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

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

3

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29

30

31 **Abstract**

32 Atmospheric particulate matter (APM) emitted by iron ore processing industries has a  
33 complex composition, including diverse metallic particles and nanoparticles. Such an  
34 APM causes air to water cross-contamination and has recently been demonstrated to have  
35 harmful sublethal impacts on fish, eliciting stress responses, affecting the immune system,  
36 and reducing blood oxygen-carrying capacity. These findings imply potential  
37 consequences for fish aerobic performance and energy allocation, particularly in their  
38 ability to tolerate respiratory challenges such as aquatic hypoxia. To assess that potential  
39 limitation, we analyzed metabolic, cardiorespiratory, and morphological responses to  
40 progressive hypoxia in tilapia, *Oreochromis niloticus*, exposed for 96h to an  
41 environmentally relevant concentration of settleable particulate matter (SePM). This level  
42 of SePM contamination initiate detectable gill damage, which reduced respiratory  
43 efficiency, increased ventilatory effort and reduced fish capacity to deal with hypoxia.  
44 The critical O<sub>2</sub> tension was increased (from 26 to 34% of O<sub>2</sub> / from 1.84 to 2.76 mg O<sub>2</sub> ·  
45 L<sup>-1</sup>). The resting normoxic respiratory frequency was elevated, limiting ventilatory  
46 adjustments throughout hypoxia. Such ventilatory inefficacy is likely to increase  
47 ventilatory cost and cause relevant alterations in energy allocation. We point up that a  
48 potential progression in gill damage is potentially problematic in the long term as it could  
49 result in infection, blood loss, ion imbalance, changes in the overall energy allocation and  
50 hypoxia tolerance. Such an effect might not cause immediate lethality, but it might  
51 threaten fish populations since the consequences limits physiological performance. This  
52 was the first investigation to evaluate the physiological responses of fish to hypoxia after  
53 SePM contamination. We suggest that the present level of environmental SePM deserves  
54 attention. The present results base the need for extensive dedicated studies on SePM  
55 effects in aquatic fauna.

56

57 **Keywords:** atmospheric particulate matter; iron; industry; metal/metalloid;  
58 nanoparticle; environmental risk; physiological responses.

59

60

## 61        **1. Introduction**

62            Recent studies have demonstrated that aquatic environments may be affected by  
63 atmospheric particulate matter (APM) derived from industrial activities for steel  
64 processing (Callén et al., 2009; Park and Kim, 2005; Tsai and Cheng, 2004). Steel  
65 industries emit a complex mixture of solid and/or liquid particles into the atmosphere  
66 which may vary in composition, shape and size, and includes metallic nanoparticles and  
67 metallic compounds (Souza et al., 2021a). Estuarine waters in Vitória Bay, Espírito Santo  
68 State - BR, are contaminated by a range of metallic particles (including Al, Cr, Mn, Fe,  
69 Ni, Cu, Zn, As, Se, Ag, Cd, Hg, Pb) derived from settleable APM (SePM) (Souza et al,  
70 2021b and 2021c). Isotopic analysis of carbon, nitrogen, lead, and strontium revealed that  
71 these SePM contaminants are emitted by a nearby steel industry complex in the city of  
72 Vitória (Souza et al., 2018b). Existing legislations in several countries do not consider air  
73 to water cross-contamination in environmental monitoring programs (e.g. CONAMA  
74 491/2018 - BRASIL, 2018; Environmental Protection Agency – EPA, European Union  
75 and Canada). It is relevant the some studies effectively assess whether SePM  
76 contamination poses a real threat to ecosystems health and if so, the magnitude of the  
77 related risk.

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

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

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

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

109

110         **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 - 20°18'32"S, 40°16'33"O, 14 km from the Tubarão industrial  
115 complex), collected between 5 and 8 g per m<sup>2</sup> each month, over an area of 5,000 m<sup>2</sup>. 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<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> 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,  $M_B = 110 \pm 87\text{g}$ ) were obtained from a local  
138 fish farm (Piscicultura Poletini, 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  $25 \pm 1^\circ\text{C}$ , under natural  
142 photoperiod (~12 h:12 h) and constant physicochemical parameters: pH 6.7–7.3, DO 6.0–  
143 7.3  $\text{mg}\cdot\text{L}^{-1}$ , hardness 48–53  $\text{mg}\cdot\text{L}^{-1}$  (as  $\text{CaCO}_3$ ), alkalinity 40–43  $\text{mg}\cdot\text{L}^{-1}$  (as  $\text{CaCO}_3$ ),  
144 ammonium 0.1  $\text{mg}\cdot\text{L}^{-1}$ ; chloride 42  $\text{mg}\cdot\text{L}^{-1}$  and conductivity 110  $\mu\text{S}\cdot\text{cm}^{-1}$ . During  
145 acclimation, fish were fed *ad libitum* daily with commercial pellets (Supra<sup>®</sup> 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 ( $1 \text{ g}\cdot\text{L}^{-1}$ ) 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  $25 \pm 1^\circ\text{C}$ . 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.

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

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

173

#### 174 *2.4. Morphological analyses*

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



186 (1)  $10^0 \sum I + 10^1 \sum II + 10^2 \sum III$ , where:

187  $PI$ ,  $PII$ , and  $PIII$  are the total number of alterations in each stage;  $10^0$ ,  $10^1$ , and  $10^2$   
188 are integrated factors according to damage severity. The average HI value was classified  
189 into four categories: 1-10, normal organ function; 11-20, from mild to moderate organ  
190 damage; 21-50, from moderate to severe damage; +100, irreparable to the tissue.

191

## 192 2.5. Responses to hypoxia

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

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

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

210 (1)  $\dot{M}O_2 = [\beta O_2 \cdot V_{RE} \cdot W_0^{-1} \cdot (\frac{\delta pO_2}{\delta t})]$

211 Where  $\beta O_2$  is the coefficient for  $O_2$  solubility in water ( $\text{mg of } O_2 \cdot \text{L}^{-1}$ );  $V_{RE}$ , the  
212 respirometer volume (L);  $W_0^{-1}$ , the animal body mass (Kg); and  $(\frac{\delta pO_2}{\delta t})$  represents  $O_2$   
213 decline over time ( $\% \text{ of } O_2 \cdot \text{h}^{-1}$ ).

214  $\dot{M}O_2$  was analyzed and plotted at each level of progressive hypoxia. Routine  
215 metabolic rate (RMR) was estimated as the mean rate of  $O_2$  consumption at rest in  
216 normoxia ( $PwO_2 = 100 \%$ ); RMR estimates the aerobic energy expended for minimal  
217 activity at rest (Krogh, 1914).  $O_2$ crit values were evaluated for each experimental group  
218 by regression (SigmaPlot 12.5, Systat Software Inc) according to Bilberg and coworkers  
219 (2010).

220

## 221 *2.6. Instrumentation and cardiorespiratory recording*

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

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

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

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

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

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

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

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

250 Oxygen extraction from the ventilatory current was calculated as:

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

252

### 253 *2.7. Statistical analysis*

254 All data were analyzed for normality (Shapiro-Wilk). Differences in morphologic  
255 parameters between experimental groups (Control and SePM) were analyzed by Student's  
256 T-test. Mean values of each cardiorespiratory variable were compared between groups  
257 and  $O_2$  tensions (normoxia and hypoxia) using TwoWay ANOVA and Student-Newman-  
258 Keuls (SNK). Tests were considered significant at the 95% level of confidence ( $P < 0.05$ ).

259 Data are presented as means  $\pm$  SE. All data were analyzed with R v.4.1.1 (R Core Team  
260 2021).

261

### 262 3. Results

#### 263 *SePM contamination*

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

269 **Table 1. Metal concentrations ( $\mu\text{g}\cdot\text{L}^{-1}$ ) in the experiment**  
270 **water in Control (clean water) and SePM (exposed) groups**  
271

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

<b>Pb</b>	<LOQ	14.79 ± 0.18
<b>Bi</b>	<LOD	<LOD

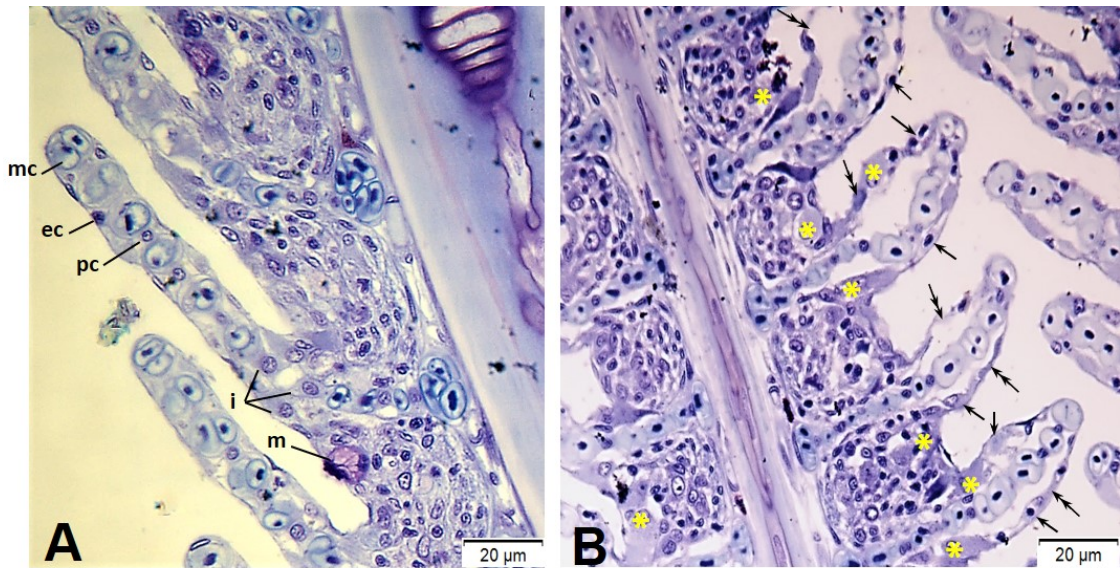
272 **Values are mean ± SEM. LOD: limit of detection. LOQ: limit of**  
273 **quantification. Limit of detection (LOD, µg· L<sup>-1</sup>): B 0.05, Mn 0.51,**  
274 **Fe 13.0, Ni 0.22, As 0.23, Se 0.52, Y 0.0, Nb 0.0, Sn 0.02, La**  
275 **0.01, Ce 0.0, Ta 0.02, Hg-201 0.06, Hg-202 0.04, and Bi 0.02.**  
276 **Limit of quantification (LOQ, µg· L<sup>-1</sup>): B 0.15, Al 12.8, Ti**  
277 **0.22, Cr 0.16, Zr 0.06, Nb 0.02, W 0.02, and Pb 0.0.**  
278

279 *SePM affected gill structure*

280 In Control group, it is possible to observed the normal aspect of gill epithelium  
281 (Figure 1A) with epithelial cells (ec), ionocytes (i), marginal channel (mc), and mucous  
282 cells (m). The mean epithelial thickness was 43.69 ± 0.69 µm in the filament (Figure 2A).  
283 The distance between lamellae mean 26.49 ± 1.41 µm (Figure 2B), and lamellar height,  
284 106.59 ± 0.46 µm (Figure 2C).

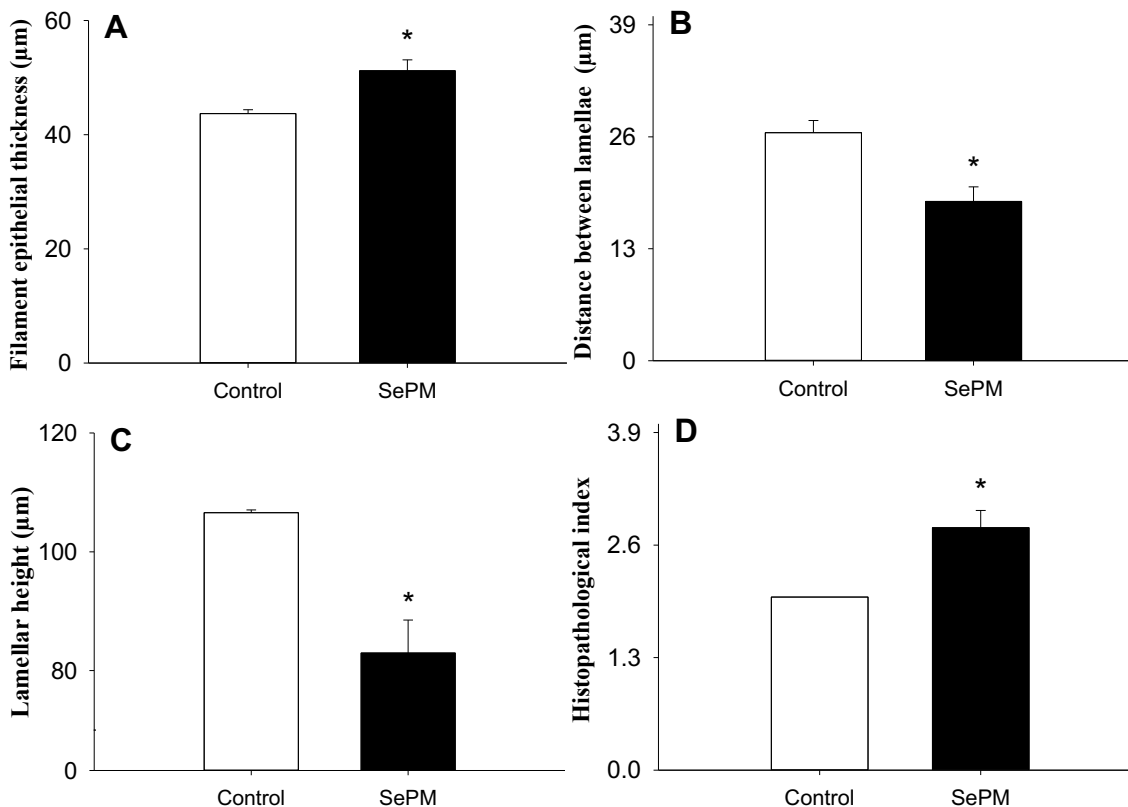
285 SePM contamination affected gill tissue and histopathological alterations were  
286 observed. The most frequent alterations were epithelial cells hypertrophy, epithelial  
287 displacement, and ionocytes proliferation (Figure 1B). The contamination resulted in  
288 15 % increase in filament epithelial thickness (51.20 ± 1.91 µm, p = 0.003 - Figure 2A);  
289 30 % reduction in distance between lamellae (18.50 ± 1.69 µm, p = 0.001 - Figure 2B);  
290 and a 22 % reduction in lamellar height (82.97 ± 5.55 µm, p = 0.001 - Figure 2C). The  
291 observed alterations resulted in higher histopathological index (Figure 2D).

292



293  
 294 **Figure 1. Morphology of Nile tilapia gills. (A) normal gill structure with their respective**  
 295 **structures; (B) single arrow indicates hypertrophy of epithelial cells, double arrow**  
 296 **indicates epithelial displacement, and asterisk indicates proliferation of ionocytes.**  
 297 **Abbreviations: pc = pillar cells, mc = marginal channel, ec = epithelial cell, m = mucous**  
 298 **cell, and i = ionocyte. Scale bar = 20µm.**

299



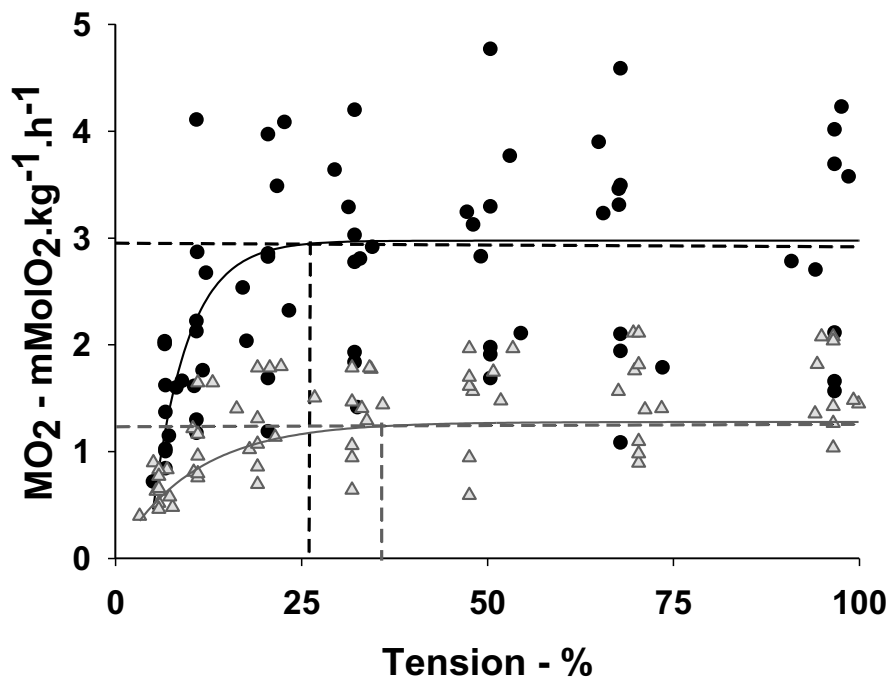
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**Figure 2. Gill morphometric variables: filament epithelial thickness (A), distance between lamellae (B), lamellar height (C) and histopathological index (D) in Nile tilapia (*Oreochromis niloticus*) exposed to settleable atmospheric particulate matter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

306 **Physiology:**

307 Resting normoxic RMR was not different between experimental groups.  
308 Regardless of treatment,  $\dot{M}O_2$  was maintained nearly constant until  $O_2$  tension was  
309 reduced to about 25-35%. In both groups, it was possible to identify an  $O_{2crit}$ , and below  
310 that,  $\dot{M}O_2$  was reduced progressively with oxygen saturation (Figure 3). The ranges of  
311 oxyregulation were different. SePM exposure affected the capacity to deal with hypoxia,  
312 and  $O_{2crit}$  was 31% higher in the SePM group than in Control group (34% and 26%,  
313 respectively - Figure 3).



314 **Figure 3. Oxygen uptake ( $MO_2$  -  $mmolO_2 \cdot kg^{-1} \cdot h^{-1}$ ) and  $O_{2crit}$  estimation in**  
315 **Nile tilapia, *Oreochromis niloticus*, in progressive hypoxia. Symbols represent**  
316 **Control (black circle) and SePM (grey triangles) groups. Horizontal dashed**  
317 **lines indicate routine metabolic rate in each group. Continuous lines indicate**  
318 **the fitted regression of the metabolic alteration during progressive hypoxia.**  
319 **Vertical dashed lines indicate the critical oxygen ( $O_{2crit}$ ) in each group (26%**  
320 **and 34% in Control and SePM group, respectively).**  
321

322  
323 SePM contamination resulted in 45% decrease in total gill ventilation in normoxia  
324 (Control  $\dot{V}_G = 36.69 \pm 4.88$  and SePM  $\dot{V}_G = 20.19 \pm 1.32$   $LH_2O \cdot kg^{-1}$ ,  $p = 0.004$  - Fig  
325 4A). Nevertheless,  $f_R$  was 12% higher in the SePM group, even in normoxia, increasing

326 it from  $59.64 \pm 2.21$  to  $66.81 \pm 2.95$  breaths  $\cdot$  min<sup>-1</sup> ( $p = 0.022$  - Fig 4B). Despite those  
327 alterations, the ventilatory tidal volumes were similar between groups in normoxia ( $\dot{V}_T =$   
328  $0.84 \pm 0.16$  and  $0.74 \pm 0.24$  LH<sub>2</sub>O  $\cdot$  Kg<sup>-1</sup>  $\cdot$  breaths min<sup>-1</sup>,  $p = 0.126$ , Fig 4C).

329 The reduction in oxygen availability induced an increase in the ventilatory effort,  
330 and therefore,  $\dot{V}_G$ ,  $f_R$ , and  $\dot{V}_T$  increased in both groups. However, SePM-contaminated  
331 fish had a limited rise in gill ventilation, -21% lower than Control fish (Control  $\dot{V}_G =$   
332  $344.62 \pm 40.33$  and SePM  $\dot{V}_G = 272.89 \pm 25.40$  LH<sub>2</sub>O  $\cdot$  kg<sup>-1</sup>,  $p < 0.001$  - Fig 4A).  
333 Nevertheless, under hypoxia, contaminated fish were capable of increasing  $f_R$  as much as  
334 Control fish (Control  $f_R = 71.64 \pm 3.00$  and SePM  $f_R = 71.44 \pm 4.28$ ,  $p = 0.491$  - Fig 4B).  
335 SePM exposition tend to reduce  $\dot{V}_T$  in hypoxia (Control  $\dot{V}_T = 6.28 \pm 1.74$  and SePM  $\dot{V}_T$   
336  $= 4.03 \pm 0.57$  LH<sub>2</sub>O  $\cdot$  Kg<sup>-1</sup> breaths min<sup>-1</sup>,  $p = 0.781$  - Fig 4C). However,  $\dot{V}_T$  variability  
337 was high and no difference we observed between experimental groups ( $p = 0.781$ ).

338 Oxygen extraction was 32% higher in SePM group than in Control in normoxia  
339 (Control  $EO_2 = 57.71 \pm 3.79$  and SePM  $EO_2 = 76.40 \pm 3.25$  %,  $p = 0.001$  - Fig 5A). Both  
340 groups had  $EO_2$  reduced after the ventilatory increase in deep hypoxia, down to  $40.10 \pm$   
341  $3.18$  and  $46.64 \pm 4.61$  %, respectively ( $p < 0.001$ , Fig 5A). However, the  $EO_2$  in SePM  
342 group was 16 % higher than Control in deep hypoxia ( $p = 0.001$  - Fig 5A). Heart rate was  
343 13% reduced in normoxic SePM fish (Control  $f_H = 39.22 \pm 1.99$  and SePM  $f_H = 34.00 \pm$   
344  $1.47$  bpm,  $p = 0.021$  - Fig 5C). Both groups developed the same level of hypoxic  
345 bradycardia (Control  $f_H = 22.35 \pm 1.67$  and SePM  $f_H = 24.21 \pm 1.53$  bpm,  $p = 0.253$  - Fig  
346 5C).

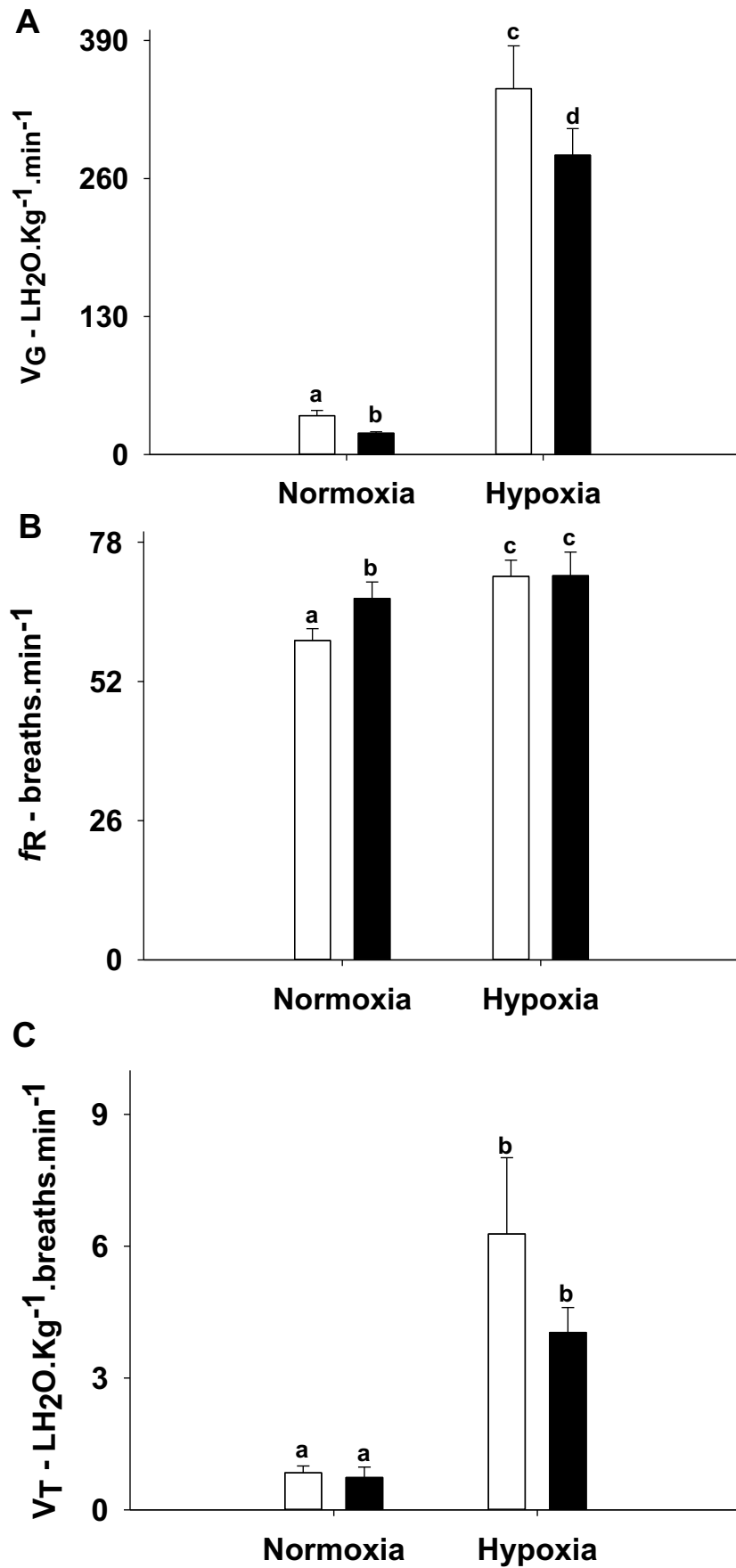
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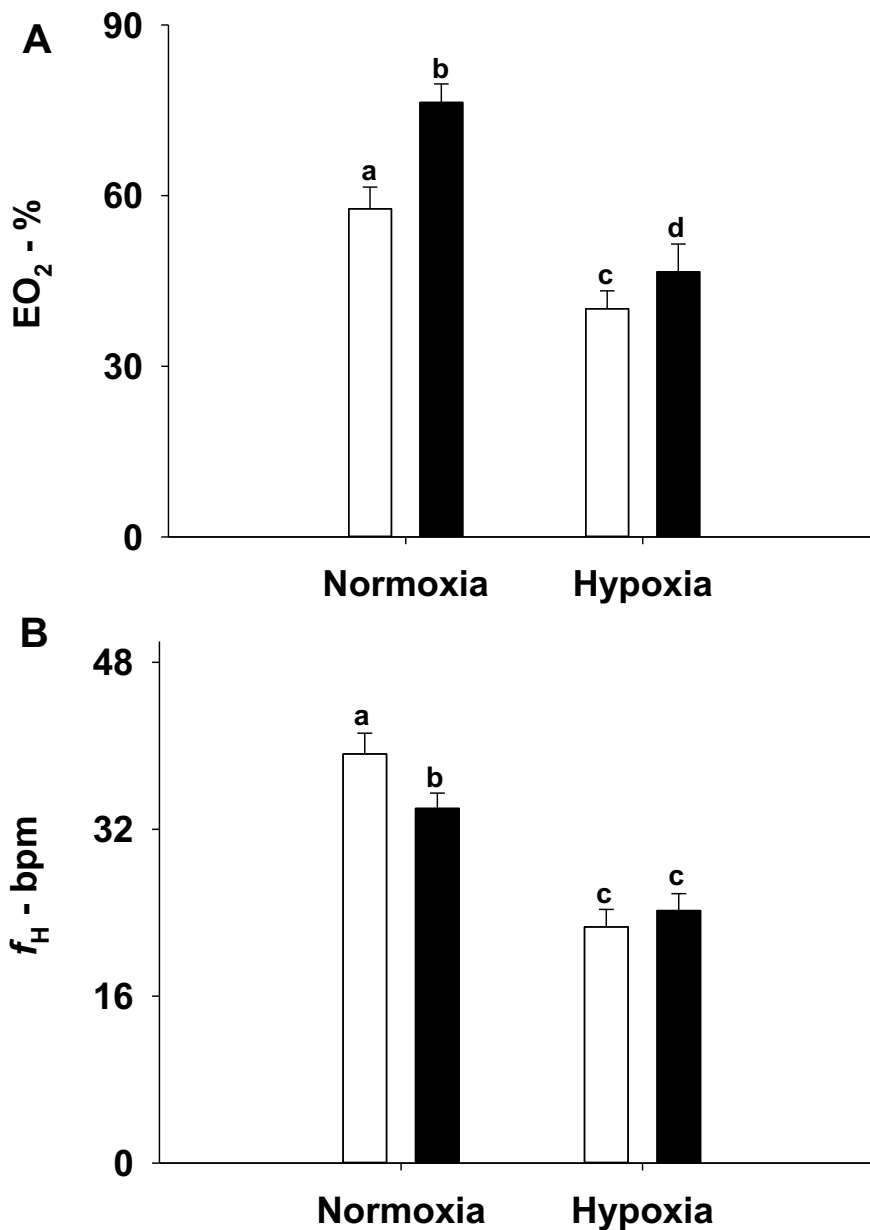
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355

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

356 (black bar) groups. Panels: A. Gill ventilation,  $\dot{V}_G - \text{LH}_2\text{O} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ; B. Respiratory  
 357 frequency,  $f_R - \text{breaths} \cdot \text{min}^{-1}$ ; C. Ventilatory tidal volume,  $V_T - \text{LH}_2\text{O} \cdot \text{kg}^{-1} \cdot \text{breaths} \cdot \text{min}^{-1}$ .  
 358 Different letters denote different means. Two-Way ANOVA and SNK,  $P < 0.05$ ,  $n = 10$ .

359



360

361

362 **Figure 5. Oxygen extraction (EO<sub>2</sub>) and heart rate (f<sub>H</sub>) values of Nile tilapia, *Oreochromis***  
 363 ***niloticus*, in normoxia and deep hypoxia. Bars represent mean values ± standard error in**  
 364 **Control (white bar) and SePM (black bar) groups. Panels: A. Oxygen extraction, EO<sub>2</sub> - %;**  
 365 **B. Heart rate, f<sub>H</sub> – bpm. Different letters denote different means. Two-Way ANOVA and**  
 366 **SNK,  $P < 0.05$ ,  $n = 10$ .**

367

368

369 **4. Discussion**

370 *Settleable Atmospheric Particulate Matter - SePM*

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

388 Recent metal identification in natural water bodies of Santa Cruz and Vitória Bay  
389 (ES-Brazil) demonstrated a variable but sustained presence of SePM deposited by air-to-  
390 water cross-contamination (Souza et al., 2018b, 2019). The APM production by the local  
391 industrial complex may vary over time; however, it is constant delivered (IBEF, 2011;  
392 Santos, et al., 2017). Most part of the SePM mass was composed by Fe and Al, yet their  
393 values in water were within  $\text{LC}_{50}$  for each metal (Fe, 1.46-1.71 and Al, 0.095-235  $\text{mg L}^{-1}$

394 <sup>1</sup>, 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<sup>-1</sup>) 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

419 fusion of some secondary lamellae are considered transitory alterations, serving as a  
420 mechanism to increase the barrier against the entrance of contaminants (Hadi and Alwan,  
421 2012). While other alterations such as necrosis, lamellar rupture, and shedding of gill  
422 epithelium are considered permanent injuries (Abdel-Khalek, et al. 2016).

423         Although this first response seems to help avoid the progression of contamination,  
424 it also results in limiting oxygen uptake since they cause the increase of the diffusion  
425 distance and the reduction of the respiratory area (Wilson and Taylor, 1993; Abdel-  
426 Khalek, et al. 2016). Filament thickening together with epithelial hypertrophy and  
427 displacement may reflect an inflammatory reaction due to tissue lesions. Such symptoms  
428 are likely to impact O<sub>2</sub> uptake. A detailed description of that effect in the water-blood  
429 barrier and its duration are beyond the scope of our study and should be considered in a  
430 dedicated investigation.

431         The observed alterations in gill tissue also complement the results previously  
432 observed by Soares and coworkers (*submitted*), who reported stress response and  
433 hematocrit reduction after SePM. Stress is likely to follow tissue lesion, mainly if pain is  
434 associated with the process. Besides, we can assume that hematocrit reduction would be  
435 an expected result of the combination of several small bleeding points in damaged gill  
436 tissue and hyperventilation due to the O<sub>2</sub> uptake impairment.

437         Resting normoxic-tilapia had no alteration in the overall oxygen uptake after 96h  
438 in SePM-contaminated water. However, SePM induced an increase in  $f_R$  and  $EO_2$  and  $f_H$   
439 decrease. Those alterations were present even at rest in normoxia and suggest a  
440 cardiorespiratory compensation for a limitation in oxygen uptake, since they are the  
441 typical alteration observed in hypoxia response. Soares and coworkers (*submitted*) have  
442 reported that the same level of SePM contamination also reduced tilapia hematocrit (-  
443 12%), hemoglobin (-26%), and red blood cell counts (-38%). This reduction in oxygen-

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

446 The combination of higher  $f_R$  with both reduced  $f_H$  and oxygen-carrying capacity  
447 is intriguing. If  $f_H$  and  $O_2$  carrying capacity are lower, fish might have a higher stroke  
448 volume to maintain routine oxygen uptake and oxygen supply to tissues (Wang and Hicks,  
449 2002; Wells, 2009). Stroke volume and pulse pressure increase are associated to hypoxic  
450 bradycardia and they are assumed to enhance lamellar perfusion to increase the effective  
451 area for gas exchange (Farrell, 2007; Reid et al., 2006). Such alterations may open poorly  
452 perfused vascular spaces in the gill lamellae (Randall, 1982), and also recruit unperfused  
453 lamellae (Farrell et al., 1980). That mechanism would take account for the observed  
454 higher  $EO_2$  in SePM-contaminated fish. Furthermore, a higher arteriovenous  $O_2$   
455 difference could also contribute to the elevated  $EO_2$  with decreased  $f_H$ . The combination  
456 among higher  $f_R$  and  $EO_2$  with lower  $f_H$  indicates that alterations might be working in the  
457 present case, even when the fish is resting in normoxia.

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

464

465 *SePM limits the performance to cope with hypoxia*

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

469 ventilatory strategy to cope with hypoxia. The Control fish evoked the expected  
470 cardiorespiratory alteration in hypoxia. The capacity to perform those adjustments is  
471 responsible to maintain  $\dot{M}O_2$  stable until  $O_{2crit}$ . Therefore, a limited capacity is expected  
472 to reduce the oxyregulation area in the progressive hypoxia trial.

473         Tilapia is a very hypoxia-resistant species, previous reports of  $O_{2crit}$  vary between  
474 16 and 30 mmHg (0.84 to 1.6  $mgO_2 \cdot L^{-1}$  – Fernandes and Rantin, 1987; 1989, and Martins  
475 et al., 2011). The variation in estimated  $O_{2crit}$  may reflect differences in animal size,  
476 temperature, hypoxia protocol, and calculation methods (Rogers et al. 2016).  
477 Nonetheless, our data demonstrate that SePM changed tilapia capacity to cope with  
478 hypoxia. We observed that SePM-contaminated fish had higher  $O_{2crit}$ , which rise from 26  
479 to 34% (1.84 to 2.76  $mgO_2 \cdot L^{-1}$ ), reflects the problem.

480         Environmental hypoxia is a common, daily naturally occurring phenomenon in  
481 freshwater ecosystems (Richards, 2009). Therefore, it is a severe and unavoidable  
482 physiological challenge that can exert strong selective effects on aquatic organisms  
483 (Graham, 1997). Hypoxic events may vary in frequency and severity according to factors  
484 such as photosynthesis, water column depth, salinity, temperature, tide, and organic  
485 matter (Nezlin et al. 2009). The higher  $O_{2crit}$  of the SePM-contaminated fish means a  
486 reduced scope for activity when the oxygen level is low in environment (Vitoria Bay -  
487 Claireaux and Chabot, 2016). This could have knock-on effects on many activities that  
488 underpin fish population dynamics, notably space use, foraging and feeding, intraspecific  
489 interactions, and so forth. The observed effect is especially relevant if we consider the  
490 short-term contamination used in our protocol. The progression in tissue damage might  
491 provide long-term gill alteration changing fish physiology or affect energy allocation due  
492 to the respiratory cost of ventilation.

493

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-contaminated 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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530

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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 ( $P_wO_2 = 5\%$ ), at 25°C, in control and SePM exposed (SePM) groups**

		Mass (g)	$\dot{M}O_2$ (mMolO <sub>2</sub> · kg <sup>-1</sup> · h <sup>-1</sup> )	$\dot{V}_G$ (mLH <sub>2</sub> O · kg <sup>-1</sup> · min <sup>-1</sup> )	$f_R$ (breaths · min <sup>-1</sup> )	$V_T$ (LH <sub>2</sub> O · Kg <sup>-1</sup> · min <sup>-1</sup> )	O <sub>2</sub> crit (%)	EO <sub>2</sub> (%)	$f_H$ (bpm)
Control	Normoxia		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	110 ± 87	1.34±0.15	356.41±38.57 <sup>#</sup>	71.64±3.00 <sup>#</sup>	6.28±1.74		40.10±3.18 <sup>#</sup>	22.35±1.69 <sup>*</sup>
SePM	Normoxia		3.20±0.24	20.19±1.32 <sup>*</sup>	66.81±2.95 <sup>*</sup>	0.74±0.24	34	76.40±3.25 <sup>*</sup>	34.00±1.47 <sup>#</sup>
	Deep Hypoxia		1.26±0.09	246.46±20.22 <sup>**</sup>	71.44±4.28 <sup>#</sup>	4.03±0.57		46.64±4.61 <sup>**</sup>	24.21±1.53 <sup>#</sup>

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<sub>2</sub>), heart rate ( $f_H$ ), and critical tension (O<sub>2</sub>crit). Asterisk denotes difference between experimental groups and hashtag denotes difference between tensions. ANOVA two way and Student-Newman-Keuls' tests ( $P < 0.05$ ).

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