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PII: S0045-6535(20)33018-6

DOI: https://doi.org/10.1016/j.chemosphere.2020.128820

Reference: CHEM 128820

To appear in: *ECSN*

Received Date: 5 June 2020

Revised Date: 1 October 2020

Accepted Date: 28 October 2020

Please cite this article as: Rivera-Ingraham, G.A., Andrade, M., Vigouroux, R., Solé, M., Brokordt, K., Lignot, J.-H., Freitas, R., Are we neglecting Earth while conquering space? Effects of aluminized solid rocket fuel combustion on the physiology of a tropical freshwater invertebrate, *Chemosphere*, https://doi.org/10.1016/j.chemosphere.2020.128820.

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CREDIT AUTHOR STATEMENT

G.A. Rivera-Ingraham, R. Vigouroux and J.-H. Lignot conceptualized the experiment. G.A. Rivera-Ingraham conducted the *in-vivo* analyses while M. Andrade, R. Freitas and M. Solé conducted the biochemical analyses. All authors contributed to data interpretation, manuscript writing, review and editing.

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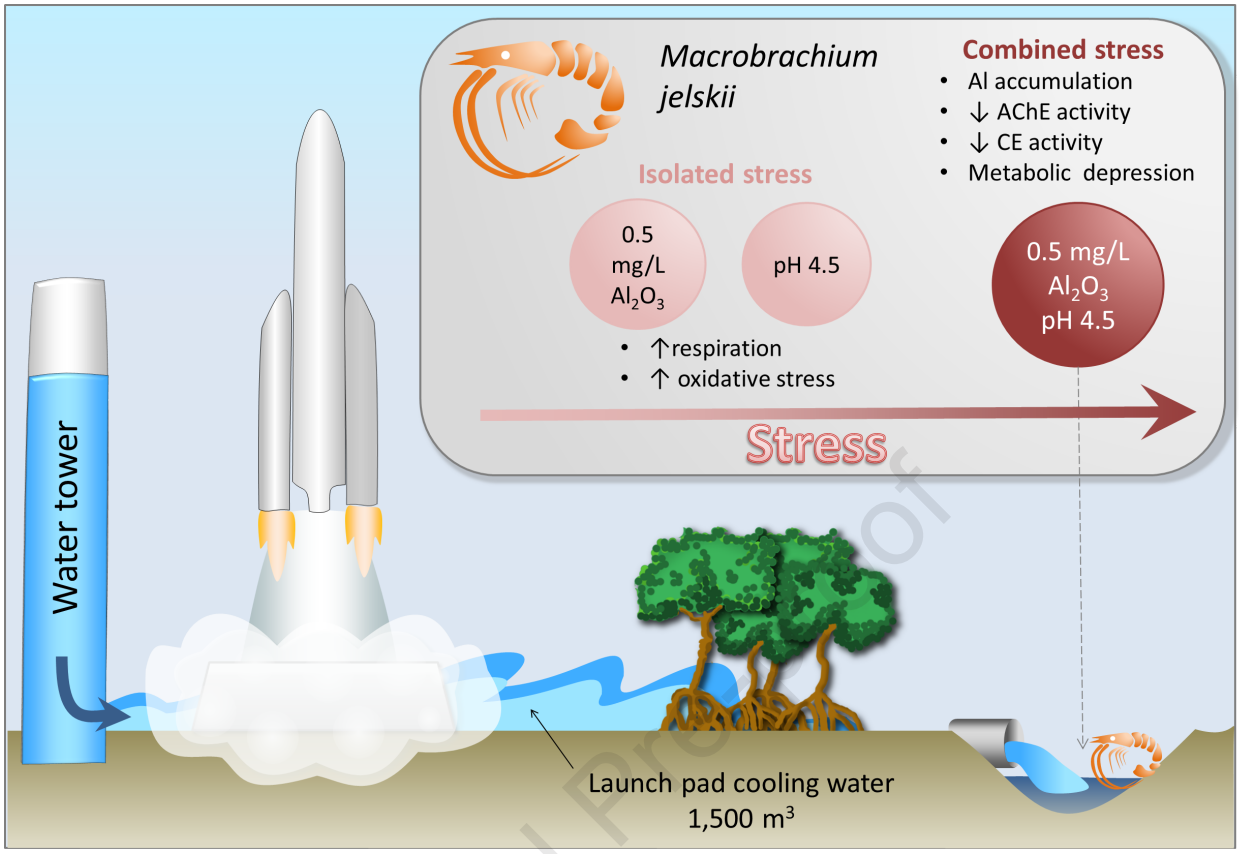

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ABSTRACT

Space launchers often use aluminized-solid fuel ("propergol") as propellant and its combustion releases tons of Al_2O_3 and HCl that sink in terrestrial and aquatic environments, polluting and decreasing water pH. We studied the impact of these events on the biochemical/physiological performance of the freshwater shrimp *Macrobrachium jelskii*, with wild specimens collected from a non-impacted site in French Guiana. In the laboratory, shrimps were exposed for one week to: i) undisturbed conditions; ii) Al_2O_3 exposure (0.5 mg L^{-1}) at normal pH (6.6); iii) decreased pH (4.5) (mimicking HCl release in the environment) with no Al_2O_3; or iv) Al_2O_3 0.5 mg L^{-1} and pH 4.5, representing the average conditions found in the water bodies around the *Ariane 5* launch pad. Results showed that shrimps bioaccumulated Al regardless of water pH. The combined effect of Al_2O_3 and low pH caused the most impact: acetylcholinesterase and carboxylesterase activities decreased, indicating neurotoxicity and reduced detoxification

capacity, respectively. Animal respiration was enhanced with Al_2O_3 and pH variations alone, but the synergic interaction of both stressors caused respiration to decrease, suggesting metabolic depression. Oxidative damage followed a similar pattern to respiration rates across conditions, suggesting free radical-mediation in Al toxicity. Antioxidant activities varied among enzymes, with glutathione reductase being the most impacted by Al_2O_3 exposure. This study shows the importance of addressing space ports' impact on the environment, setting the bases for selecting the most appropriate biomarkers for future monitoring programs using a widespread and sensitive crustacean in the context of an increasing space-oriented activity across the world.

Keywords: acidification, aluminum oxide, biomarkers, crustaceans, homeostasis, propergol toxicity.

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1. INTRODUCTION

In the context of space exploration, there is an increasing activity of space ports throughout the world. In 2017, the most active space ports were Cape Canaveral (USA), Kourou (French Guiana) and Baïkonour (Russia) in that order (Bousquet, 2017), counting with 5, 3 and 5 active launch pads, respectively. There are another 8 other important space ports located in the USA, Russia, China, India, Japan and New Zealand, but there are numerous and smaller other facilities worldwide. Though having certainly a limited spatial impact, the environmental pollution caused by such activities, and including in the context of global climate change, calls for the urgent need to determine its consequences on nearby environments and human populations, the final goal being to better assess policy options.

Most commonly, launchers use aluminized solid propellant, known as "propergol", which is composed of about 68 % of ammonium perchlorate (NH_4ClO_4), 18 % aluminum (Al) and 14 % de polybutadiene. After each launch, a large (contrail) cloud resulting from the combustion of propergol during the flight is released over several km^3 into the atmosphere, and combustion compounds deposit into soils and water bodies (Cencetti et al., 2007; Voigt et al., 2016). For the specific case of Ariane 5, this cloud has been estimated to contain 149 Tn of Al_2O_3 (resulting from the combustion of the highly reactive Al particles) (Gonçalves de Miranda, 2000), 120 Tn of CO and CO_2 and 90 Tn of HCl (De Lacour, 2011). But the launch pad itself is left with most of the pollutants, which derive from the acid (ground) cloud produced during the take-off (Richard and Chemoul, 2012). This is composed of unneglectable accumulations of Al_2O_3 microparticles and HCl, which reach nearby aquatic compartments when over 1,500 m^3 of water are released on the launch pad surface to cool down ground installations (Harvey, 2003). For the case of Kourou's Space Port, this cloud has been estimated to most significantly pollute the 1 km^2 around the launch pad and to affect an area of up to 8 km^2 (De Lacour, 2011) and its particles have been detected at least several weeks after a launch (Vigouroux, pers.

obs). Even if up to date the quality of the freshwater bodies located roughly around space ports has been monitored from a physico-chemical perspective, to the authors knowledge there are no studies addressing the impact of these launches on the fitness and physiology of the macrofauna present around the launch pads and connecting waters.

Even if certain molecules released from the combustion of propergol may be degraded or become biologically unavailable, others may have an important ecotoxicological impact and interfere with physiological processes in the short, medium and long terms. Among these, Al_2O_3 particles stand out, and upon combustion these reach the environment in the micro-size range (with a size of 3-4 µm for the smallest particles and up to 50-60 µm for particle agglomerates) (Gonçalves de Miranda, 2000). In general terms, aluminum-based compounds are of particular interest given that, as far as it is known, this element has no physiological role (reviewed by Nayak, 2002). It is often responsible for adverse physiological effects to humans: it is a known neurotoxic agent (Kaizer et al., 2008) and is believed to be responsible for neurodegenerative diseases (Halatek et al., 2005). Aluminum has also been long recognized to be a toxicant for aquatic species, particularly in gill breathing fauna. Gills are osmoregulatory organs, and Al may accumulate in its tissues and compromise the activity of enzymes involved in ion uptake, leading to loss of plasma/haemolymph ions and eventually causing osmoregulatory failure (Rosseland et al., 1990). Aluminum toxicity could be accentuated when environmental pH is reduced, as happens around the launch pads due to the concomitant release of HCl. In such cases, aquatic fauna is increasingly impacted, because: i) low pH increases the solubility of Al in water (e.g. Rejeki, 2003); ii) a decrease in pH in the environment may cause metals to enter tissues in an ionic state, having higher toxic effects than if these compounds remained in a neutral state (Rendal et al., 2011) and iii) because gills, already impacted by Al bioaccumulation, could be increasingly solicited in ion pumping to maintain intracellular acid-base homeostasis (Henry and Wheatly, 1992).

In this context, there is thus an urgent need to characterize the effect of the generated wastewaters (with Al_2O_3 microparticles and with a low pH) that infiltrate the nearby sediments and which may enter the freshwater network, especially in the context of increasing activity of space ports. To our knowledge, this is the first study addressing the impact of Al_2O_3 microparticles under acidic conditions in the aquatic environment. This is a relatively insoluble compound when compared with other Al-based molecules, which have been extensively used in the literature to address the interacting effects of Al and low pH in temperate fish and invertebrates. Nevertheless the information on tropical species remains scarce (Rejeki, 2003). Because space ports are located in tropical and subtropical regions of the world, to minimize the amount of fuel required for launchers to reach space, this work aims to fill in the gap of knowledge on the consequences of these activities on tropical environments. We addressed this issue using a crustacean species because they are known to be especially sensitive to low pH and Al pollution (Herrmann, 2001). We selected the freshwater shrimp *Macrobrachium jelskii* (Miers, 1877) Chace and Holthuis, 1948 (Caridea, Palaemonidae) as a model species. This species inhabits the roots and vegetation of margin freshwater environments of the Atlantic coast of Central and South America, roughly from Costa Rica to Argentina (see Collins, 2000 and references therein). The relevance of this model relies on it being of ecological and economical importance, used in fishing, fishkeeping and food (Vera-Silva et al., 2017). It may also potentially become an interesting bioindicator, because *Macrobrachium* is the largest genus of the family Palaemonidae and that *Macrobrachium* species are present in waters of every continent except Europe (Holthuis and Ng, 2009). Using this freshwater shrimp, the present study focused on the consequences of launching activities on animal physiological and biochemical performance, given that these key endpoints have been rarely considered, further justifying the interest of this work. Furthermore, most previous studies have addressed Al toxicity using soluble aluminum compounds. Given that the Al_2O_3 resulting from the propergol combustion is sparingly soluble at circumneutral pH, the need to characterize its impact on

animal physiology is further required. For the case of Al_2O_3 nanoparticles, a previous study using the freshwater branchiopod *Ceriodaphnia dubia* suggests that its toxicity is due to free radical formation, but also to a perturbation of the energy budget of the cells (Li et al., 2011). Hence, for the first time in an invertebrate species, we address in the present study the effects of pH on Al_2O_3 toxicity from an energy-redox perspective. We focused, on the one hand, on energy use, because all cellular processes (even at basal conditions but especially under environmental changes) have an energetic cost. On the other hand, we analyzed redox balance as the equilibrium between antioxidant defenses and oxidative damage. This is because mitochondria, in their role of cell energy suppliers, consume O_2 and consequently produce free radicals (reactive oxygen and nitrogen species) (RONS) which need to be neutralized by (energy-costly) antioxidant defenses. When antioxidant and other detoxification mechanisms are overwhelmed by these compounds, cell structures such as membrane, proteins or DNA may be damaged and eventually induce mutations and cell death.

The present study was conducted in French Guiana, hosting the Guiana Space Centre (CSG) which was here selected as a study case. It is from CSG that most of the European and allied satellites are launched. It covers an area of about 700 km^2 and it is composed of three active launch pads for Ariane 5, Vega and Soyouz rockets, with the first two using propergol as propellant. Using *M. jelskii*, which is commonly found in the water courses around CSG, the final goal of the study is to infer on the physiological and biochemical effects of the combined effects of Al_2O_3 microparticles and decreased pH on this representative crustacean species of the freshwater macrofauna of French Guiana. Ultimately, this study aims to identify suitable early warning biomarkers of launchers' impact in a worldwide represented family of crustaceans to better aid policy managers and advisors in the management of increasing numbers of launch activities worldwide.

2. MATERIALS AND METHODS

138 *2.1 Animal procurement and maintenance conditions*

139 A total of 150 *Macrobrachium jelskii* juveniles were collected from Bois Diable Lake
140 (5º10'41.2''N; 52º39'28.8''W), located outside the space port's impact zone and within the
141 outskirts of Kourou, French Guiana. Care was taken to use animals of similar weight (0.45 ±
142 0.03 g). Water parameters at the collection site were the following: 92% air saturation (WTW
143 Oxi 3205), 0.0 ppt salinity, 213 µS cm^{-1} conductivity (WTW ProfiLine Cond 3110), 30.3 ˚C
144 temperature and a pH of 6.6 (WTW ProfiLine pH 1970i). Water samples were collected at the
145 site for Al content determinations.

146 Animals were immediately transported to the laboratory facilities at Hydreco-Guyane, where
147 they were maintained in aquaria equipped with aeration systems and maintained with fresh
148 water from the collection site. Animals were allowed to acclimate to laboratory conditions for
149 5 days. Water was changed each 48h using freshly collected water from Bois Diable Lake and
150 Al_2O_3 and pH were reconstituted accordingly.

151 *2.2 Experimental design*

152 After the acclimation period, animals were exposed to one of the following conditions: i)
153 unaltered conditions (for control purposes); ii) Al_2O_3 microparticle exposure (0.5 mg L^{-1}) at
154 natural pH (6.6) (serving as an Al_2O_3 control), iii) decreased pH (4.5) (serving as a decreased pH
155 control) and iv) Al_2O_3 microparticle exposure (0.5 mg L^{-1}) under low pH (4.5). The later
156 represents the average Al_2O_3 concentrations and pH values found in the Karouabo stream
157 (where the 1,500m^3 of cooling water ends up pouring) around 7 days after an Ariane 5 launch
158 (Monchaux et al., 2015; Clavier et al., 2017). This particular stream has little exchange rates
159 (especially during dry season), ensuring that our laboratory conditions reasonably mimic those
160 found in the environment. All treatments were carried out in water freshly obtained from the
161 animal collection site. Al_2O_3 was purchased from Sigma (purity 99.9%). This compound is
162 sparingly hydro-soluble at pH 6.6 but its solubility increases under the acidified conditions here

used. Despite the degree of solubility, the particles were maintained in suspension through the effect of the aquaria aeration systems. In each case, pH was achieved by the addition of HCl since it is a major component of the propergol combustion. Three aquaria were used per condition, each containing 5 L of freshwater (changed each 48h) and 10 animals, making a total of 30 shrimps per conditions. Total exposure time was 7 days. After this period, 6 shrimps per condition (2 per aquarium) were used to carry out respirometric analyses and were later preserved in liquid nitrogen to determine Al_2O_3 bioaccumulation in tissues. Another 21 animals per condition (7 per aquarium) were sacrificed through immersion in liquid nitrogen for biochemical determinations (see below). The 3 remaining animals per condition were fixed in Bouin's fixative for further histological analyses in the frame of another study.

2.3 Respirometric analyses

To quantify respiration rates (RR), 10-ml glass metabolic chambers previously equipped with an oxygen sensor spots (OXSP5, sensor code SD7-541-207, Pyro-Science GmbH, Aachem, Germany) glued to the inner side of the chamber were used. For each measurement, a single animal was introduced in the chamber, containing 10 mL of freshly-prepared medium at the pH and Al_2O_3 conditions to which the animal was exposed during the experiment. Chambers were then closed, ensuring the absence of any air bubbles within the chamber and measurements were carried out at room temperature (25ºC) using a four-channel fiber optic oxygen meter (Firesting, Pyro-Science GmbH). All measurements started in fully oxygenated water and oxygen concentration was registered each 5 sec through the Pyro Oxygen Logger software as a functioning of declining O_2 partial pressure (pO_2). Four measurements were recorded in parallel, in all cases 1 being a blank (containing no animals and serving for determining background (microbial) respiration). When possible, animals were allowed to breathe until oxygen was completely consumed in the chamber to estimate the respiratory behavior of these organisms and how water conditions affected hypoxia tolerance. The critical

188 pO_2 (p_cO_2), as defined by Tang (1933) and indicating the onset of anaerobic metabolism, was
189 calculated using the equation by Duggleby (1984).

190 After each measurement, all animals within the chamber were weighed and preserved in liquid
191 nitrogen for further quantification of Al accumulation (n=6 per condition). RR results were
192 expressed as nmol O_2 $min^{-1} \cdot g^{-1}$ fresh weight (FW).

193 *2.4 Metal content*

194 Aluminum bioaccumulation in whole soft tissues was assessed for each condition. All 6 animals
195 per condition preserved for this purpose were pooled into a single sample (with two technical
196 samples per pool). Total Al concentrations were quantified by inductively coupled plasma mass
197 spectrometry (ICP-MS, X-Series, Thermo Scientific), after microwave assisted acid digestion.
198 After freeze-drying, 100–200 mg of the samples were digested in a CEM MARS 5 microwave,
199 with 1 mL HNO_3 65%, 2 mL H_2O_2 30% and 1 mL H_2O, by increasing temperature to 180 ˚C in 10
200 min, which was then maintained for 10 min. After cooling, the obtained digests were
201 transferred into 25 mL polyethylene vessels and the volume made up with ultrapure water.
202 Quality control was made through the use of blanks, certified reference material NIST 2976
203 (Mussel Tissue) and duplicates. Blanks were below the quantification limits for Al, the
204 coefficient of variation of samples duplicates was 18% and mean percentage of recovery was
205 128%.

206 Dissolved aluminum in the water from the collection site (n=4) was also determined using
207 inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 7400 Duo, Thermo
208 Scientific) according to the NF EN ISO 11885 method.

209 *2.5 Biochemical analyses*

210 All biochemical analyses were conducted on 4 pools per experimental condition, each
211 composed of 5 animals (i.e. 20 randomly selected animals out of the 21 preserved for this

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purpose). Animals were homogenized using a manual potter and liquid nitrogen. Each resulting homogenate was separated into three subsamples of about 0.2 g of grounded tissue each. One subsample was diluted in a 50mM phosphate buffer (pH 7.0 with 1mM ethylene diamine tetraacetic acid tetrasodium salt hydrate, 1% (v/v) Triton X-100 and 1mM dithiothreitol) and was used to assess: i) energy reserves (glycogen (GLY) and protein (PROT) contents); ii) antioxidant enzyme activities; iii) cellular damage (protein carbonyl content (PC)); iv) neurotoxicity and metabolism (acetylcholinesterase (AChE) and carboxylesterase (CE) activities). A second subsample was diluted at a 1:2 ratio (w:v) in a 0.1M Tris-HCl buffer (containing 15% (w/v) PVP, 153 mM magnesium sulfate ($MgSO_4$) and 0.2% (v/v) Triton X-100) and served to quantify electron transport chain (ETC) activity. The third and last subsample was diluted in 20% (v/v) trichloroacetic solution and was used to quantify lipid peroxidation (LPO), also indicative of oxidative damage.

GLY content was assessed using the protocol described by Dubois et al. (1956) and using glucose to build the standard curve, which ranged from 0 to 2 mg per mL. Protein content was assessed following the Lowry et al. (1951) method, with bovine serum albumin as standard (100 µg mL^{-1}). In both cases, measurements were carried out spectrophotometrically at 492 and 750 nm for GLY and PROT content, respectively. Results were expressed in mg per g FW.

Antioxidant and biotransformation/detoxification capacity of shrimps was assessed through the quantification of the activity 5 different enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferases (GSTs) and glutathione reductase (GR). SOD activity was determined in samples following the protocol original described by Beauchamp and Fridovich (1971). Data were obtained using a curve of SOD standards which ranged between 0.25 to 60 units (U) mL^{-1}, where one U corresponds to the amount of enzyme causing a 50% inhibition of nitroblue tetrazolium reduction under assay conditions. CAT activity was measured spectrophotometrically at 540 nm according to

238 Johansson and Borg (1988) and using a formaldehyde standard curve ranging from 0 to 150
239 µM. CAT activity was expressed as U per g FW, where one U of enzyme activity corresponds to
240 the formation of 1 nmol of formaldehyde per min under assay conditions. GPx was determined
241 spectrophotometrically at 340 nm (Ɛ = 6.22 nM^{-1} cm^{-1}) (Paglia and Valentine, 1967) and
242 results were expressed as U per g FW (U=nmol min^{-1}), where U represent the number of
243 enzymes that caused the formation of 1.0 µmol nicotinamide adenine dinucleotide phosphate
244 (NADPH) per min. GSTs were also quantified spectrophotometrically (340 nm, Ɛ = 9.6 mM^{-1}
245 cm^{-1}) using a method adapted from Habig et al. (1974). Results were expressed as U of GSTs
246 activity per g FW, where one U corresponds to the quantity of GSTs that catalyzes the
247 conversion of 1 µmol of 1-chloro-2,4-dinitrobenzene per min. GR activity was measured as the
248 oxidation of NADPH following the protocol described by Carlberg and Mannervik (1985).
249 Measurements were carried out spectrophotometrically at 340 nm (Ɛ = 6.22$mM^{-1}cm^{-1}$) and
250 expressed as U per g FW, were U in this case represents the µmol of NADPH oxidized per min.

251 Oxidative damage was assessed through the quantification of protein carbonyls (PC) and
252 peroxidized lipid (LPO) content. PC levels were determined using the DNPH alkaline method
253 (Mesquita et al., 2014), results were read at an absorbance of 459 nm and expressed as nmol
254 of protein carbonyl groups formed per gFW. LPO was determined as TBARS (thiobarbituric acid
255 reactive substances) content using the protocol described by Buege and Aust (1978). Briefly,
256 this method consists in adding 2-thiobarbituric acid (TBA), which reacts with lipid peroxidation
257 by-products (such as malondialdehyde). The resulting TBARS were quantified by absorbance at
258 532nm and results were expressed in nmol of MDA equivalents per gFW.

259 ETC activity was assessed using the protocol by Packard (1974) with the modifications
260 described by De Coen and Janssen (1997). The absorbance was read at 490 nm during 10 min
261 at 25 sec intervals using a microplate reader. ETC (i.e Q-cytochrome B complex) activity was

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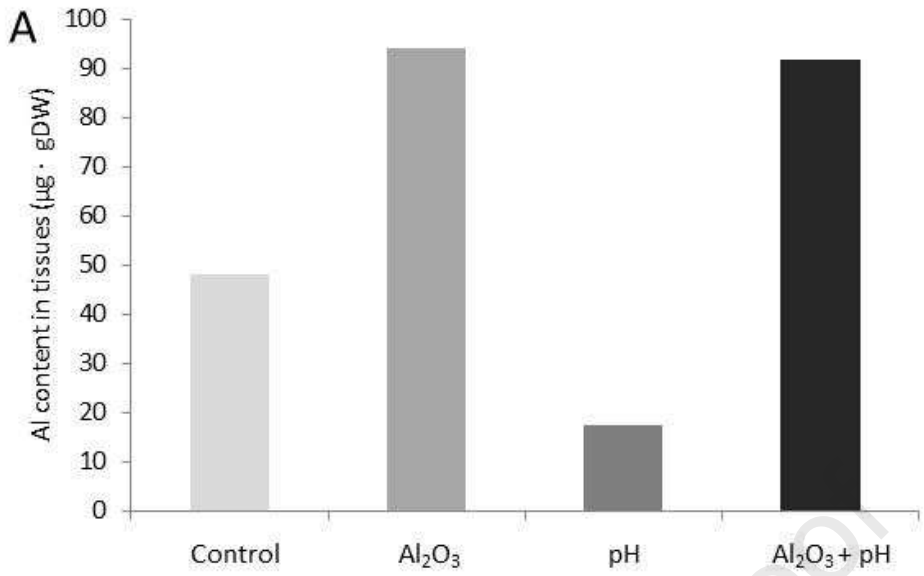
262 calculated as the amount of formazan formed in each well and the results expressed in nmol ·
263 min^{-1} per g FW.

264 Neurotoxicity and detoxification capacities were assessed through the quantification of AChE
265 and CEs activities, respectively. For AChE activity determinations we followed the method of
266 Ellman et al. (1961) and modifications by Mennillo et al. (2017). Enzyme activities, measured as
267 the formation of dianion of 5-thio-2-nitrobenzoic acid, were recorded spectrophotometrically
268 for 5 min at 412 nm and expressed in nmol per min per g FW, using the molar extinction
269 coefficient (ε) 13,600 nM^{-1} cm^{-1}. CEs were measured using 2 different commercial substrates:
270 ρ-nitrophenyl acetate (ρNPA) and ρ-nitrophenyl butyrate (ρNPB). Activity was measured
271 spectrophotometrically at 405 nm as the formation of ρ-nitrophenol from ρNPA and ρNPB as
272 described by Hosokawa and Satoh (2005). Activities were expressed nmol min^{-1} per g FW.
273
274 *2.6 Stastistical analyses*
275 All data sets were tested for normality (Kolmogorov-Smirnov test) and homocedasticity
276 (Levene test). When the assumptions for parametric statistics were met, one-way ANOVA tests
277 were carried out, followed by a Student-Newman-Keuls *a-posteriori* multiple comparison test.
278 For the rest of the cases, a Kruskal-Wallis test was performed followed by U-Mann Whitney
279 pairwise comparison tests. All these analyses were carried out using SPSS 15.0 (SPSS Inc., IL,
280 USA). All data and figures are expressed as mean ± standard error of mean (SEM). Significant
281 differences among conditions were represented with different lower and upper case letters in
282 the graphs.
283
284 **3. RESULTS**

285 No mortality was recorded throughout the experiment, suggesting no acute toxicity.

286 *3.1 Aluminum content in water and bioaccumulation levels*



287

288 **Figure 1: Bioaccumulation levels of Al registered in *M. jelskii* under the different treatments**

289 Water at Bois Diable Lake, which was here used for the *in-vitro* experimentation, showed
290 values below 0.05 mg Al per ml. Animals exposed for 7 d to the treatment containing Al_2O_3
291 microparticles in the water (under normal pH) showed Al contents in their tissues that were 2-
292 fold higher than the controls (Figure 1). These values were similar to those registered in
293 animals exposed to the combined effect of Al_2O_3 microparticles and decreased pH. Animals
294 exposed to acidic water conditions (with no Al_2O_3 addition) showed half the content of the
295 control animals.

296

297 *3.2 Energy related parameters*

298 Whole animal respiration rates (RR) were higher in those exposed to high Al_2O_3 concentration
299 or low pH conditions respect to the control ($F=10.011$; $p<0.001$) (Figure 2A). In contrast,
300 animals exposed to the combination of Al_2O_3 plus low pH showed the lowest RR values. The
301 p_cO_2 values roughly followed the RR pattern, but differences were not statistically significant
302 among treatments ($F=1.418$; $p=0.263$).

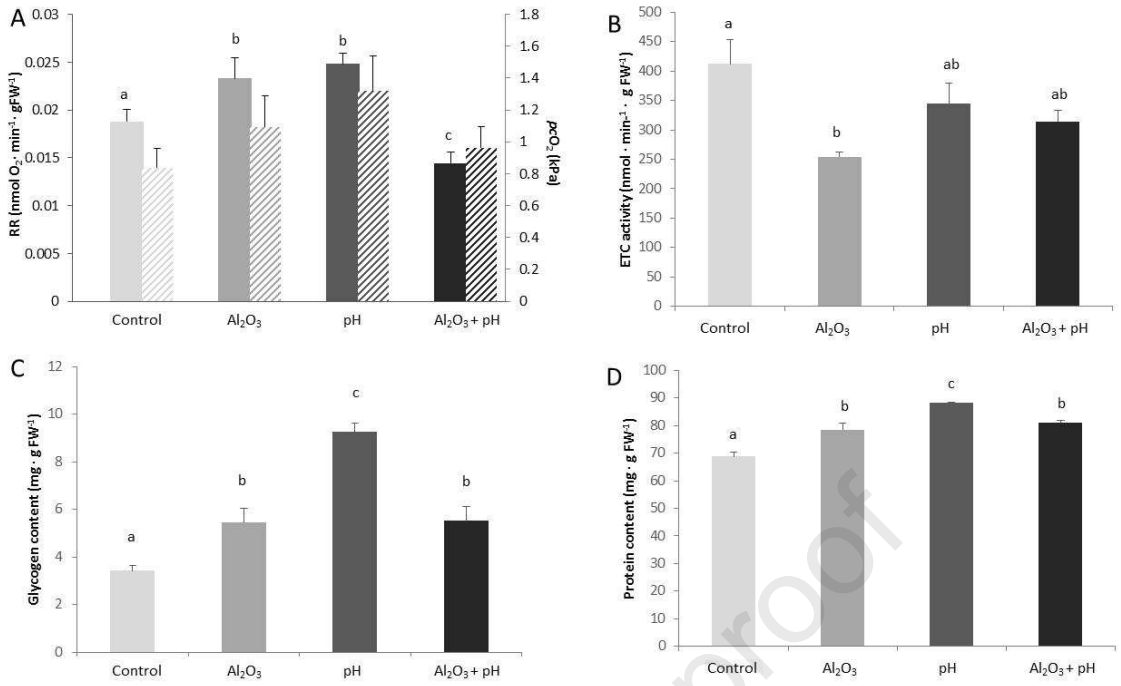


Figure 2: Energetic parameters (means ± SEM) measured in *M. jelskii* under the different treatments. A) Respiration rates (RR) and critical pO_2 (p_cO_2), shown in plain-colored and striped columns, respectively (n=6); B) Mitochondrial electron transport chain (ETC) activity; C) Glycogen (GLY) content; D) Protein (PROT) content. Values associated to different letters are statistically different from each other, according to a one-way ANOVA test followed by a Student-Newman-Keuls *a-posteriori* multiple comparison test (for results shown in subpanels A and C), and a Kruskal-Wallis followed by U-Mann Whitney pair-wise comparisons (for results shown in subpanel B and D).

The maximum ETC activity was shown by control animals, while Al_2O_3 alone induced a 1.6-fold decrease. However, overall ETC activities did not match the RR results (Figure 2B). The only significant difference was registered between controls and animals exposed to Al_2O_3 under normal pH ($F=4.132$; $p=0.038$).

Both GLY and PROT content were significantly affected by changes in water conditions (GLY: $F=26.048$; $p< 0.001$. PROT: $K=10.072$; $p=0.018$) (Figures 2C and 2D). In both cases data showed a similar pattern: the presence of Al_2O_3 in the water (with or without decreased pH) caused an increase in GLY and PROT contents by about 1.6- and 1.2-fold, respectively. Animals subjected to a decrease in pH showed the highest values (2.7- and 1.3-fold over control animals for GLY and PROT contents, respectively).

3.3 Antioxidants

All the antioxidant activities analyzed in this study showed significant differences among

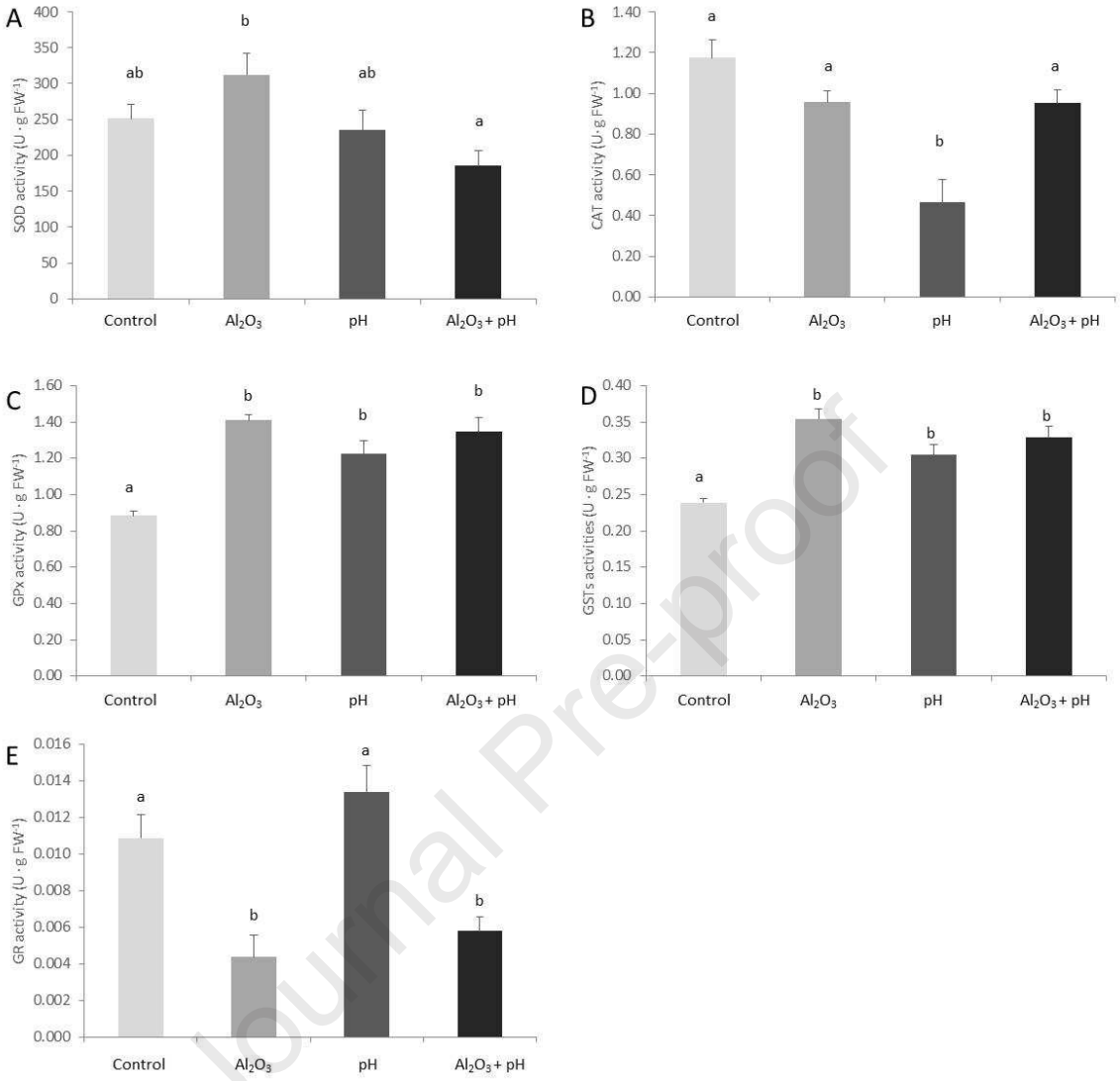


Figure 3: Antioxidant and biotransformation activities (means ± SEM) measured in *M. jelskii* under the different treatments: A) Superoxide dismutase (SOD); B) Catalase (CAT); C) Glutathione peroxidase (GPx); D) Glutathione S-transferases (GSTs); E) Glutathione reductase (GR). Values associated to different letters are statistically different from each other, according to a one-way ANOVA test followed by a Student-Newman-Keuls *a-posteriori* multiple comparison test.

conditions, although the patterns varied among the enzymes measured. SOD activity showed the highest values in animals exposed to increased Al_2O_3 concentrations, while the lowest values were shown by animals exposed to the combination of Al_2O_3 and reduced pH ($F=4.335$; $p=0.034$) (Figure 3A). CAT activity, however, showed similar values across conditions, for the

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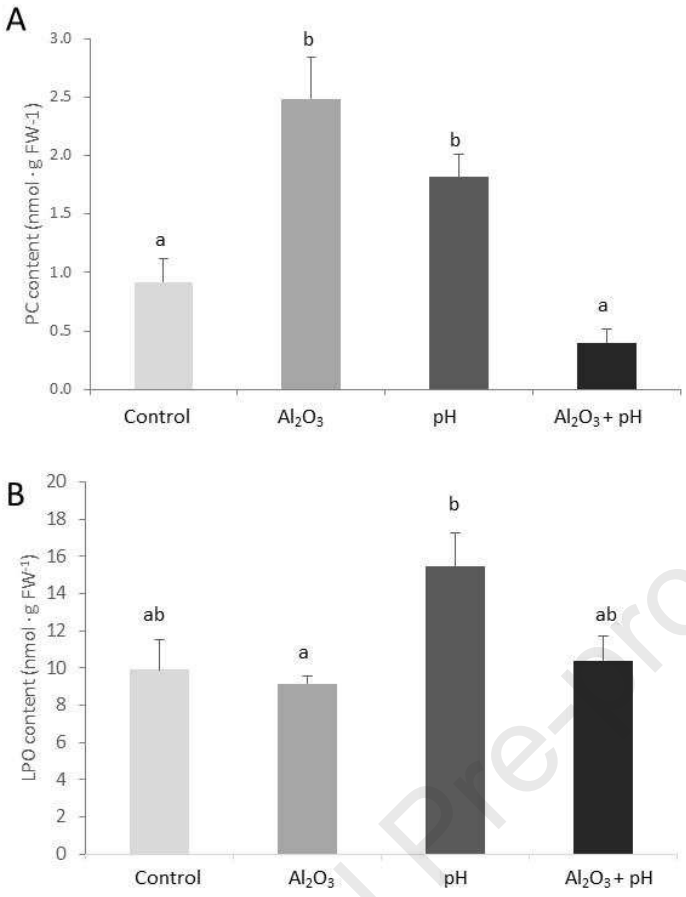


Figure 4: Oxidative damage markers (means ± SEM) measured in *M. jelskii* under the different treatments: A) protein carbonyl (PC) content; B) peroxidized lipid (LPO) (malondialdehyde-like compounds) content. Values associated to different letters are statistically different from each other, according to a one-way ANOVA test followed by a Student-Newman-Keuls *a-posteriori* multiple comparison test.

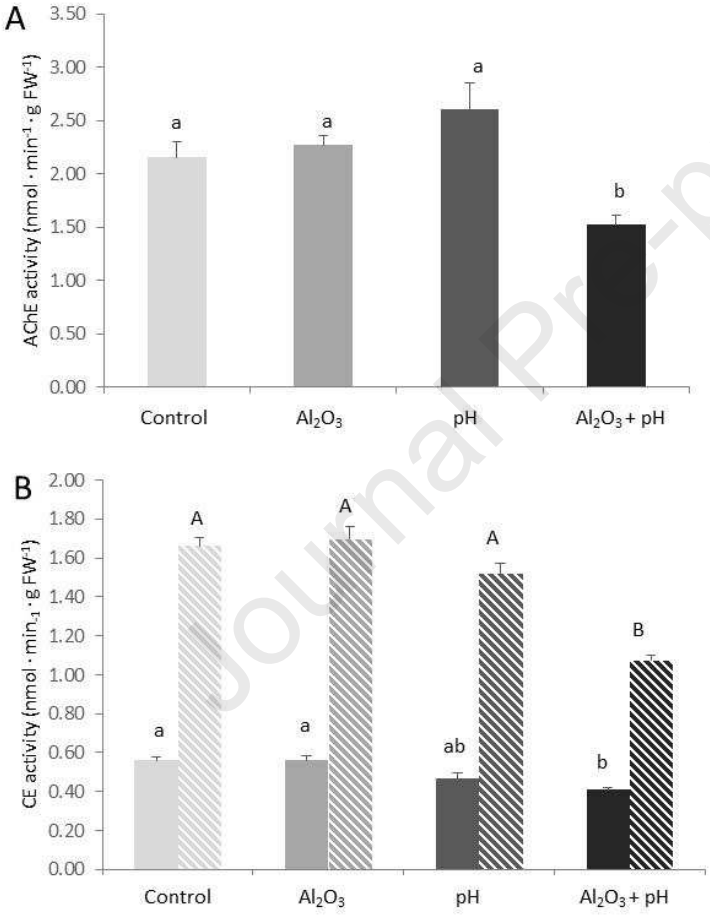
sole exception of animals exposed to reduced pH, for which values were in average 2-fold lower (F=11.585; p=0.001) (Figure 3B). GPx (Figure 3C) and GSTs activities (Figure 3D) showed precisely the same pattern, with all conditions showing significantly increased values compared to controls (GPx: F=17.323; p=0.001. GSTs: F=17.228; p<0.001). GR activity was significantly lower in those animals exposed to increased Al_2O_3 in the water, accompanied or not with a decrease in environmental pH (F=11.917; p=0.001) (Figure 3E).

3.4 Oxidative damage

Both protein and lipid damage values showed significant differences among conditions. Protein carbonyl content was highest in animals exposed to decreased pH and increased Al_2O_3 alone

343 (F=18.906; *p*<0.001). However, animals subjected to the combination of these two factors
344 showed values that did not differ from controls (Figure 4A). Contrarily, MDA content
345 (associated with lipid peroxidation) was most affected by low pH, with animals exposed to
346 decrease environmental pH showing 1.5-fold higher MDA content than controls (F=6.697;
347 *p*=0.045) (Figure 4B).

348 *3.5 Neurotoxicity and detoxification capacity*



349

350 **Figure 5: Activity values as (means ± SEM), corresponding to: A) acetylcholinesterase (AChE) activity and B)**
351 **Carboxylesterase activity, measured using ρNPA and ρNPB as substrates (plain-colored and stripped bars,**
352 **respectively). Values associated to different letters are statistically different from each other, according to a one-**
353 **way ANOVA test followed by a Student-Newman-Keuls *α-posteriori* multiple comparison test.**

354 AChE activity was significantly lower (by 1.5-fold) in organisms exposed to Al_2O_3 at low pH in
355 comparison to the remaining conditions (F=7.657; *p*=0.005) (Figure 5A). CEs showed a similar
356 pattern, although activities measured with ρNPB as substrate yielded higher hydrolysis rates

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than with ρNPA. In both cases, activities were the lowest under Al_2O_3 exposure at low pH (ρNPA: F= 7.820; p=0.006. ρNPB: F=27.161; p <0.001) (Figure 5B).

4. DISCUSSION

The present study documents the hazard of anthropogenic acidification and micron-sized Al_2O_3 pollution in the context of space port launching activities. To the authors' knowledge, our study is the first to address this subject using an energy-redox approach in an aquatic invertebrate. Ecological studies carried out around the Ariane 5 launch pad in French Guiana revealed that the biodiversity and abundance of aquatic invertebrates such as Diptera larvae are comparable to control values three weeks after a launch (Vigouroux, pers. obs.). However, it is known that compared to such ecological approaches (consisting on taxonomical or trait-based metrics), eco-physiological parameters can significantly reduce the threshold at which stress can be detected. This study provides evidence that launch activities impact the shrimps at a biochemical and physiological levels. Thus, it would therefore be advisable to reproduce the same studies over a time step closer to a launch and to integrate these physiological analyses *in-situ* to select a set of early-warning biomarkers for their potential implementation in future monitoring programs.

Al_2O_3 exposure impairs aquatic respiration and leads to oxidative damage

Our results show that even if Al_2O_3 is only scarcely hydrosoluble at circumneutral pH, *M. jelskii* exposed to 0.5 mg L^{-1} of Al_2O_3 accumulate Al in their body, with values reaching almost double the concentrations of control (undisturbed) animals. Given its low solubility at normal pH (6.6), we hypothesize that that this accumulation must occur mostly through particle ingestion.

Regardless of the uptake pathway, Al_2O_3 exposure under normal pH caused animals to significantly increase their RRs. Such a response has also been observed by Herrmann and Andersson (1986) in two species of lotic mayflies exposed to increasing amounts of aluminum

sulfate in the water (in the range of the values here used). Another study carried out in rainbow trout also registered an increase in RR within the first 3 days of exposure to aluminum sulfate although values decreased after that time (Neville, 1985). But despite the increased RR, ETC activity was 1.6 times lower than the control group, leading us to hypothesize that respiration was impaired. "The gills are the major site of interactions between waterborne toxic metals and the organism in aquatic Crustacea" (Henry et al., 2012) and it has been observed that aquatic organisms exposed to Al-rich waters accumulate Al on their gills (reviewed by Rosseland et al., 1990). When dealing with more soluble Al compounds (aluminum sulfates, nitrates, etc.), this occurs in the form of aluminum hydroxide ($Al(OH)_3$) and presumably due to the negative charge of the gill mucus (McDonald, 1983). But the scarcely hydrosoluble soluble micro-particles like the ones used in this study, are nevertheless recognized by the immune system as foreign bodies and leading to the induction of the mucosal immune response. In consequence, exposure to Al_2O_3 may produce, as Al does, inflammation of gill tissues and proliferation of mucus cells (reviewed by Rosseland et al., 1990). An overproduction of mucus may block oxygen uptake and eventually lead to gill clogging and a decrease of respiration efficiency through a "mechanical impact route" (Herrmann and Andersson, 1986). Without further confirmation through histological examination, we hypothesize that animals may be hyperventilating under these conditions, as has been seen to happen in other organisms (Malte and Weber, 1988), in an attempt to increase O_2 uptake and fight an increasing functional hypoxia.

Despite a decreased ETC activity, we can presume that there is enhanced RONS production, inducing oxidative stress as evidenced by the 2.7-times higher PC levels registered in the Al_2O_3-exposed group. In the nematode *Caenorhabditis elegans*, exposure to Al_2O_3 nanoparticles indeed increased RONS formation, as shown by the 2',7' dichlorofluorescein fluorescence assay (Li et al., 2012). The source of these RONS could be various: i) due to direct exposure to Al_2O_3 and/or alterations on mitochondrial activity; ii) because Al_2O_3 may, as Al does, be

407 increasing intracellular Fe concentrations through various pathways (e.g. Wu et al., 2012) and
408 thus promoting the Fenton reactions; iii) as the result of the immune response, carried out
409 mainly by haemocytes which produce RONS during the phagocytic process of foreign particles
410 (Oyanedel et al., 2016).

411 The activities of GSTs and GPx were significantly increased upon changes in environmental
412 conditions. Their synthesis, as well as that of other new molecules to face the stress, may
413 explain the increased PROT levels. But these defense mechanisms would require additional
414 energetic demands (e.g. Novais et al., 2013). This has been observed in other freshwater
415 invertebrates exposed to heavy metal pollution, which resulted in depleted GLY reserves (e.g.
416 Rajalekshmi and Mohandas, 1993). However, in the present study GLY contents were higher
417 compared to control values (as seen in other models) (e.g. Chinoy and Memon, 2001), leading
418 us to hypothesize that sources of energy other that GLY are being mobilized. Among the
419 antioxidants analyzed, GR activity was the most clearly affected by Al_2O_3, with decreased
420 activities under both normal and acidified conditions. GR is a key enzyme in the maintenance
421 of the redox homeostasis. It is responsible for reducing oxidized glutathione (GSSG) to renew
422 the reduced glutathione (GSH) pool, "the heart of one of the most important cellular
423 antioxidant systems" (Couto et al., 2016). Similar GR depletions when exposed to heavy metals
424 have been registered in freshwater bivalves (Guidi et al., 2010) or fish (Giguère et al., 2005).
425 The latter fish study suggested that this could be due to the metal binding to the enzyme
426 functional group. Some reports demonstrate that metal cations such as Zn^{+}, Cd^{2+}, Cu^{2+}or Fe^{2+}
427 (this varying among animal species) can induce redox inactivation of GR (Christie and Costa,
428 1984; Peinado et al., 1991; Cardoso et al., 2008). However, to the authors knowledge there is
429 no information that Al cations could induce similar effects. Another possibility is that GR is
430 inactivated due to the lack of NADPH, a co-enzyme that is required to catalyze the reduction of
431 GSSG. The major source of NADPH in the cell is the pentose phosphate pathway through the
432 glucose-6-phosphate dehydrogenase (G6PDH), which activity has been reported to be

433 inhibited by Al in various models, such as *Saccharomices cerevisiae* (Cho and Joshi, 1989) and
434 human erythrocytes (Bulat et al., 2008).

435 Altogether, and despite the increase of antioxidant activities, the results obtained are
436 suggestive of an excess of RONS formation, causing a misbalance between oxidant and anti-
437 oxidant pathways in favor of the first, and induce oxidative stress. This stress would be mainly
438 affecting the protein fraction of the cells, increasing PC levels significantly. Similar results have
439 been recorded for fish species using aluminum sulfate (García-Medina et al., 2010). Altogether,
440 these results suggest a link between Al and protein damage through the mediation of Al- or
441 respiratory-induced RONS formation.

442 *A decrease in pH reduces baseline Al but increases energetic requirements and oxidative stress*

443 Aluminum is the third most abundant element on the Earth's crust, and is commonly present
444 in continental water bodies. Water acidification promotes the mobilization of Al from the
445 edaphic to the aquatic environment, further increasing the concentrations to which aquatic
446 fauna are exposed. In the present study low pH alone actually reduced the presence of Al in
447 tissues by half (compared to controls). However, the increased RR showed by animals under
448 low pH could be contributing to fuel higher detoxification rates (which may potentially be
449 facilitated by the higher solubility of Al under acidified conditions), causing its accumulation to
450 be reduced compared to control values. As it will be discussed further below, this would be
451 possible under lower (natural) Al concentrations, and not being the case at increased levels
452 when animals are under higher levels of stress.

453 A decrease in environmental pH had important effects on the energy-redox parameters in *M.*
454 *jelskii.* This condition caused RRs to increase in the shrimps compared to controls, and though
455 maintaining comparable ETC activities, this would be supplying the cells with the necessary
456 energy to maintain pH homeostasis. Maintenance of intracellular acid-base balance is essential
457 for normal physiology and metabolic function, and it is achieved through various processes,

458 among which we find two energy-consuming processes. The first, the transfer of acid and/or
459 base equivalents across the cell membrane (namely electroneutral exchanges of HCO_3^- for Cl^-
460 and Na^+ for H^+) (Henry and Wheatly, 1992), which depends on the ion gradient produced by
461 active transporters such as the Na^+/K^+- and the H^+-ATPases (reviewed by Whiteley, 2011). And
462 second, transmembrane protein synthesis and activity (Deigweiher et al., 2010), which under
463 decreased pH has been estimated to require an allocation of up to 84% of the total energy
464 available (Pan et al., 2015). Thus, such compensatory processes may well be explaining the
465 increased RR observed in the present study. Such RR increases have also been documented for
466 marine organisms, although freshwater examples are scarce. Many studies already addressed
467 this in the context of climate change and CO_2-induced seawater acidification. Compared to
468 freshwater systems, pH changes in the marine environment are much smaller but they provide
469 a source of comparison. For example, at small pH changes (decrease in 0.4-1.2 units), some
470 studies have shown increases in RR in echinoderms (e.g. Wood et al., 2008; Stumpp et al.,
471 2011) or marine gastropods (e.g. Thomsen and Melzner, 2010). However, larger pH shifts in
472 the marine environment are often needed to lead to metabolic depression (e.g. Pörtner et al.,
473 2004). In freshwater systems, water acidification also occurs, with pH shifts being considerably
474 larger than in marine systems. For the particular case of the water bodies of French Guiana, pH
475 values are often as low as 5.5-6.0 and in some very lentic water courses it may decrease down
476 to 4.5 (Dedieu et al., 2015; Crespy et al., 2019), i.e. the pH value used in this study. Thus,
477 adapted to such acidic values, *M. jelskii* is capable of maintaining high RRs (at low pH alone)
478 and avoiding metabolic depression.

479 Nevertheless, the observed increase in RR could also be responsible for an increase in RONS
480 formation, depleting specific antioxidant reserves such as CAT. The same was observed for a
481 marine shrimp (*Litopenaeus vannamei*) exposed to decreased pH (6.7), where CAT was
482 decreased after 7 days of exposure (Han et al., 2018). Overall, this CAT depletion may be partly
483 contributing to the induction of oxidative stress, with the resulting excess in RONS formation

484 damaging both the protein and the lipid fractions of the cells. This agrees with previous studies
485 revealing that, under similar conditions, the yeast *Saccharomyces cerevisae* suffered also from
486 cellular membrane damage (García-Saucedo et al., 2011), or in the shrimp *L. vannamei* which
487 also showed increased MDA under decreased pH conditions (Han et al., 2018).

488

489 Al_2O_3 *microparticle pollution at low pH causes a synergic effect, inducing neurotoxicity and*
490 *metabolic depression.*

491 Usually, at lower pH Al becomes more bioavailable and crustaceans tend to accumulate higher
492 concentrations (Rejeki, 2003). Despite Al_2O_3 not being as hydrosoluble as aluminum sulfates
493 and nitrates commonly used in other Al-toxicity studies, its solubility still increases under acidic
494 conditions. This may be determining different uptake pathways than when Al_2O_3
495 microparticles are under less soluble form. Despite this, the Al concentration values registered
496 in animals exposed to Al_2O_3 at normal (6.6) and acid (4.5) pH conditions were similar. We may
497 consider two possible reasons for this: i) technically speaking, because the method here used
498 to quantify Al in the tissues may have certain technical limitations avoiding us to register
499 higher accumulation under acidic conditions; and ii) biologically, probably due to the animals
500 presenting lower ventilation rates (decreased scaphognathite beating) under the context of a
501 probable metabolic depression under this combined pressure.

502 Even if we did not register higher Al bioaccumulation at lower pH, it is evident from the
503 present results that the combined effect of Al under acidified conditions impacted animal
504 physiology at higher levels. In the present study, one of the most relevant conclusions is that
505 Al_2O_3 under low pH had neurotoxic impact on *M. jelskii.* This is shown by both decreased AChE
506 and CE activities in the animals. Al is a known neurotoxic contaminant (Kaizer et al., 2008), and
507 AChE activity is a good biomarker for this purpose in aquatic invertebrates (e.g. Forget et al.,
508 2003). CEs, which have already been characterized for *M. jelskii* (Lima et al., 2013), are also

509 good indicators of water quality, namely pesticides (e.g. Solé et al., 2018), but there are also
510 works reporting their decrease as a result of exposure to trace metals in other aquatic species
511 (de Lima et al., 2013). In the context of this study, the decrease of both esterases (AChE and
512 CEs activities) were a clear indicator of the synergic impact that pH and Al_2O_3 exposure had on
513 *M. jelskii.* Given that animals under these conditions did not show higher Al accumulation than
514 animals exposed to Al_2O_3 alone, we hypothesize that this is a genuine biochemical synergism
515 and not just the effect of increased Al availability under acidic conditions.

516 In a bioenergetics framework, exposure to low pH or Al_2O_3 alone would constitute a moderate
517 stress situation for *M. jelskii.* Under such conditions ATP would be still supplied by aerobic
518 metabolism alone and this energy would be devoted to fulfilling the increasing needs of
519 maintenance processes (e.g. extra-cellular protection and repair) in detriment of others such
520 as growth or reproduction ("pejus range") (see Fig. 1 in Sokolova et al., 2012). But the
521 combination of low pH and increased Al_2O_3 would be an extreme stress situation ("pessimum
522 range") where animals will need to rely partly on anaerobic metabolism (nil aerobic scope) and
523 all available energy would be directed towards maintenance alone (Sokolova et al., 2012). Such
524 a metabolic depression is far from uncommon in nature, and countless examples can be found
525 in the literature. For example, for cyprinid fish exposed to trace metals, metabolic depression
526 has been determined to occur when metal concentrations in the environment overpass 40% of
527 the maximum sub-lethal level (Peles et al., 2012). The fact that PC concentrations were
528 significantly lower than when animals were exposed to Al_2O_3 or pH alone, further support the
529 hypothesis that *M. jelskii* enters a state of reduced metabolism, and while no mortality rates
530 were registered, we may hypothesize that this provides a way for animals to prolong survival
531 until the return of more tolerable environmental conditions.

532 To the authors' knowledge there are no studies addressing the specific pathways in which
533 Al_2O_3 harms aquatic species under low pH. For general Al toxicity, this equally remains

relatively unknown, but it has been attributed to Al decreasing the tolerance of benthic invertebrates to water acidification through the impairment of osmoregulation processes (reviewed by Herrmann, 1987). Indeed, negative effects caused by inorganic Al exposure under acidified conditions have been observed for freshwater fish and invertebrates (e.g. Herrmann, 1987; Leino and McCormick, 1993). In the freshwater fish *Micropterus salmoides,* Leino and McCormick (1993) observed that under 30 µg L^{-1} monomeric Al and a pH of 4.5, key osmoregulatory organs such as gills were obliterated by hyperplasia of the interlamellar epithelium and showed over a 2-fold decrease in the amount of chloride (mitochondria-rich) cells. Leivestad et al. (1987), on the other hand, demonstrated in salmon that Al exposure can reduce the activity of Na^+-K-ATPase, key transporter in ensuring osmoregulation. Altogether, results suggest that the Al-induced respiratory disability may additionally be leading to osmoregulatory impairment when exposure occurs under acidified conditions.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In the Anthropocene era, it has become imperative to identify appropriate indicators to predict biodiversity changes. While taxonomy- or trait-based approaches can provide means of "drawing the rough edges", ecophysiological metrics often provide early warning signs because they are detectable at lower stress threshold levels (Branquinho et al., 2019). By applying such tools, altogether, our results support the hypothesis that Al_2O_3 (through direct exposure or even ingestion of undissolved particles) impairs respiration and oxidative status of *M. jelskii*. However, under acidified conditions, Al_2O_3 induces neurotoxicity and metabolic depression in this shrimp species. Further works should aim to confirm if this is due to the disruption of the acid-base homeostasis through either mechanistic (e.g. inhibition of certain transporters, alterations of the gill function) or energetic pathways (failure to deliver enough oxygen to deal with the energetic requirements for osmoregulation).

559 Regarding the combined effect of pH and Al_2O_3 exposure (which are the real conditions found
560 around the launch pads), the biomarkers that resulted most informative to reach our
561 conclusions were the neurotoxicity parameters (AChE and CEs), GR activities and RRs. From a
562 practical point of view these are important conclusions, given that the identification of
563 biomarkers to assess environmental quality is a major issue in the field of ecotoxicology. By
564 using a shrimp species belonging to a world-distributed family, our results set the bases for
565 providing the metrics and reference values to be used with an active biomonitoring approach.
566 This would ultimately allow understanding the complexity of the biochemical and physiological
567 responses to such contaminants. Even though the results of this study must be validated in the
568 field, the inclusion of these biomarkers in future monitoring programs of space port launching
569 activities based on propergol fuel should be considered.

570 Future works should focus on how and where Al_2O_3 bioaccumulation is occurring in *M. jelskii*
571 under normal and acidified conditions, and if as for other species, gills are impacted.
572 Additionally, the consequences that the combined ecotoxicological effects with other
573 pollutants (metals, plastics, fuels, etc.) or even with eutrophication and other climate-change
574 related stressors may have on macrofauna and the trophic chain remain completely unknown.
575 Given that detoxification capacity was reduced under Al_2O_3 exposure at low pH (low CE
576 activities), this subject should be urgently addressed in both aquatic and terrestrial ecosystems
577 to better predict the impact of launchers in the context of increasing activities of space ports
578 worldwide.

579

580 **6. ACKNOLEDGEMENTS**

581 The authors would like to thank Carla Leite (University of Aveiro, Portugal) and Stéphanie
582 Raffestin (Institute Pasteur, French Guiana), for carrying out the quantification of Al in shrimp
583 tissues and water samples, respectively. Special thanks go to Eduarda Pereira (University of

Aveiro) for her advice on the chemical behavior of Al_2O_3. Thanks also go to Delphine Bouvier and Damien Monchaux (Hydreco Guyane) for their support throughout the experimental setup, to Greg Quartarollo (Hydreco Guyane) for doing the taxonomic identification of the experimental animals, and to Manon Guilaut, Pauline Le Page and Candice Thomas for the help in the animal collection. We are also grateful to the two anonymous referees for their constructive comments which helped improving the original version of this manuscript.

7. AUTHORS CONTRIBUTIONS

G.A. Rivera-Ingraham, R. Vigouroux and J.-H. Lignot conceptualized the experiment. G.A. Rivera-Ingraham conducted the *in-vivo* analyses while M. Andrade, R. Freitas and M. Solé conducted the biochemical analyses. All authors contributed to data interpretation, manuscript writing, review and editing.

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Are we neglecting Earth while conquering space? Effects of aluminized solid rocket fuel combustion on the physiology of a tropical freshwater invertebrate

Georgina A. Rivera-Ingraham[1,4], Madalena Andrade[2], Regis Vigouroux[1], Montserrat Solé[3], Katherina Brokordt[4], Jehan-Hervé Lignot[5], Rosa Freitas[2]

HIGHLIGHTS

- Propergol fuel releases Al_2O_3 and hydrochloric acid (HCl) upon combustion.
- No physiological assessments on their impact have been carried out so far.
- Simultaneous exposure to these two compounds produces toxicity in tropical shrimps.
- The impact is mediated by respiration impairment and loss of acid-base regulation.
- Esterases and glutathione reductase activities are good indicators for this impact.

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Are we neglecting Earth while conquering space? Biomarkers for the effects of aluminized solid rocket fuel combustion on the physiology of a tropical freshwater invertebrate

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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