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Settleable atmospheric particulate matter affects cardiorespiratory responses to hypoxia in Nile tilapia (*Oreochromis niloticus*)

De Angelis C.F. [1, *], Soares M.P. [1], Cardoso I.L. [1], Filogonio R. [1], Taylor E.W. [2], McKenzie D.J. [3], Souza I.C. [1], Wunderlin D.A. [4], Monferrán M.V. [4], Fernandes M.N. [1], Leite C.A.C. [1]

[1] Department of Physiological Sciences Department, Federal University of São Carlos, Rod Washington Luis km 235, 13565-905 São Carlos, SP, Brazil
[2] School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
[3] MARBEC, Université de Montpellier, CNRS, Ifremer, IRD, Montpellier 34095, France
[4] ICYTAC: Instituto de Ciencia y Tecnología de Alimentos Córdoba, CONICET, Departmento de Química Orgánica, Universidad Nacional de Córdoba, Bv. Medina Allende s/n, Ciudad Universitaria, 5000 Córdoba, Argentina

* Corresponding author : C. F. De Angelis, email address : caroldeangelis3@hotmail.com

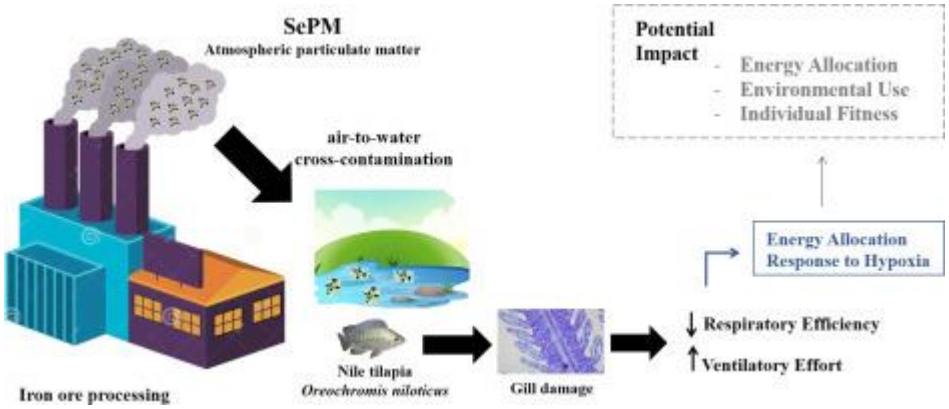
david.mckenzie@cnrs.fr ; iara@ufscar.br ; mmonferran@unc.edu.ar ; dmnf@ufscar.br ; cleo.leite@ufscar.br

Abstract :

Atmospheric particulate matter (APM) emitted by iron ore processing industries has a complex composition, including diverse metallic particles and nanoparticles. Settleable APM (SePM) causes air to water cross-contamination and has recently been demonstrated to have harmful sublethal impacts on fish, eliciting stress responses, affecting the immune system, and reducing blood oxygen-carrying capacity. These findings imply potential consequences for fish aerobic performance and energy allocation, particularly in their ability to tolerate respiratory challenges such as aquatic hypoxia. To assess that potential limitation, we analyzed metabolic, cardiorespiratory, and morphological alterations after exposing tilapia, Oreochromis niloticus, to an environmentally relevant concentration of SePM (96 h) and progressive hypoxia. The contamination initiated detectable gill damage, reducing respiratory efficiency, increasing ventilatory effort, and compromising fish capacity to deal with hypoxia. Even in normoxia, the resting respiratory frequency was elevated and limited respiratory adjustments during hypoxia. SePM increased O2crit from 26 to 34% of O2 (1.84 to 2.76 mg O2·L−1). Such ventilatory inefficacy implies higher ventilatory cost with relevant alterations in energy allocation. Progression in gill damage might be problematic and cause: infection, blood loss, ion imbalance, and limited cardiorespiratory performance. The contamination did not cause immediate lethality but may threaten fish populations due to limitations in physiological performance. This was the first investigation to evaluate the physiological responses of fish to hypoxia after SePM contamination. We suggest that the present level of environmental SePM

deserves attention. The present results demonstrate the need for comprehensive studies on SePM effects in aquatic fauna.

Graphical abstract



Highlights

► Industrial activity produces Settleable atmospheric particulate matter (SePM). ► SePM has been pointed out as potential source of air to water cross-contamination. ► SePM caused gill damage and increased ventilatory effort in fish. ► SePM-exposed fish changed ventilatory strategy under hypoxia. ► SePM might limited fish capacity to deal with common environmental challenges.

Keywords : Atmospheric particulate matter, Iron, Industry, Metal/metalloid, Nanoparticle, Environmental risk, Physiological responses

1. Introduction

Recent studies have demonstrated that aquatic environments may be affected by atmospheric particulate matter (APM) derived from industrial activities for steel processing (Callén et al., 2009; Park and Kim, 2005; Tsai and Cheng, 2004). Steel industries emit a complex mixture of solid and/or liquid particles into the atmosphere which may vary in composition, shape and size, and includes metallic nanoparticles and metallic compounds (Souza et al., 2021a). Estuarine waters in Vitória Bay, Espírito Santo State - BR, are contaminated by a range of metallic particles (including Al, Cr, Mn, Fe, Ni, Cu, Zn, As, Se, Ag, Cd, Hg, Pb) derived from settleable APM (SePM) (Souza et al, 2021b and 2021c). Isotopic analysis of carbon, nitrogen, lead, and strontium revealed that these SePM contaminants are emitted by a nearby steel industry complex in the city of Vitória (Souza et al., 2018b). Existing legislations in several countries do not consider air to water cross-contamination in environmental monitoring programs (e.g. CONAMA 491/2018 - BRASIL, 2018; Environmental Protection Agency – EPA, European Union and Canada). It is relevant the some studies effectively assess whether SePM contamination poses a real threat to ecosystems health and if so, the magnitude of the related risk.

Most studies on the effects of metals and metalloids in fishes have focused on tissue bioaccumulation and on establishing lethal doses (Fernandes et al., 2008; Vicente-Martorell et al., 2009; Demirak et al., 2006; Souza et al., 2013; Arantes et al., 2016), with little known about potential sub-lethal functional impacts. The study of sub-lethal contamination can provide valuable information on the potential ecological risks related to SePM contamination. Such studies allow observing the development of functional problems before they cause lethality or will enable the observation of functional damage that affects the performance of individuals to cope with specific environmental

conditions, such as swimming, hypoxia tolerance, temperature acclimation, or others. Functional impairment, even if developed at a sublethal level, can lead to population damage by altering the use of the environment or by generating energy reallocation and thus influencing growth, reproduction, and offspring size. However, the identification of sublethal damages are the first step to establish the need of a broader investigation. We argue that physiological measurements may be useful to reveal functional impairments, since it provides direct measurements to assess how animals cope with relevant environmental conditions.

Recent investigation observed that short-term SePM exposition affected Nile tilapia, *Oreochromis niloticus*. SePM reduced blood oxygen-carrying capacity, exerted immunotoxic effects and elicited humoral stress response (Soares et al., *submitted*). The authors suggested that such effects would impair homeostasis maintenance and limit fish performance. Therefore, SePM may limit fish capacity to cope with ecophysiological challenges such as environmental hypoxia, temperature variations, or routine activities such as swimming, foraging, courtship, or agonistic encounters.

In this investigation, we have analyzed the effect of short-term exposure (96 h) of an environmentally relevant SePM concentration on the Nile tilapia response to hypoxia. We accessed SePM impact on the cardiorespiratory and metabolic responses to progressive hypoxia and on gills morphology. Tilapia is a globally widespread invasive species, which is native from northern Africa and inhabits the SePM-contaminated locations in the state of Espírito Santo, Brazil. Hypoxia is a common environmental stressor in freshwater and estuarine environments and, therefore, effective physiological adjustment will be significant for population survival and fitness.

2. Materials and Methods

111 *2.1. Settleable atmospheric particulate matter - SePM*

112 The SePM was collected as described in Souza and coworkers (2021a). Containers
113 placed on roofs at a height of 20 m, in proximity to the steel industrial complex in Vitória
114 city – Brazil (Ilha do Boi - 20º18'32"S, 40º16'33"O, 14 km from the Tubarão industrial
115 complex), collected between 5 and 8 g per m^2 each month, over an area of 5,000 m^2. The
116 SePM comprises a complex mixture of organic and inorganic (chiefly metallic)
117 substances (Souza et al., 2021c). Its composition may vary over time (Santos et al, 2017).
118 Therefore, in order to assure repeatability, SePM was sampled from pooled material
119 collected over a year. The SePM was aliquoted and stored at the Comparative
120 Zoophysiology and Biochemistry Lab (LZBC) in the Department of Physiological
121 Sciences at the Federal University of São Carlos (UFSCar, Brazil). SePM sampling was
122 stored as testimony for any further analyses and evaluations.

123 The pooled SePM was analyzed for its main components and particle size. We
124 used granulometry, muffle furnace and solubilization with HNO_3/H_2O_2 in a microwave
125 digester to access particle size (Gee and Or, 2002), proportion of organic materials
126 (Goldin, 1987; Suguio, 1973), and contents of inorganic materials (Environmental
127 Protection Agency – EPA, 3052 - USEPA, 1996). Aquarium water samples were
128 analyzed by mass spectrometry with inductively coupled plasma (Q-ICPMS, Agilent
129 7500 Series CX technology) equipped with an ASX-100 autosampler (CETAC-
130 technologies, Omaha, NE, USA), at the Brazilian Agricultural Research Corporation
131 (EMBRAPA, Brazil) and Food Science and Technology Institute of Córdoba (YCITAC
132 - Universidad Nacional de Cordoba, Argentina). The analyses of water samples are
133 reported here to attest the ecologically relevant level of contamination. Detailed analyses
134 and composition of the pooled SePM was previously published (Soares et al, *submitted*).
135

136 *2.2. Fish supply and acclimation*

137 Juveniles of *O. niloticus* (body mass, M_B =110 ± 87g) were obtained from a local
138 fish farm (Piscicultura Polettini, São Paulo State, Brazil). Exposure protocols and
139 experiments were therefore performed on animals raised in unpolluted conditions. Prior
140 to experimentation, fish were maintained for 30d in 1,000L holding tanks equipped with
141 a continuous supply of well-aerated and dechlorinated water, at 25 ± 1°C, under natural
142 photoperiod (~12 h:12 h) and constant physicochemical parameters: pH 6.7–7.3, DO 6.0–
143 7.3 $mg.L^{-1}$, hardness 48–53 $mg.L^{-1}$ (as $CaCO_3$), alkalinity 40–43 $mg.L^{-1}$ (as $CaCO_3$),
144 ammonium 0.1 $mg.L^{-1}$; chloride 42 $mg.L^{-1}$ and conductivity 110 $\mu S.cm^{-1}$. During
145 acclimation, fish were fed *ad libitum* daily with commercial pellets (Supra® Acqua line
146 specific for tilapia: 5-8 mm; 12% moisture; 32% protein; 6% fat). Fish were fasted for
147 24h prior to contaminant exposure and all experimental procedures. These procedures
148 were approved by the Committee of Ethics in Animal Experimentation (CEUA-UFSCar).
149

150 *2.3. Experimental Design*

151 After laboratory acclimation, each fish was individually maintained for 96h in an
152 aquarium (200 L) containing either clean biofilter water (control group, n = 10) or water
153 exposed to SePM (SePM group, n = 10), with a fish:water volume ratio of 1g:1L (OECD
154 203, 2019). The SePM exposure dose (1 $g{\cdot}L^{-1}$) refers to raw SePM mass and represents
155 an environmentally relevant contamination in the estuarine area close to Vitória city,
156 Espírito Santo State - BR, (Santos et al, 2017, IEMA, 2021). Water was continually
157 aerated and mixed by air stone and maintained at 25 ± 1°C. The aquariums tanks were
158 shielded with black plastic to avoid visual disturbance, but light cycle was artificially
159 preserved. At 48h, 50% of the water was replaced to counter waste ammonia
160 accumulation, and to re-establish initial contaminant concentrations for the SePM group.

Water samples (5 mL) were collected before and after the replacement procedure, to analyze composition. After 96h, each fish was instrumented and underwent exposure to progressive hypoxia (P_wO_2 100 – 5%) in clean water, during which we measured oxygen uptake. We also recorded cardiorespiratory parameters in normoxia and deep hypoxia to assess the SePM impact on them (see below).

Each fish was subjected to the complete experimental sequence: SePM/blank exposure for 96h; instrumentation and 24h recovery; progressive hypoxia protocol; euthanasia, and dissection to sample gill tissue. After each experimental trial, respirometer chamber and experimental tank were cleaned with uncontaminated biofilter water. Background tests of oxygen uptake were performed during the process. Each fish underwent the whole protocol. The topics below were organized according to data description in results and discussion sections.

2.4. Morphological analyses

After hypoxia, each fish was euthanized by benzocaine overdose (1 $g \cdot L^{-1}$) followed by medullary section. Samples of the second gill arch in both groups (Control and SePM, n = 5) were carefully dissected out and fixed in Bouin (24 h), washed with water for 24 hours, dehydrated in alcohol (70 – 95%), and embedded in historesin (Leica, Germany). Histological longitudinal sections (3 μm thickness - Micron HM 360) were stained with toluidine blue. Gill tissues were analyzed by light microscopy (Olympus BX51, Denmark and Motic Image Plus 2.0 software, China) and quantified in 4 random microscope fields/section (n = 20) for each animal. Gill morphometry was examined (Image J Pro-plus 6.0 software, United States), such as filament epithelial thickness, distance between lamellae, and lamellar height. The level of histopathology was calculated using a histopathological index (HI) (Poleksic; Mitrovic-Tutundzic, 1994):

(1) $10^0 \sum I + 10^1 \sum II + 10^2 \sum III$, where:

P*I*, P*II*, and P*III* are the total number of alterations in each stage; 10^0, 10^1, and 10^2 are integrated factors according to damage severity. The average HI value was classified into four categories: 1-10, normal organ function; 11-20, from mild to moderate organ damage; 21-50, from moderate to severe damage; +100, irreparable to the tissue.

2.5. Responses to hypoxia

The exposure protocol was followed by anesthesia and instrumentation for cardiorespiratory recording. After instrumentation each fish was placed in a polyethylene respirometer (5L chamber) that was immersed in a polyethylene tank (75 L). Fish were recovered for 24h in clean normoxic water (PwO_2 of 100%), at 25 ± 1°C.

The protocol was designed to record metabolic rate in normoxic water and during progressive hypoxia (PwO_2 = 70, 50, 30, 20, 10 and 5 %), which was developed at acclimation temperature, 25 ± 1°C in clean water. The normoxic and each hypoxic level were controlled by bubbling either compressed atmospheric air or N_2 in the water. Each level of PwO_2 was maintained during 30 minutes. Water oxygen saturations (% of atmospheric O_2) were measured using optodes (PreSens OXY-4 mini probes) and associated software. The 100% saturation was set by calibrating the electrode using fully aerated water.

The rates of oxygen uptake ($\dot{M}O_2$ - $mMolO_2 \cdot kg^{-1} \cdot h^{-1}$) in each tension were calculated using intermittent stopped-flow respirometry (measure time – 6 minutes, flush time – 4 minutes, see McKenzie et al., 2012 for general protocol) with the optode in the respirometer and associated PreSens software. The $\dot{M}O_2$ was calculated for the periods of intermittent stopped flow (Rosewarne et al. 2016) as follows:

(1) $$\dot{M}O_2 = [\beta O_2 \cdot V_{RE} \cdot W_0^{-1} \cdot (\frac{\delta pO2}{\delta t})]$$

Where βO_2 is the coefficient for O_2 solubility in water (mg of $O_2 \cdot L^{-1}$); V_{RE}, the respirometer volume (L); Wo^{-1}, the animal body mass (Kg); and $(\frac{\delta pO2}{\delta t})$ represents O_2 decline over time (% of $O_2 \cdot h^{-1}$).

$\dot{M}O_2$ was analyzed and plotted at each level of progressive hypoxia. Routine metabolic rate (RMR) was estimated as the mean rate of O_2 consumption at rest in normoxia (PwO_2 = 100 %); RMR estimates the aerobic energy expended for minimal activity at rest (Krogh, 1914). O_2crit values were evaluated for each experimental group by regression (SigmaPlot 12.5, Systat Software Inc) according to Bilberg and coworkers (2010).

2.6. Instrumentation and cardiorespiratory recording

As a reference for the physiological adjustments to deal with hypoxia, the cardiorespiratory parameters were recorded during normoxia (PwO_2 =100%) and deep hypoxia (PwO_2 = 5 %). The instrumentation followed SePM/blank exposure. For that, each fish was anaesthetized by immersion in 0.1% benzocaine in aerated water until loss of equilibrium and spontaneous ventilatory activity. Fish were transferred to an operating table where the gills were continuously irrigated with aerated anesthetic (0.05% benzocaine). A polyethylene catheter (PE 60) was placed dorsally in the buccal cavity and a second catheter (PE 60) was implanted in the distal portion of the opercular cleithrum. Two needle type electrocardiogram (ECG) electrodes were implanted ventrally: one laid between the gills and the heart, and the other close to the pelvic fins. The reference electrode remained in the water of the experimental chamber.

Following recovery, the buccal catheter was connected to a pressure transducer (MLT0380/D - ADInstruments) to amplify and continuously record buccal pressure signals (Bridge Amp/Powerlab - ADInstruments) and derive instantaneous respiratory

frequency (f_R, on Labchart - ADInstruments). Inspired water (PiO_2) was sampled from the respirometry chamber and expired water (PeO_2) was sampled continuously from the opercular catheter. The ECG leads were connected to an Animal bioAmp/Powerlab (ADInstruments), to amplify and continuously record ECG, to calculate heart rate (f_H).

Gill ventilation volume ($\dot{V}_G$ – $LH_2O \cdot kg^{-1} \cdot min^{-1}$) was calculated using the Fick principle:

(2) $\dot{V}_G = [\dot{M}O_2 / (C_iO_2 - C_eO_2)]$, where:

C_iO_2 represents the inspired O_2 concentration (the average oxygen level in the respirometer chamber); and C_eO_2 represents the expired O_2 concentration (the average oxygen level recorded in the water from operculum cannula – Cech and Brauner, 2011).

The mean respiratory frequency (f_R - breaths $\cdot$ min^{-1}) was measured from the buccal pressure variation during 1 minute. Ventilatory tidal volume (V_T - $L \cdot kg^{-1} \cdot breath^{-1}$) was calculated as the ratio between gill ventilation and the respiratory frequency

(3) $V_T = \dot{V}_G / f_R$

Oxygen extraction from the ventilatory current was calculated as:

(4) $EO_2 = (PiO_2 - PeO_2) / PiO_2 \cdot 100$

2.7. Statistical analysis

All data were analyzed for normality (Shapiro-Wilk). Differences in morphologic parameters between experimental groups (Control and SePM) were analyzed by Student's T-test. Mean values of each cardiorespiratory variable were compared between groups and O_2 tensions (normoxia and hypoxia) using TwoWay ANOVA and Student-Newman-Keuls (SNK). Tests were considered significant at the 95% level of confidence ($P<0.05$). Data are presented as means ± SE. All data were analyzed with R v.4.1.1 (R Core Team 2021).

3. Results

SePM contamination

Contamination with raw SePM resulted in the presence of a wide variety of metal species in the water. Metal concentrations in the contaminated water were mainly constituted by iron ores and aluminum, plus titanium and cerium (Table 1). Despite that, in our experiment, 96h exposure to environmentally relevant concentration of SePM did not result in fish death.

Table 1. Metal concentrations (µg· L^{-1}) in the experiment water in Control (clean water) and SePM (exposed) groups

Metal	Control	SePM
B	<LOD	<LOQ
Al	<LOQ	645.82 ± 1.11
Ti	<LOQ	114.66 ± 2.81
V	1.05 ± 0.02	4.36 ± 0.007
Cr	<LOQ	1.51 ± 0.02
Mn	<LOD	44.12 ± 0.31
Fe-56	<LOD	627.44 ± 5.72
Ni	<LOD	0.50 ± 0.005
Cu	4.73 ± 0.07	18.79 ± 0.08
Zn	7.12 ± 0.05	35.73 ± 0.09
As	<LOD	<LOD
Se	<LOD	<LOD
Rb	10.10 ± 0.08	10.49 ± 0.07
Sr	22.12 ± 0.31	26.95 ± 0.15
Y	<LOD	1.51 ± 0.01
Zr	<LOQ	1.65 ± 0.05
Nb	<LOD	<LOQ
Ag	0.22 ± 0.004	0.19 ± 0.004
Cd	7.18 ± 0.16	<LOD
Sn	<LOD	<LOD
Ba	0.57 ± 0.01	0.33 ± 0.001
La	<LOD	1.64 ± 0.02
Ce	<LOD	3.57 ± 0.02
Ta	<LOD	<LOD
W	0.10 ± 0.01	<LOQ
Hg-201	<LOD	<LOD
H-202	<LOD	<LOD

Pb	<LOQ	14.79 ± 0.18
Bi	<LOD	<LOD

Values are mean ± SEM. LOD: limit of detection. LOQ: limit of quantification. Limit of detection (LOD, µg· L^{-1}): B 0.05, Mn 0.51, Fe 13.0, Ni 0.22, As 0.23, Se 0.52, Y 0.0, Nb 0.0, Sn 0.02, La 0.01, Ce 0.0, Ta 0.02, Hg-201 0.06, Hg-202 0.04, and Bi 0.02. Limit of quantification (LOQ, µg· L^{-1}): B 0.15, Al 12.8, Ti 0.22, Cr 0.16, Zr 0.06, Nb 0.02, W 0.02, and Pb 0.0.

SePM affected gill structure

In Control group, it is possible to observed the normal aspect of gill epithelium (Figure 1A) with epithelial cells (ec), ionocytes (i), marginal channel (mc), and mucous cells (m). The mean epithelial thickness was 43.69 ± 0.69 µm in the filament (Figure 2A). The distance between lamellae mean 26.49 ± 1.41 µm (Figure 2B), and lamellar height, 106.59 ± 0.46 µm (Figure 2C).

SePM contamination affected gill tissue and histopathological alterations were observed. The most frequent alterations were epithelial cells hypertrophy, epithelial displacement, and ionocytes proliferation (Figure 1B). The contamination resulted in 15 % increase in filament epithelial thickness (51.20 ± 1.91 µm, p = 0.003 - Figure 2A); 30 % reduction in distance between lamellae (18.50 ± 1.69 µm, p = 0.001 - Figure 2B); and a 22 % reduction in lamellar height (82.97 ± 5.55 µm, p = 0.001 - Figure 2C). The observed alterations resulted in higher histopathological index (Figure 2D).

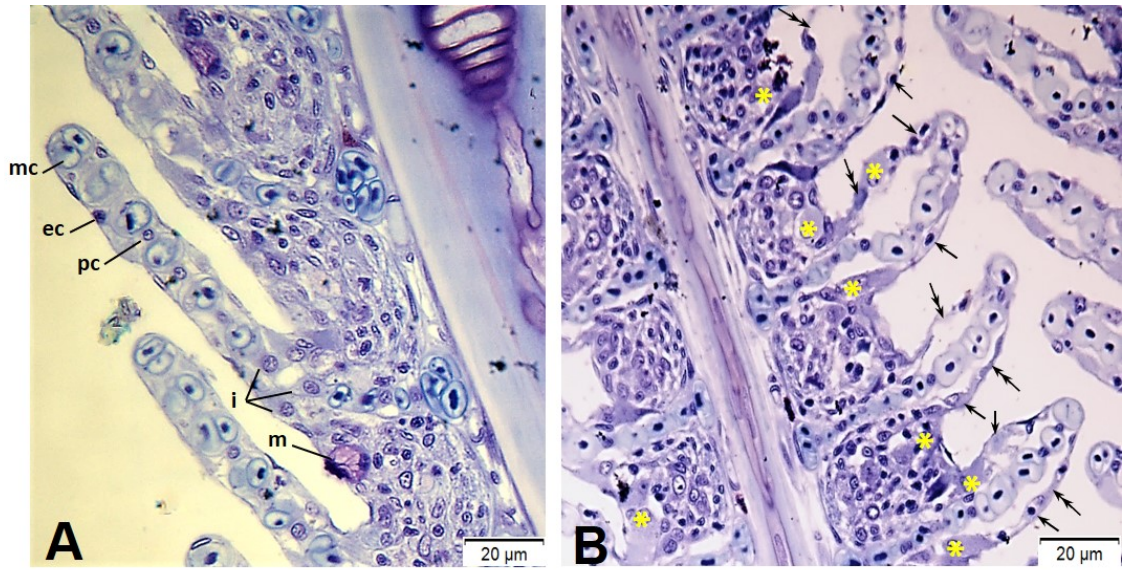


Figure 1. Morphology of Nile tilapia gills. (A) normal gill structure with their respective structures; (B) single arrow indicates hypertrophy of epithelial cells, double arrow indicates epithelial displacement, and asterisk indicates proliferation of ionocytes. Abbreviations: pc = pillar cells, mc = marginal channel, ec = epithelial cell, m = mucous cell, and i = ionocyte. Scale bar = 20µm.

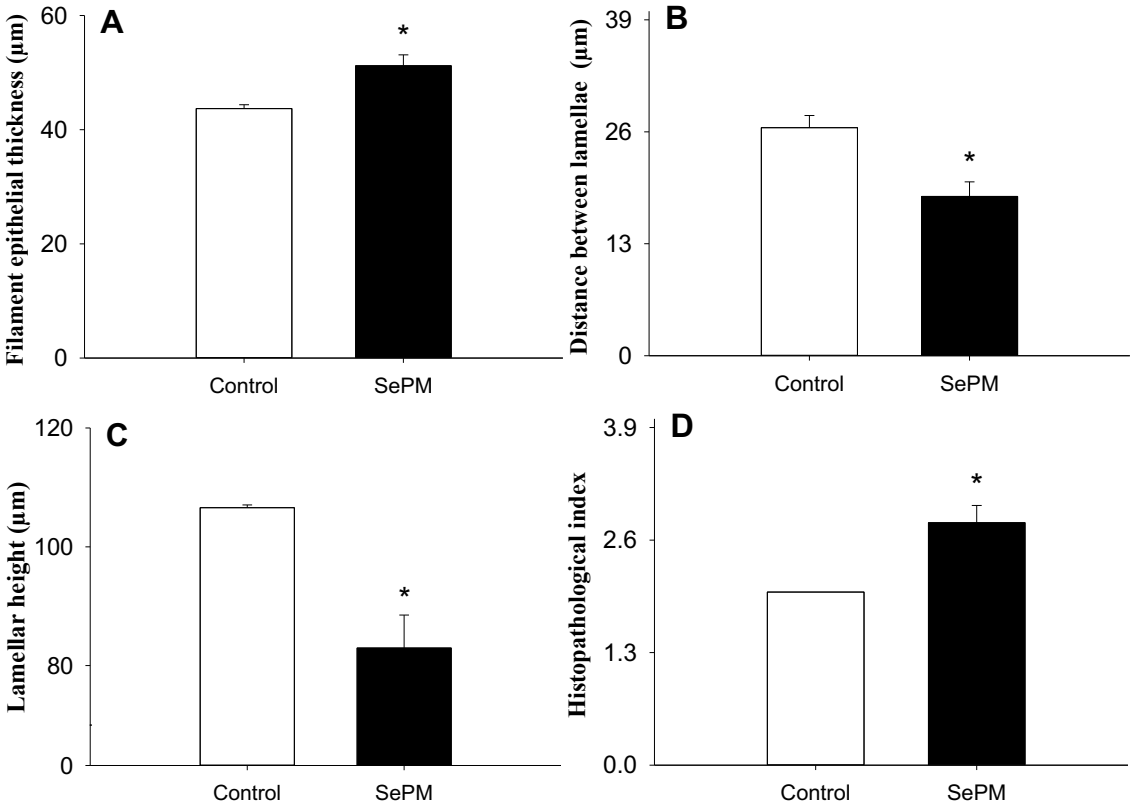


Figure 2. Gill morphometric variables: filament epithelial thickness (A), distance between lamellae (B), lamellar height (C) and histopathological index (D) in Nile tilapia (*Oreochromis niloticus*) exposed to settleable atmospheric particulate matter (SePM) for 96 h. Bars represent means ± standard error. * denotes mean difference (Student's t-test, P < 0.05).

Physiology:

Resting normoxic RMR was not different between experimental groups. Regardless of treatment, $\dot{M}O_2$ was maintained nearly constant until O_2 tension was reduced to about 25-35%. In both groups, it was possible to identify an O_2crit, and below that, $\dot{M}O_2$ was reduced progressively with oxygen saturation (Figure 3). The ranges of oxyregulation were different. SePM exposure affected the capacity to deal with hypoxia, and O_2crit was 31% higher in the SePM group than in Control group (34% and 26%, respectively - Figure 3).

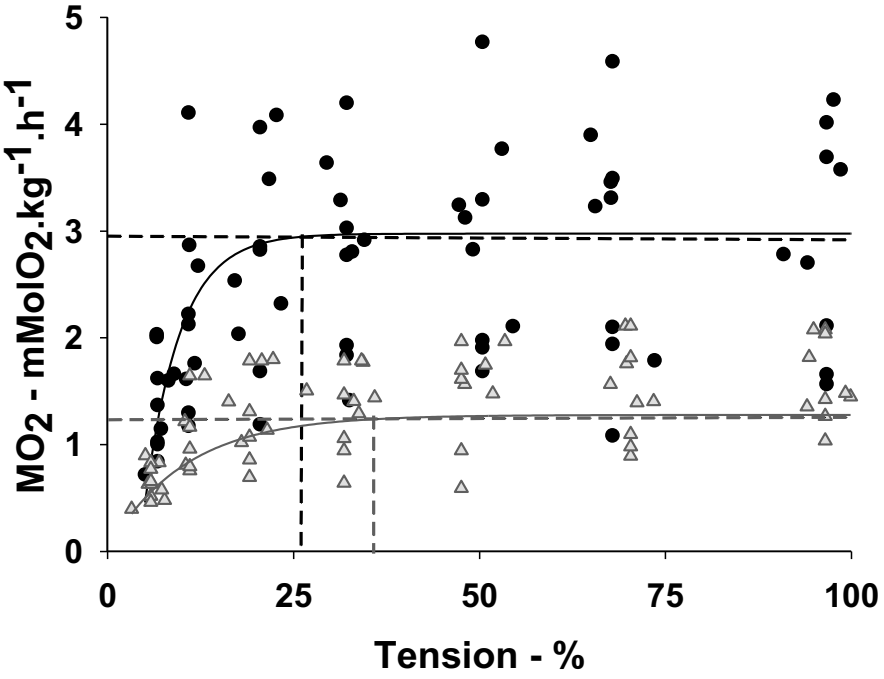


Figure 3. Oxygen uptake (MO_2 - $mmolO_2 \cdot kg^{-1} \cdot h^{-1}$) and O_2crit estimation in Nile tilapia, *Oreochromis niloticus*, in progressive hypoxia. Symbols represent Control (black circle) and SePM (grey triangles) groups. Horizontal dashed lines indicate routine metabolic rate in each group. Continuous lines indicate the fitted regression of the metabolic alteration during progressive hypoxia. Vertical dashed lines indicate the critical oxygen (O_2crit) in each group (26% and 34% in Control and SePM group, respectively).

SePM contamination resulted in 45% decrease in total gill ventilation in normoxia (Control $\dot{V}_G$ = 36.69 ± 4.88 and SePM $\dot{V}_G$ = 20.19 ± 1.32 $LH_2O \cdot kg^{-1}$, p = 0.004 - Fig 4A). Nevertheless, f_R was 12% higher in the SePM group, even in normoxia, increasing

it from 59.64 ± 2.21 to 66.81 ± 2.95 breaths · min^{-1} (p = 0.022 - Fig 4B). Despite those alterations, the ventilatory tidal volumes were similar between groups in normoxia ($\dot{V}_T$ = 0.84 ± 0.16 and 0.74 ± 0.24 $LH_2O \cdot Kg^{-1}\cdot$ breaths min^{-1}, p = 0.126, Fig 4C).

The reduction in oxygen availability induced an increase in the ventilatory effort, and therefore, $\dot{V}_G$, f_R, and $\dot{V}_T$ increased in both groups. However, SePM-contaminated fish had a limited rise in gill ventilation, -21% lower than Control fish (Control $\dot{V}_G$ = 344.62 ± 40.33 and SePM $\dot{V}_G$ = 272.89 ± 25.40 $LH_2O \cdot kg^{-1}$, p < 0.001 – Fig 4A). Nevertheless, under hypoxia, contaminated fish were capable of increasing f_R as much as Control fish (Control f_R = 71.64 ± 3.00 and SePM f_R = 71.44 ± 4.28, p = 0.491 - Fig 4B). SePM exposition tend to reduce $\dot{V}_T$ in hypoxia (Control $\dot{V}_T$ = 6.28 ± 1.74 and SePM $\dot{V}_T$ = 4.03 ± 0.57 $LH_2O \cdot Kg^{-1}$ breaths min^{-1}, p = 0.781 - Fig 4C). However, $\dot{V}_T$ variability was high and no difference we observed between experimental groups (p = 0.781).

Oxygen extraction was 32% higher in SePM group than in Control in normoxia (Control EO_2 = 57.71 ± 3.79 and SePM EO_2 = 76.40 ± 3.25 %, p = 0.001 - Fig 5A). Both groups had EO_2 reduced after the ventilatory increase in deep hypoxia, down to 40.10 ± 3.18 and 46.64 ± 4.61 %, respectively (p < 0.001, Fig 5A). However, the EO_2 in SePM group was 16 % higher than Control in deep hypoxia (p = 0.001 - Fig 5A). Heart rate was 13% reduced in normoxic SePM fish (Control f_H = 39.22 ± 1.99 and SePM f_H = 34.00 ± 1.47 bpm, p = 0.021 - Fig 5C). Both groups developed the same level of hypoxic bradycardia (Control f_H =22.35 ± 1.67 and SePM f_H =24.21 ± 1.53 bpm, p = 0.253 - Fig 5C).

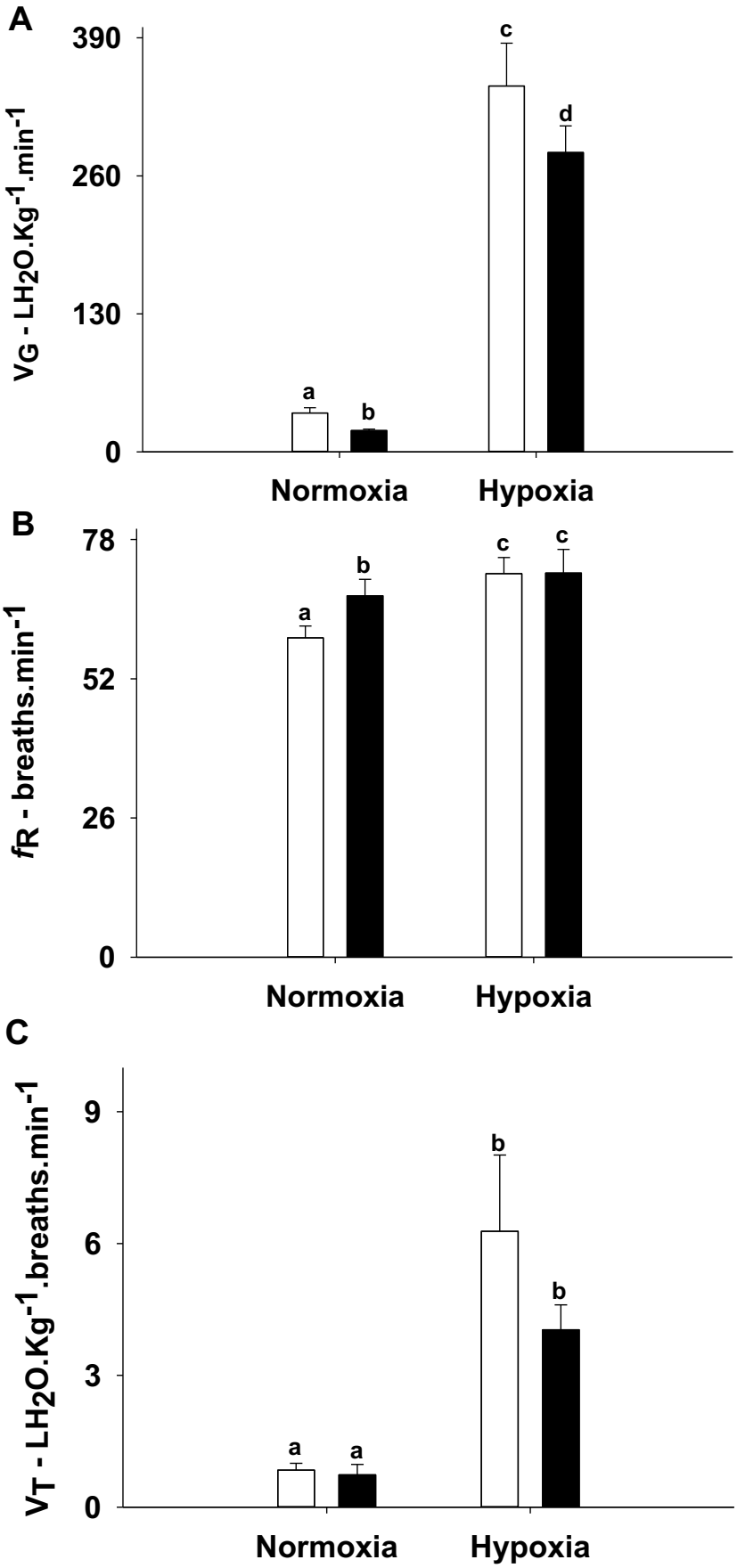


Figure 4. Ventilatory variables of Nile tilapia, *Oreochromis niloticus*, in normoxia and deep hypoxia. Bars represent mean values ± standard error in Control (white bar) and SePM

(black bar) groups. Panels: A. Gill ventilation, $\dot{V}_G$ – $LH_2O \cdot kg^{-1} \cdot min^{-1}$; B. Respiratory frequency, f_R – breaths · min^{-1}; C. Ventilatory tidal volume, V_T – $LH_2O \cdot kg^{-1} \cdot$ breaths.min^{-1}. Different letters denote different means. Two-Way ANOVA and SNK, P < 0.05, n = 10.

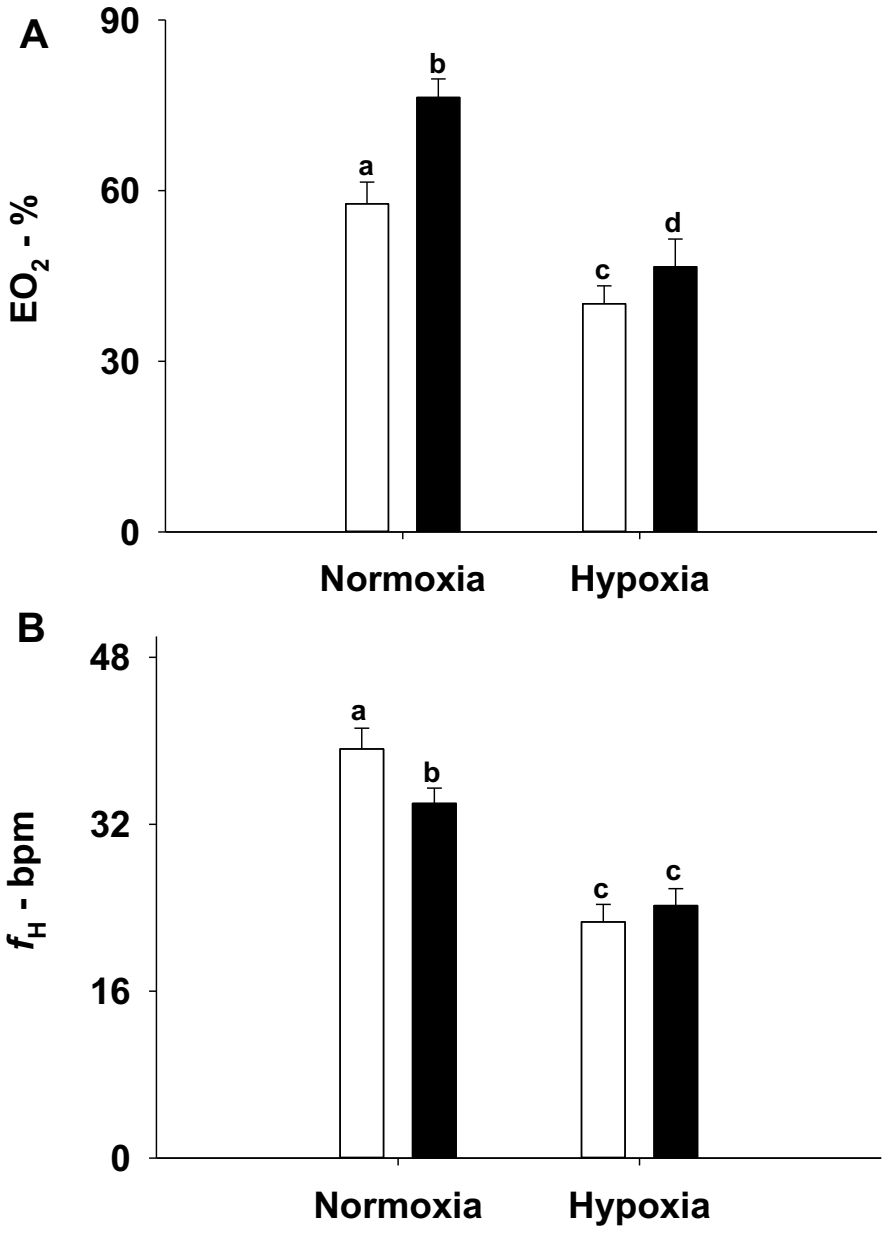


Figure 5. Oxygen extraction (EO_2) and heart rate (f_H) values of Nile tilapia, *Oreochromis niloticus*, in normoxia and deep hypoxia. Bars represent mean values ± standard error in Control (white bar) and SePM (black bar) groups. Panels: A. Oxygen extraction, EO_2 - %; B. Heart rate, f_H – bpm. Different letters denote different means. Two-Way ANOVA and SNK, P < 0.05, n = 10.

4. Discussion

Settleable Atmospheric Particulate Matter - SePM

In this study, we observed that an ecologically relevant concentration of SePM caused functional impacts in fish. SePM is composed by a highly complex mixture of different-sized particles varying in shape and composition. The particles are composed by both organic and inorganic materials with the latter including diverse metallic elements (Souza et al., 2021b, 2021a, Soares et al., *submitted*). It is hard to predict the final effect of such a complex mixture or even identify the main component causing the observed alterations, even considering the knowledge regarding individual metal contamination. The difficulty is caused by the combination of multiple factors: the number of metallic species, the multiple possible combinations among metallic/metalloid elements, and the fact that some metals are present at nanometric scale (Salgado, 2003). At the moment, the only way to replicate that contamination and assess its effect is by using raw SePM samples. In order to standardize our evaluation of the toxic effects of SePM, we have followed a previously used methodology (Soares et al., *submitted*). We used samples from pooled SePM (collected over a year), and SePM concentration that mimics the level of water contamination (1 $g \cdot L^{-1}$ of the raw SePM) reported for the aquatic environment near Vitoria city ES-Brazil (Institute of Environment and Hydric Resources – IEMA, 2021; Souza et al., 2021b, 2021a).

Recent metal identification in natural water bodies of Santa Cruz and Vitória Bay (ES-Brazil) demonstrated a variable but sustained presence of SePM deposited by air-to-water cross-contamination (Souza et al., 2018b, 2019). The APM production by the local industrial complex may vary over time; however, it is constant delivered (IBEF, 2011; Santos, et al., 2017). Most part of the SePM mass was composed by Fe and Al, yet their values in water were within LC_{50} for each metal (Fe, 1.46-1.71 and Al, 0.095-235 mg L^{-}

394 [1], Kennedy, 2011). They were below the maximum limits established by the Brazilian
395 regulatory agency CONAMA (357/2005 – Fe, 0.3-5.0; Al, 0.1-0.2 mg. L^{-1}) for Brazilian
396 freshwater bodies of classes 1 to 3, referred to as 'protected for aquatic communities,
397 aquaculture, fishing, and supply'.
398
399 *SePM affects fish gills and ventilation*
400 There was 100% fish survival following 96h of exposure to SePM, confirming
401 that the experimental protocol was sublethal. However, the SePM exposition led to an
402 early but evident process of gill damage, which started a significant reduction in the
403 performance to cope with hypoxia.
404 Gills are commonly the first target of diverse water pollutants due to its
405 characteristic contact with the external environment (Perry and Laurent, 1993). Gill
406 damage usually leads to relevant harm in fish physiology since it would affect diverse
407 functional systems and might inflict severe long-term impact. Metals have been reported
408 to cause alterations on mucous cells, chloride cells (Mallatt, 1985), and epithelial edema
409 (Sola et al., 1995; Campbell et al., 1999). Both dissolved metals and metallic
410 nanoparticles have also been reported to cause gills damage (Shaw, 2011), affecting
411 diverse functions such as respiratory functions (Ni – Pyle et al., 2002; Cu – Wilson and
412 Taylor, 1993), acid–base balance, ammonia excretion (Cu – Wilson and Taylor, 1993;
413 Taylor et al., 1996), and osmoregulation (Cd – Mccarty; Houston, 1975; Giles, 1984).
414 The gill alterations observed in the present experiment are early indicatives of
415 histopathology, such as the reduction of lamellar structure (distance and height) and may
416 work as a mechanism to increase the tissue barrier. The gill alterations typically
417 associated with metal contamination have generally been described as both transitory and
418 permanent. Epithelial lifting, hyperplasia, hypertrophy of the epithelial cells, and partial

fusion of some secondary lamellae are considered transitory alterations, serving as a mechanism to increase the barrier against the entrance of contaminants (Hadi and Alwan, 2012). While other alterations such as necrosis, lamellar rupture, and shedding of gill epithelium are considered permanent injuries (Abdel-Khalek, et al. 2016).

Although this first response seems to help avoid the progression of contamination, it also results in limiting oxygen uptake since they cause the increase of the diffusion distance and the reduction of the respiratory area (Wilson and Taylor, 1993; Abdel-Khalek, et al. 2016). Filament thickening together with epithelial hypertrophy and displacement may reflect an inflammatory reaction due to tissue lesions. Such symptoms are likely to impact O_2 uptake. A detailed description of that effect in the water-blood barrier and its duration are beyond the scope of our study and should be considered in a dedicated investigation.

The observed alterations in gill tissue also complement the results previously observed by Soares and coworkers (*submitted*), who reported stress response and hematocrit reduction after SePM. Stress is likely to follow tissue lesion, mainly if pain is associated with the process. Besides, we can assume that hematocrit reduction would be an expected result of the combination of several small bleeding points in damaged gill tissue and hyperventilation due to the O_2 uptake impairment.

Resting normoxic-tilapia had no alteration in the overall oxygen uptake after 96h in SePM-contaminated water. However, SePM induced an increase in f_R and EO_2 and f_H decrease. Those alterations were present even at rest in normoxia and suggest a cardiorespiratory compensation for a limitation in oxygen uptake, since they are the typical alteration observed in hypoxia response. Soares and coworkers (*submitted*) have reported that the same level of SePM contamination also reduced tilapia hematocrit (-12%), hemoglobin (-26%), and red blood cell counts (-38%). This reduction in oxygen-

carrying capacity likely generated the due hypoxemia and caused the increase in ventilatory drive (Leite et al., 2007; Zeraik et al., 2013).

The combination of higher f_R with both reduced f_H and oxygen-carrying capacity is intriguing. If f_H and O_2 carrying capacity are lower, fish might have a higher stroke volume to maintain routine oxygen uptake and oxygen supply to tissues (Wang and Hicks, 2002; Wells, 2009). Stroke volume and pulse pressure increase are associated to hypoxic bradycardia and they are assumed to enhance lamellar perfusion to increase the effective area for gas exchange (Farrell, 2007; Reid et al., 2006). Such alterations may open poorly perfused vascular spaces in the gill lamellae (Randall, 1982), and also recruit unperfused lamellae (Farrell et al., 1980). That mechanism would take account for the observed higher EO_2 in SePM-contaminated fish. Furthermore, a higher arteriovenous O_2 difference could also contribute to the elevated EO_2 with decreased f_H. The combination among higher f_R and EO_2 with lower f_H indicates that alterations might be working in the present case, even when the fish is resting in normoxia.

Another relevant aspect is that the expected ventilatory adjustment to support a higher ventilatory drive is $\dot{V}_G$ increasing (Fernandes and Rantin, 1989). The fact that f_R was increased while $\dot{V}_G$ was reduced is a functional evidence of gill damage. In such a case, increasing $\dot{V}_T$ would have limiting advantages since breathing area would be reduced. Therefore, the hyperventilation of the healthy areas is required, and f_R increasing would reflect that mechanism.

SePM limits the performance to cope with hypoxia

An impaired respiratory surface is likely to require higher ventilatory work and blood perfusion to satisfy tissue demands for oxygen, particularly during environmental hypoxia. Since SePM exposure affected routine ventilation, it also impacted the

ventilatory strategy to cope with hypoxia. The Control fish evoked the expected cardiorespiratory alteration in hypoxia. The capacity to perform those adjustments is responsible to maintain $\dot{M}O_2$ stable until O_2crit. Therefore, a limited capacity is expected to reduce the oxyregulation area in the progressive hypoxia trial.

Tilapia is a very hypoxia-resistant species, previous reports of O_2crit vary between 16 and 30 mmHg (0.84 to 1.6 $mgO_2 \cdot L^{-1}$ – Fernandes and Rantin, 1987; 1989, and Martins et al., 2011). The variation in estimated O_2crit may reflect differences in animal size, temperature, hypoxia protocol, and calculation methods (Rogers et al. 2016). Nonetheless, our data demonstrate that SePM changed tilapia capacity to cope with hypoxia. We observed that SePM-contamined fish had higher O_2crit, which rise from 26 to 34% (1.84 to 2.76 $mgO_2 \cdot L^{-1}$), reflects the problem.

Environmental hypoxia is a common, daily naturally occurring phenomenon in freshwater ecosystems (Richards, 2009). Therefore, it is a severe and unavoidable physiological challenge that can exert strong selective effects on aquatic organisms (Graham, 1997). Hypoxic events may vary in frequency and severity according to factors such as photosynthesis, water column depth, salinity, temperature, tide, and organic matter (Nezlin et al. 2009). The higher O_2crit of the SePM-contamined fish means a reduced scope for activity when the oxygen level is low in environment (Vitoria Bay - Claireaux and Chabot, 2016). This could have knock-on effects on many activities that underpin fish population dynamics, notably space use, foraging and feeding, intraspecific interactions, and so forth. The observed effect is especially relevant if we consider the short-term contamination used in our protocol. The progression in tissue damage might provide long-term gill alteration changing fish physiology or affect energy allocation due to the respiratory cost of ventilation.

494 *SePM may affect energy allocation*

495 SePM exposure increased f_R. Since such compensation was associate to
496 maintenance of resting metabolism, the energy allocation might be different. We can
497 safely assume that a higher portion of resting energy might be allocated to support
498 breathing in SePM-contamined fish. Modulating f_R to meet oxygen demands is
499 energetically expensive (Rantin et al. 1992). The cost of water ventilation has been
500 experimentally estimated and may vary from 2 to 50% according to fish species and their
501 morphophysiological characteristics (Shelton, 1970; Boehlert et al., 1991). In tilapia,
502 ventilation has been estimated to consume about 3% of routine aerobic metabolism, even
503 under normoxic conditions. In hypoxia, this can rise to 10% of total aerobic energy use
504 (Fernandes and Rantin, 1994) therefore accounting for significant energy loss.

505 A permanently higher ventilation cost in normoxia and its increasing during
506 hypoxia events potentially have an important long-term impact on energy usage for body
507 maintenance and also, to support high demand challenges, such as growth, reproduction,
508 post-prandial metabolic increment, swimming, and others. We can predict that unless
509 other relevant functional compensations restore ventilatory capacity in fish under chronic
510 contamination, this will potentially affect animal fitness and the stability of the population
511 in the natural environment. We suggest that despite sublethal, the level of contamination
512 tested is not innocuous.

513

514 *Final considerations*

515 This was the first investigation to evaluate the physiological responses of fish to
516 hypoxia after of air-to-water cross-contamination by SePM from the iron ore processing
517 industry. We observed that after 96h, SePM exposure caused significant alterations on gill
518 tissue, leading to changes in cardiorespiratory variables, which caused a direct impact on

519 the efficiency to deal with hypoxia. That limitation might extend to other challenges such
520 as swimming, foraging, species interaction, and others. Therefore, SePM exposure,
521 although not leading to immediate death, can potentially impact fish populations. The
522 current result substantiates the need for extensive dedicated studies on the effects of SePM
523 on aquatic fauna.
524
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Supplementary material

Table 1. Cardiorespiratory and metabolic variables of resting Nile tilapia, *Oreochromis niloticus*, in normoxia (P_wO_2 = 100%) and deep hypoxia (PwO_2 = 5%), at 25°C, in control and SePM exposed (SePM) groups

		Mass (g)	$\dot{M}O_2$ ($mMolO_2 \cdot kg^{-1} \cdot h^{-1}$)	$\dot{V}_G$ ($mLH_2O \cdot kg^{-1} \cdot min^{-1}$)	f_R (breaths · min^{-1})	V_T ($LH_2O.Kg^{-1}.breaths.min^{-1}$)	O_2crit (%)	EO_2 (%)	f_H (bpm)
Control	Normoxia	110 ± 87	2.82±0.32	36.69±4.88	59.64±2.21	0.84±0.16	26	57.71±3.79	39.22±1.99
	Deep Hypoxia		1.34±0.15	356.41±38.57$^{\#}$	71.64±3.00$^{\#}$	6.28±1.74		40.10±3.18$^{\#}$	22.35±1.69*
SePM	Normoxia		3.20±0.24	20.19±1.32*	66.81±2.95*	0.74±0.24	34	76.40±3.25*	34.00±1.47$^{\#}$
	Deep Hypoxia		1.26±0.09	246.46±20.22$^{\#*}$	71.44±4.28$^{\#}$	4.03±0.57		46.64±4.61$^{\#*}$	24.21±1.53$^{\#}$

Values are mean ± SE. Refer to oxygen consumption ($\dot{M}O_2$), gill ventilation volume ($\dot{V}_G$), respiratory frequency (f_R), ventilatory tidal volume (V_T), oxygen extraction from the ventilatory current (EO_2), heart rate (f_H), and critical tension (O_2crit). Asterisk denotes difference between experimental groups and hashtag denotes difference between tensions. ANOVA two way and Student-Newman-Keuls' tests ($P < 0.05$).