻¹ copper for 24–72 h, while no alterations were observed in *A. agassizii* (Braz-Mota et al., 2018). Such a response may indicate that *P. axelrodi* is more vulnerable to nB₂O₃ exposure under natural conditions, as such additional aerobic demands would leave less scope available for activities such as foraging, reproduction, or predator avoidance.

Gill Na⁺ uptake mechanisms differ between *A. agassizii* and *P. axelrodi* and may contribute to their distinct (putative) behavioral responses to nB₂O₃, although no differences in gill NKA activity were noted in either species in response to nB₂O₃ exposure. Although qualitative, these findings, along with the temporary loss of equilibrium of both Amazonian species at high exposure doses, warrant further investigation. Other ENM formulations significantly alter fish behavior with few other signs of overt toxicity (Botha et al., 2019) and these effects may translate to greater population-level impacts than changes in biochemical markers of toxicity alone (Scott and Sloman, 2004).

Exposure to BA or nB₂O₃ triggered significant liver lactate accumulation in *F. diaphanus*, while nB₂O₃ had no effect on whole body lactate in *P. axelrodi* or *A. agassizii*. If lactate accumulation was restricted to liver in *P. axelrodi* or *A. agassizii*, it may not have been detected in whole body homogenates. Glucose levels were not affected in any species under the conditions tested. Lactate usually accumulates in the absence of sufficient oxygen, but disruption of a metabolic pathway could also cause such a response. In the liver of white sucker, zinc oxide ENMs oxidatively inhibits aconitase (Dieni et al., 2014), the enzyme responsible for isomerizing citrate

to isocitrate in the tricarboxylic acid (TCA) cycle. Decreased activity of this enzyme could theoretically inhibit flux through the TCA cycle and trigger the activation anaerobic metabolism even in the presence of abundant oxygen. This explanation is not well supported however, since we found no evidence of oxidative stress (see below). The observed increase in gill NKA activity in *F. diaphanus* hints at the possibility that BA or nB₂O₃ may have damaged the gill epithelium. If this occurred, impaired O₂ uptake and hypoxemia may trigger hepatic lactate accumulation. This explanation is equally dubious though, as $\dot{M}O_{2max}$, which is dependent upon a functional O₂ transport cascade, was not affected by BA or nB₂O₃ in *F. diaphanus*.

Gill NKA activity in fish is impacted by exposure to a variety of ENM formulations (Federici et al., 2007; Farmen et al., 2012; Schultz et al., 2012; Katuli et al., 2014; Bessemer et al., 2015; Black et al., 2017). Both BA and nB₂O₃ exposure significantly stimulated gill NKA activity in *F. diaphanus*, but nB₂O₃ exposure had no effect on activity in either Amazonian species. ENM-induced gill membrane damage may also indirectly stimulate NKA activity (Bessemer et al., 2015), but the maintenance of $\dot{M}O_{2min}$ and $\dot{M}O_{2max}$ suggests the gill is healthy, at least with respect to its respiratory functions. As mentioned above, it is possible that an increase in gill blood flow resulting from pseudo-hypoxic response could enhance diffusive Na⁺ losses to the environment, stimulating NKA activity as a compensatory response to maintain Na⁺ balance in this species. Boron also interacts with vertebrate membranes and alters their biophysical properties (Verstraeten et al., 2005), which may affect NKA function. The molecular activity of NKA increases as lateral membrane pressures increase (Wu et al., 2004), so it is possible that boron stimulated NKA activity by altering the structure of the gill basolateral membrane. Clearly, more work is necessary to confidently assign a mechanism to this observation and to evaluate the potential of BA and nB₂O₃ to disrupt Na⁺ homeostasis in *F. diaphanus*.

nB₂O₃ Exposure Does Not Result in Oxidative Stress

Oxidative stress is a common mechanism of toxicity across a wide variety of ENM formulations and is frequently observed in ENM-exposed fish (Handy et al., 2011; Callaghan and MacCormack, 2017). The activities of G6PDH and GR were examined in liver tissue of *F. diaphanus* and GST activity was assessed in whole body homogenates of *P. axelrodi* or *A. agassizii*; these enzymes play important roles in the defense against oxidative stress (Tian et al., 1999; Hellou et al., 2012). G6PDH produces the NADPH required by GR to reduce oxidized glutathione, which is a key anti-oxidant molecule responsible for scavenging ROS, while GST conjugates reduced glutathione to xenobiotics to defend against oxidative stress. None of these enzymes were impacted by nB₂O₃ exposure, nor were LPO or MDA concentrations, which are biomarkers of oxidative damage. Under the conditions tested, nB₂O₃ exposure does not illicit a significant oxidative stress response in any of the species tested.

Bioactivity Is Similar Between Boric Acid and nB₂O₃

In most instances, BA and nB₂O₃ exposure triggered similar biological responses, with a few exceptions. In isolated rainbow trout hepatocytes, nB₂O₃ impacted cellular $\dot{M}O_2$ at a lower exposure concentration than BA but this effect did not translate to *in vivo* $\dot{M}O_2$ responses in *F. diaphanus*, where neither compound had an effect. At a lower exposure concentration, nB₂O₃ also triggered a significantly greater accumulation of lactate in *F. diaphanus* but the general response to BA was still similar. Lastly, BA significantly increased brain AChE activity, while nB₂O₃ had no effect. These subtle differences in bioactivity could relate to the relative bioavailability of each compound at the target site of action. Cellular boron uptake occurs via specific transporters (Ocampo-Néstor et al., 2017) while ENMs are often internalized and processed via the lysosomal system (Wang et al., 2013). It is unclear how these mechanisms may impact the downstream bioavailability and bioactivity of BA and nB₂O₃. Overall, there are few clear nano-specific effects of nB₂O₃ but the bioactivity that is noted is realized at lower exposure doses than BA.

CONCLUSION

nB₂O₃ is one of a number of boron ENM formulations with potential industrial, consumer, and clinical applications but little information is available on their potential ecotoxicity. Our results illustrate that nB₂O₃ exhibits species-specific bioactivity toward north-temperate and Amazonian freshwater fish. To our knowledge, environmental nB₂O₃ concentrations have not been studied, but based on modeling for other ENM formulations (Gottschalk et al., 2009; Keller and Lazareva, 2014), the exposure doses required to generate bioactivity (1–10 mg L⁻¹) likely far exceeded environmental relevance. A lower nB₂O₃ exposure (0.1 mg L⁻¹) dose had no effect on cellular $\dot{M}O_2$ in hepatocytes but additional concentrations should be assessed in whole animal exposures to rule out the possibility of a hormetic response, which is observed with other ENM formulations (Iavicoli et al., 2014).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Mount Allison University Animal Care Committee.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication

FUNDING

TM and SW were supported by Natural Sciences and Engineering Research Council of Canada Discovery grants. PG was supported by a New Brunswick Innovation Foundation (NBIF) Research Assistantship and by a Mount Allison University President's Research and Creative Activity Award. LT was supported by NBIF and the Canadian Foundation for Innovation (Grant No. 35589). AV and VA-V received a research fellowship from the Brazilian Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). SB-M received an MSc fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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ACKNOWLEDGMENTS

We thank Mr. Wayne Anderson and Mr. James Ehrman for assistance with animal care and electron microscopy analyses, respectively. We also thank the staff and students of the Laboratory of Ecology and Molecular Evolution at INPA for logistical assistance.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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