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# Effects of chronic exposure of metals released from the dissolution of an aluminium galvanic anode on the Pacific oyster *Crassostrea gigas*

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1                                   **Effects of chronic exposure of metals released from the dissolution of an**  
2                                   **aluminium galvanic anode on the Pacific oyster *Crassostrea gigas***

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9    **Abstract**

10    Among the anthropogenic sources releasing metallic species into the marine environment, the  
11    galvanic anode cathodic protection system (GACP) is widely used to protect submerged metallic  
12    structures from corrosion. Galvanic anodes are an alloy of metals of which the main component is  
13    aluminum or zinc. Very few studies were performed to study their potential biological effects. We  
14    investigated the chronic toxicity of an aluminum-based galvanic anode on the Pacific oyster,  
15    *Crassostrea gigas*. Oysters were exposed for three months to three concentrations of aluminum (50,  
16    100 and 300  $\mu\text{g L}^{-1}$ ) obtained with an electrochemical experimental device simulating the dissolution  
17    of a galvanic anode. At different exposure times, we studied a battery of biomarkers of the immune  
18    system, reproductive parameters and the metabolic state of the oysters. Results demonstrated a  
19    sensitivity of oysters at the highest concentration and some biological effects were observed  
20    especially for the malondialdehyde content in the digestive gland after 84 days of exposure. In  
21    addition to these biomarkers, the bioaccumulation of the different metals composing the anode was  
22    measured in oysters' tissues. Bivalves bioaccumulated more zinc than aluminum, even if aluminium  
23    was present in greater concentrations during exposures. Moreover, exposure time did not influence  
24    the bioaccumulation of aluminum in contrast to zinc.

25    **Keywords:** galvanic anode, aluminium, oyster, *Crassostrea gigas*, biological effects, bioaccumulation

26                                   **1) Introduction**

27    Coastal ecosystems are areas subject to significant pressure due to their anthropization, and they  
28    have become particularly vulnerable. Indeed, they are impacted by maritime transport, fishing,  
29    shellfish farming, sediment dredging, noise pollution, chemical pollution and many others. Among  
30    the anthropo-constructions affecting biodiversity, functioning and resilience of aquatic  
31    environments, merchant ships, port structures, wind farms or oil platforms are of particular interest.

32 It is common practice to connect galvanic anodes on these steel structures as a corrosion protection  
33 system. This type of cathodic protection (GACP for Cathodic Protection by Galvanic Anode) consists  
34 in controlling the corrosion of a metallic surface by means of an anode made of a metallic alloy of  
35 which electrochemical potential is lower than that of the metal to be protected (Guibert, 2009). The  
36 galvanic anode undergoes oxidation in the marine environment and releases various metals in the  
37 forms of ions or oxy-hydroxides into the environment. The principal metals composing the anodes  
38 are aluminium (Al), zinc (Zn) or magnesium because of their negative corrosion potential. Historically,  
39 zinc-based alloys were used first, but the use of aluminum-based galvanic anodes for the cathodic  
40 protection of metallic structures has been practiced since the early 1960s (Roy, 1968). The main  
41 advantages of Al are its availability, low cost, low density and uniform current distribution, which  
42 explains why it is more used in offshore structures (Guibert, 2009). As a metal alloy, galvanic anodes  
43 are known to significantly release toxicological elements (Reese et al., 2020). Depending on their  
44 physical and chemical behaviours in the solution phase, these elements are present in soluble  
45 fraction of the water or associated with suspended particulate matters, resulting in the deposition of  
46 these elements in sediments at proximity of the equipped installations when hydrodynamic  
47 conditions are low (Caplat et al., 2020). Along the marine coasts, more and more ports are equipping  
48 themselves with galvanic anodes to protect their metallic structures such as sheet piles or  
49 dolphin (pilings). Private individuals are also equipping the hulls of their pleasure boats for better  
50 protection over time. In addition, in the frame of the energy transition, the increasing installation of  
51 offshore wind farms also involves the installation of galvanic anodes. In an offshore wind farm of  
52 about 75 wind turbines, the galvanic anodes composed by Al alloy can represent a mass of about 15  
53 tons per foundation, which corresponds to an estimated transfer of metals into water of  $105 \text{ kg day}^{-1}$   
54 or approximately annually 45 tons of Al and 2 tons of Zn, contributing to the enrichment of aquatic  
55 environments with metals (Kirchgeorg et al., 2018).

56 It is therefore essential to study the impact that such a metal transfer can have on marine  
57 ecosystems. Dissolved aluminium concentrations in open sea are typically below  $0.7 \mu\text{g L}^{-1}$  (Tria et al.,  
58 2007). In coastal waters, Al concentrations vary from 0.1 to  $39.4 \mu\text{g L}^{-1}$  and can reach  $83 \mu\text{g L}^{-1}$  in  
59 heavily polluted ports (Angel et al., 2016; Golding et al., 2015). In the open ocean, zinc is present at  
60 concentrations of the order of  $0.01 \mu\text{g L}^{-1}$  and concentrations are about  $10 \mu\text{g L}^{-1}$  in coastal waters  
61 such as the English Channel with measurements about 3 à  $12 \mu\text{g L}^{-1}$  (Sheahan et al., 2007). However,  
62 levels reaching  $1800 \mu\text{g L}^{-1}$  have already been recorded in some areas (Barcellos, 1995). Overall, the  
63 contribution of anodes to metal concentrations in marine waters appears to be low, except in  
64 confined areas with low hydrodynamics and shallow depths where aggregation and precipitation  
65 processes are observed in surface sediments (Caplat et al., 2010; Deborde et al., 2015). It has been

66 recently shown that the dissolution of an aluminum-based galvanic anode induces a higher  
67 enrichment in Zn than Al in the sediment (Caplat et al., 2020). Few studies have focused on the  
68 metals released from galvanic anodes and their potential effects on marine organisms. Recently, Bell  
69 et al (2020) observed slight effects on algal growth. Caplat et al. (2010) showed that Al and Zn from  
70 the dissolution of the galvanic anode were less toxic than sulphate salts on the sea urchin,  
71 *Paracentrotus lividus*. Further investigations are required to assess the impact of galvanic anodes on  
72 the marine environment.

73 Therefore, the main objective of our study was to investigate the chronic toxicity of aluminum-based  
74 galvanic anode on the Pacific oyster, *Crassostrea gigas* (Thunberg, 1793), also known as *Magallana*  
75 *gigas*. Due to their feeding habits, bivalves concentrate environmental contaminants in their tissues  
76 by filtering large volumes of water and are therefore bioaccumulative species. This particularity and  
77 its economic importance make the pacific oyster a very useful model for the evaluation and  
78 monitoring of environments. In this work, oysters were exposed for 84 days to three concentrations  
79 of aluminium using an experimental device simulating the dissolution of a galvanic anode. At  
80 different exposure times, we analysed a battery of biomarkers of the immune system, reproductive  
81 parameters and the metabolic state of the oysters. The bioaccumulation of the different metals  
82 composing the anode was also measured in oysters' tissues.

## 83 **2) Materials and Methods**

### 84 **2.1) Organisms**

85 Pacific oysters of 18 months were collected by the experimental center « Synergie mer et littoral »  
86 (SMEL) from the shellfish farm "Thalassa" of Blainville-sur-mer (West coast of Normandy, France);  
87 oysters were from natural catchment. At the beginning of the experiment, homogeneous (ANOVA,  $p$   
88  $> 0.05$ ) in size and weight (averaged  $24.58 \pm 0.39$  g in weight and  $65.83 \pm 0.45$  mm in length) oysters'  
89 batches were constituted. The experiment was realized at Blainville-sur-mer in SMEL center, which  
90 dedicated an air-conditioned room to this objective. Oysters were acclimated for 1 month and the  
91 room temperature was 16°C.

### 92 **2.2) Experimental structure and design**

93 An electrochemical experimental device simulated the dissolution of an aluminum-based galvanic  
94 anode (GACP) in controlled conditions to obtain a transfer of metals with the same chemical  
95 speciation as in the marine environment (Figure 1). The device was equipped with reference  
96 electrode (saturated calomel electrode - SCE), a counter electrode (CE) made of a platinize-titanium  
97 alloy and an aluminum anode (Al Bera15, Bac Corrosion® Control). The assembly was electrically

98 connected to a galvanostat as already realized in other studies (Caplat et al., 2012, 2010; Mao et al.,  
99 2011; Mottin et al., 2012). Aluminum-based galvanic anode is mainly composed of seven elements  
100 and the mass percentages of each metal were given by the supplier as following: 93.2% to 97.1% of  
101 Al, 2.5% to 6.5% of Zn, 0.13% max of iron, 0.1% max of silicon, 0.01% to 0.03% of indium, 0.01% max  
102 of manganese and 0.006% max of copper. This experimental device provided 130 L of contaminated  
103 water with a high concentration of metals ( $[Al] = 11\ 131 \pm 2\ 764\ \mu\text{g L}^{-1}$ ,  $[Zn] = 500 \pm 123\ \mu\text{g L}^{-1}$ ) every  
104 24 hours. Concentrations in water of the other anode constituents were all lower than the  
105 quantification limit.

106 The exposure was performed during 84 days (from June 2 to August 28, 2020). Regarding the oysters,  
107 23 individuals were collected before the beginning of the experiment and 120 oysters per tank were  
108 randomly distributed in 4 experimental systems: 1 control and 3 aluminium nominal concentrations  
109 of 50, 100 and 300  $\mu\text{g L}^{-1}$  got after decantation process (for 24 h). Each experimental system was  
110 composed of 3 tanks (2 of 500 L and 1 of 160 L) (Figure 1). The first 500 L tank was used for daily  
111 mixing of contaminated water, phytoplankton and natural seawater filtered to 50  $\mu\text{m}$ , as follows:  
112 every day, a volume of contaminated water, produced by the experimental device with the galvanic  
113 anode, was added to the mixing 500 L tank according to the targeted Al concentration. A mixture of  
114 phytoplankton (*Isochrysis galbana* and *Chaetoceros calcitrans* in ratio 2:1) was daily added at a  
115 concentration of  $1.10^6$  cells per oyster. The volume of the mixing tank was completed to 500 L with  
116 natural seawater. Then, the mixture of the first tank fell into the container tank located below the  
117 first one, which served as a reserve and for the decantation of metals. A pump ( $700\ \text{L h}^{-1}$ ) installed in  
118 this container tank sent the contaminated water into the exposure tank, in which the oysters were  
119 placed on a sieve. Every two days the exposure tank was cleaned to remove all oyster faeces in the  
120 aim to limit the water quality degradation. Salinity ( $34.44 \pm 1.04\ \text{PSU}$ ), temperature ( $16.27 \pm 0.27\ ^\circ\text{C}$ ),  
121 carbonates ( $3.18 \pm 0.74\ \text{meq L}^{-1}$ ) and pH ( $8.06 \pm 0.08$ ) were continuously recorded with a  
122 multiparameter probe (NKE<sup>®</sup>, model STYPS+30-SI) and revealed stable under the 4 conditions.  
123 Oysters were sampled at five dates: 1 day and then 7, 29, 47 and 84 days. At each sampling date, 23  
124 individuals per condition were randomly picked, 13 for biomarkers analyses and 10 for  
125 bioaccumulation assessments.

### 126 **2.3) Biometric and histological biomarkers**

127 In each experimental condition, the oysters were daily checked to discard dead oysters and calculate  
128 mortality rates. On each sampling date, all oysters collected were measured in length, width and  
129 thickness (to the nearest 0.01 mm), and weighted (to the nearest 0.01 g) to determine potential  
130 growth. The weight of the drained visceral mass and the empty shell are also recorded. These same

131 oysters are used to determine two conditions index: the “Association Française de Normalisation”  
132 (AFNOR) condition index (CI) and the Walne an Mann index (Walne and Mann, 1975) respectively  
133 calculated as follows:

$$134 \text{ CI}_{\text{AFNOR}} = (\text{flesh weight}/\text{total weight}) \times 100$$

$$135 \text{ CI}_{\text{Walne and Mann}} = (\text{dry flesh weight} / \text{dry shell weight}) \times 100$$

136 For histology analysis, oyster cross-sections (5 mm thick) were cut behind the labial palps and fixed in  
137 Davidson’s solution (1 vol. glycerol, 2 vol. 37% formaldehyde, 3 vol. 95% ethanol and 3 vol. filtered  
138 seawater). Tissue samples were then routinely processed, and the 3  $\mu\text{m}$  paraffin-embedded sections  
139 were stained according to the trichromatic protocol of Prenant Gabe (Gabe, 1968). For each  
140 individual, the sex and stage of gametogenesis were determined. A Mean gonad Index (MGI)  
141 reflecting the global cyclic changes in the reproductive tissues of oysters was calculated (Gosling,  
142 2003) and a coefficient was assigned to each stage as follow: stage 0: sexual rest = 0; stage I:  
143 beginning of gametogenesis = 1; stage II: active gametogenesis = 2; stage IIIa: beginning of sexual  
144 maturity = 3; stage IIIb: beginning of spawning = 3.5; stage IIIc: advanced spawning = 1.5.

$$145 \text{ MGI} = (\text{number of individuals at stage } n \times \text{coefficient of the stage } n) + \dots + (\text{number of individuals at} \\ 146 \text{ stage } n_{+1} \times \text{coefficient of the stage } n_{+1}) / (\text{total number of individuals}).$$

#### 147 **2.4) Measures of metallic concentrations and bioaccumulation**

148 Seawater samples were collected every week to determine total concentrations of Al and Zn by  
149 atomic absorption spectrometry (AAS). The aluminum was analyzed by a Zeeman Atomic Absorption  
150 Spectrometer Z-AAS (AA240Z – Varian) equipped with a graphite tube atomizer (FTA 120, Varian) and  
151 a programmable sample dispenser (PSD 120, Varian). Analyses were performed with a matrix  
152 modifier solution (2 g L<sup>-1</sup> of Mg (NO<sub>3</sub>)<sub>2</sub>). Each digested sample was analysed five times, and the mean  
153 of values was used for evaluation. Zn was analysed with air–acetylene flame atomic absorption  
154 spectrometry (F-AAS) using a Varian AA240F apparatus equipped with a deuterium lamp to correct  
155 non-specific absorption. Analyses were conducted on each sample in triplicate, and mean values  
156 were used for evaluation. The limit of detection for determination of each metallic concentration  
157 was defined as mean of blank analyses plus three times standard deviation (SD) of the blank  
158 analyses. A SD of 3.1  $\mu\text{g L}^{-1}$  (n = 30) was given for Zn and 6.6  $\mu\text{g L}^{-1}$  (n = 45) for Al.

159 Ten oysters for each condition and sampling date were dissected to measure metals in tissues. The  
160 visceral mass was weighed and then stored at -20°C. The samples were freeze-drying then crushed  
161 with Teflon balls and mineralized with prior microwave acid sample digestion (Multiwave ECO, Anton  
162 Paar, Les Ulis, France) (800  $\mu\text{l}$  of concentrated and purified HNO<sub>3</sub> (70%), 200  $\mu\text{l}$  of purified H<sub>2</sub>O<sub>2</sub> and 1

163 ml of ultrapure water for 40 mg DW). All samples were spiked with three internal-standard solutions  
164 of Gallium, Rhodium and Iridium for final concentrations of 5, 1 and 1 mg L<sup>-1</sup>, respectively, diluted to  
165 50 ml with Ultrapure water to obtain solutions containing 2.0 % (v/v) of nitric acid, and then filtered  
166 at 0.45 mm using a teflon filtration system (Digifiltre, SCP Science, Villebon-sur-Yvette, France). Total  
167 concentrations of several elements (Al, Ag, As, Ca, Cd, Cu, Fe, K, Mg, Mn, Ni, Pb and Zn) were  
168 measured by High Resolution Inductively Coupled Plasma -Mass Spectrometry (HR ICP-MS Thermo  
169 Scientific™ Element 2™, Bremen, Germany and Element XR™). To assess the quality of the biota  
170 processing procedure, a standard reference material (SRM NIST-1566b oyster tissue from the  
171 National Institute of Standards and Technology, Gaithersburg, MD, USA) was analysed with a series  
172 of samples. The relative errors were less than 10% for all metal concentrations recorded in both  
173 standard reference materials, excepted for Pb and Al concentrations which ranged between 10% and  
174 40%. Appropriate blanks were also analyzed in series with all the samples. The limit of quantification  
175 (LQ) for each element was calculated from 10 times the standard deviation of 25 measures of blanks.  
176 The limits of quantification for determination of the Al and Zn concentrations measured in seawaters  
177 (10 measures of blanks) were respectively of 6.4 µg L<sup>-1</sup> for and 7.7 µg L<sup>-1</sup>.

178 Bioconcentration factors (BCFs) were used to assess the capacity of oysters to accumulate Al and Zn  
179 from environment (Adams et al., 2000). BCF was calculated as follow:

180  $BCF = [\text{Total Al or Zn concentration in oysters' flesh (mg kg}^{-1}) / \text{Average of the total Al or Zn in}$   
181  $\text{seawater (mg L}^{-1})] * 0.12$  (dry weight/fresh weight in bivalves, (Papp, 2011)).

## 182 **2.5) Immune parameters of the hemolymph**

### 183 *Hemolymph removing and hemocyte counting*

184 The hemolymph was withdrawn from the posterior adductor muscle using a 25-gauge needle  
185 mounted on a 5mL plastic syringe. The hemolymph from each oyster was transferred into an  
186 individual tube on ice. For each sample, a fraction was examined under light microscopy to check the  
187 quality of the sample and determine the concentration of cells. A volume of hemolymph was  
188 calculated in order to have 100,000 cells per sample.

### 189 *Phagocytic activity and reactive oxygen species*

190 The phagocyte capacity of hemocytes was determined by adding fluorescent beads (Fluorospheres  
191 F8823, Carboxylate, 1 µm diameter yellow-green fluorescent, Invitrogen®) to the medium. These  
192 beads are coupled with fluorescein (FITC) which allows, after phagocytosis, their detection by flow  
193 cytometry. From a stock solution of 3.6.10<sup>10</sup> beads mL<sup>-1</sup>, these were added to the medium at a rate of  
194 100 beads per cell. A stock solution was prepared using 5.6 µl of fluorescent beads added to 194.4 µl

195 of Molluscan Physiological Saline (MPS) ( $4 \cdot 10^{-1} \text{M}$  NaCl;  $1 \cdot 10^{-1} \text{M}$   $\text{MgSO}_4$ ;  $2 \cdot 10^{-2} \text{M}$  HEPES;  $1 \cdot 10^{-2} \text{M}$   
196  $\text{CaCl}_2$ ;  $1 \cdot 10^{-2} \text{M}$  KCl). After 40 min incubation at  $17^\circ\text{C}$ , Hoechst 33342 (H3570, Trihydrochloride,  
197 Trihydrate, Invitrogen<sup>®</sup>) was added to  $2 \mu\text{g mL}^{-1}$ . This compound binds to the DNA of living cells and  
198 can be detected by fluorescence allowing the elimination of debris from the cytogram. After 20 min  
199 incubation time, cells were fixed with 3% formaldehyde. All samples were stored at  $4^\circ\text{C}$  until analysis  
200 by flow cytometry (Beckman<sup>®</sup> Coulter CytoFLEX, Flow Cytometry platform SF4206 ICORE). In total, 10  
201 000 events were counted for each sample, and the phagocytosis efficiency of cells was evaluated as  
202 the percentage of hemocytes that had engulfed at least three beads (Delaporte, 2003).

203 The determination of reactive oxygen species (ROS) was also performed by flow cytometry on the  
204 same individual hemolymph samples. For this, 2,7-dichlorofluorescein diacetate (DCFH-DA, Sigma<sup>®</sup>,  
205 final concentration =  $10 \mu\text{M}$ ) which diffuses into the cells during the 1-hour incubation time was  
206 used. In the cytoplasm, the acetate (-DA) groups are eliminated by esterase and the DCFH is thus  
207 trapped in the cells. Intracellular DCFH, which is a non-fluorescent fluorescein analogue, is oxidized  
208 to dichlorofluorescein (DCF) by hemocytes to produce ROS. The oxidation of intracellular DCFH is  
209 quantitatively related to the oxidative metabolism of hemocytes and is mediated by hydrogen  
210 peroxide ( $\text{H}_2\text{O}_2$ ). After 1 hour of incubation, the samples were fixed with 3% formaldehyde and  
211 stored at  $4^\circ\text{C}$  until flow cytometry analysis. The production of DCFH results in green fluorescence,  
212 measurable on the FL1 detector of the flow cytometer. The results of ROS production were  
213 expressed as the percentage of cells showing fluorescence.

#### 214 *Lysosomal membrane stability*

215 The lysosomal system was evaluated with the neutral red retention assay (NRRA) according to the  
216 protocol used by Minguez et al. (2014). The hemolymph was deposited in the wells of a 24-well plate.  
217 After 30 min. of incubation at  $17^\circ\text{C}$  to allow the hemocytes to adhere to the well bottom,  $200 \mu\text{L}$  of  
218 NR solution were added in each well. The solution was prepared with  $10 \mu\text{g}$  of NR (N7005, Sigma  
219 Aldrich<sup>®</sup>) diluted in  $347 \mu\text{L}$  of DMSO and filtered with a  $0.22 \mu\text{m}$  syringe. Then  $10 \mu\text{L}$  of filtrate were  
220 diluted with 5 mL of MPS. NR is absorbed into the cells by membrane diffusion where it is trapped in  
221 the lysosomal compartment (Lowe and Pipe, 1994). An alteration in its absorption reflects damage to  
222 the plasma membrane. Dead cells or cells with damaged membranes cannot accumulate NR and as a  
223 result, wells containing dead or damaged cells will be less stained. After 1 hour of incubation at  $17^\circ\text{C}$ ,  
224 wells were rinsed twice with  $300 \mu\text{L}$  of MPS to remove the excess NR not incorporated in the  
225 lysosomes. Two hundred microliters of lysis buffer (1:50:49 v/v/v acetic acid, absolute ethanol, Milli-  
226 Q water) were added to each well and the plate was darkened for 30 min. at room temperature to  
227 lysis the cells and release the neutral red from the lysosomes. Absorbance measurement was



228 performed with the microplate reader at 540 nm (Flexstation 3, Molecular Device®, Sunnyvale, CA,  
229 USA, Proteogen platform SF4206 ICORE).

## 230 **2.6) Malondialdehyde content and protein content**

231 Malondialdehyde (MDA) accumulation in the gills and digestive gland was used as a lipid  
232 peroxidation marker. Samples were homogenized with a Potter-Elvehjem homogenizer for 1 min at  
233 1400 rpm in PBS (NaCl 500 mM, Na<sub>2</sub>HPO<sub>4</sub> 10 mM, KH<sub>2</sub>PO<sub>4</sub> 3.1 mM, pH 7.4) containing butylated  
234 hydroxytoluene (antioxidant agent). Samples were then centrifuged (10,000 x g, 10 min, 4°C) and the  
235 supernatants were aliquoted and stored at -80°C until analysed. The same sample was used for  
236 determining both proteins levels and the MDA content. The MDA assay was performed with a test kit  
237 (MDA-586 Oxis-Research®) following manufacturer's instructions. The plate was read with the  
238 microplate reader at 586 nm. Results were expressed in nmol MDA mg protein<sup>-1</sup>.

## 239 **2.7) Energy reserves**

240 Glycogen reserves were used as a marker of energy reserves in oysters. After removing the mantle  
241 edge and gills, a sample of the remaining visceral mass was taken, frozen in liquid nitrogen and  
242 stored at -80°C. After defrosting on ice, ultra-pure water was added to each tube in a proportion by  
243 volume: weight (100 µL for 0.01g). Tissues were homogenized with a Potter-Elvehjem for 1 min at  
244 1400 rpm. The samples were then boiled for 5 min to inactivate the enzymes and then centrifuged  
245 (3000 x g, 5 min, 4°C). The supernatant was recovered and centrifuged again (13 000 x g, 5 min, 4°C).  
246 Finally, the supernatants were aliquoted and stored at -80°C until assayed. The glycogen assay was  
247 performed with glycogen test kit (MAK016-1KT Sigma-Aldrich®) following manufacturer's  
248 instructions. The plate was read with the microplate reader at 570 nm. Results were expressed in mg  
249 glycogen. g flesh<sup>-1</sup>.

250 In addition, hemolymph was used to measure the Brix index which is an indicator of the nutritional  
251 status, strongly correlated to the concentrations of proteins, triglycerides, cholesterol, calcium and  
252 phosphorus in the hemolymph (Berry et al., 2019). Hemolymph of 10 oysters per condition and  
253 sampling date was individually analyzed. To this end, 100 µL of hemolymph were centrifuged (3000 x  
254 g, 5 min, 4°C) and supernatant was deposited on the optical reader of a digital refractometer with  
255 automatic temperature compensation (Hanna Instruments, HI96801). Results were expressed in  
256 °Brix. Ultrapure water was used as a blank between each sample.

## 257 **2.8) Statistical analyses**

258 Statistical analyses were performed using R software version 3.4.4. The normality of the data was  
259 tested with the Shapiro-Wilk test and homoscedasticity with F-test. If the data followed a normal

260 distribution and homoscedasticity was respected, analyses of variance (ANOVAs) followed by Tukey  
261 post-hoc tests were used to determine homogeneous groups. When the data did not follow a normal  
262 distribution, non-parametric Kruskal-Wallis (K-W) tests followed by Dunn's tests were performed.  
263 Differences were considered significant at  $p$ -value  $< 0.05$ . In addition to these analyses, a correlation  
264 matrix (Pearson test) and a principal component analysis were performed on the biomarkers and  
265 metals concentrations data. All results are presented as mean  $\pm$  SEM (Standard Error of the Mean).

### 266 **3) Results**

267 For C1, C2 and C3 conditions, effective concentrations of metals measured in exposure tanks during  
268 the experiment were about respectively 65, 125 and 296  $\mu\text{g. L}^{-1}$  for Al and 15, 22 and 35  $\mu\text{g. L}^{-1}$  for Zn  
269 (Table I).

#### 270 **3.1) Oyster mortality and biometry**

271 For the 3 months of exposure, 1 % on the 120 oysters of the control died and there was no difference  
272 between the control and the 3 concentrations (*data not shown*). Regardless of the conditions or  
273 time, oysters did not show variations in the different biometric parameters measured during the  
274 experiment (ANOVA,  $p > 0.05$ ). In the same way there was no variation for condition index which  
275 were on average of 17.61 for the AFNOR condition index and 5.79 for the Walne and Mann index.

#### 276 **3.2) Bioaccumulation**

277 At the beginning of the experiment, the aluminum and zinc levels in the oysters are respectively 19.7  
278  $\pm 5.7$  and 672.5  $\pm 96.4$   $\text{mg kg}^{-1}$ . Figure 2 shows the bioaccumulation kinetics of Al and Zn during the  
279 84 days of exposure for the 4 experimental conditions. After 24 hours of exposure, significant  
280 differences in aluminum levels in oysters were observed. For condition C3, it reached 109.0  $\pm 25.2$   
281  $\text{mg kg}^{-1}$  and subsequently it is necessary to wait 47 days to recover a similar content. Indeed, after 1  
282 week of exposure, a decrease of the aluminum concentration in oysters was observed in all the  
283 experimental conditions. Table II lists elements concentrations measured in the total tissues of  
284 oysters ( $n = 10$ ) collected for each condition after 84 days. Results for all exposure times are included  
285 in supplementary 1. For Al, the contents in oysters were respectively 14.6  $\pm 6.8$ , 26.8  $\pm 3.5$ , 27.6  $\pm 3.3$   
286 and 103.7  $\pm 10.3$   $\text{mg kg}^{-1}$  for the control, C1, C2 and C3. The bioaccumulation kinetics of Zn was  
287 different from that of Al. After 29 days, significant differences in the flesh concentrations were  
288 observed between the C3 condition and the control, with Zn content of respectively 1532  $\pm 131.9$   $\text{mg}$   
289  $\text{kg}^{-1}$  and 908  $\pm 46.3$   $\text{mg kg}^{-1}$ . The concentration differences between all the conditions gradually  
290 increased after 84 days. Then, Zn levels in oysters were respectively 1 052  $\pm 108$ , 1 328  $\pm 132$ , 1 952  $\pm$   
291 82 and 2 754  $\pm 419$   $\text{mg kg}^{-1}$  for the control, C1, C2 and C3. For both metals, the concentration in

292 oyster tissue was significantly higher for condition C3. The other toxicologically important elements  
293 analyzed such as copper, lead or cadmium did not show significant variations in oysters and a similar  
294 result was obtained for potassium, magnesium and calcium (ANOVAs,  $p > 0.05$ ). Figure 3 presents  
295 kinetics of bioconcentration factors of Al and Zn during the 3 months of exposure for the 4  
296 experimental conditions. For the two metals, highest BCF values were linked to lowest and naturally  
297 exposure concentrations. For the three concentrations of exposures (C1, C2 and C3), Al presents  
298 close and lowest BCF values, whereas Zn BCF values increased in relation to the decrease of exposure  
299 concentrations.

### 300 **3.3) Gametogenesis stages**

301 Histological analysis (n=13 per condition) did not reveal any significant differences in the progress of  
302 gametogenesis between the 4 exposure conditions (Kruskal-Wallis,  $p > 0.05$ ). After 84 days, MGI  
303 were, respectively, for the control, C1, C2 and C3 of  $3.35 \pm 0.15$ ,  $3.04 \pm 0.24$ ,  $3.27 \pm 0.22$  and  $3.35 \pm$   
304  $0.16$  (*data not shown*).

### 305 **3.4) Immune parameters**

306 Figure 4 shows four immune parameters of oyster hemocytes after 84 days. For all parameters (i.e.  
307 hemocyte count, phagocytic activity, ROS production and lysosomal system), we did not record any  
308 significant differences between individuals exposed to the four conditions (Kruskal-Wallis,  $p > 0.05$ ).  
309 However, some decreasing trends could be observed, particularly for phagocytic efficiency with a  
310 40% decrease for the C3 condition compared to the control. By contrast, there was an increase of  
311 50% in the lysosomal system for condition C2 and 25% for condition C3. The individual variability  
312 induced large deviations which probably explains the absence of significance.

### 313 **3.5) Proteins and MDA levels**

314 Protein concentrations in the digestive gland ranged from 34.55 to 54.73 mg mL<sup>-1</sup> and in the gills  
315 from 15.34 to 27.24 mg mL<sup>-1</sup>. There were no significant differences between the different exposure  
316 conditions throughout the exposure (*data not shown*). After 84 days, MDA levels in the digestive  
317 gland of oysters showed variations with a significant decrease only for oysters exposed to C3 (figure  
318 5). The values averaged  $3.61 \pm 0.79$ ,  $1.92 \pm 0.24$ ,  $1.62 \pm 0.18$ ,  $1.15 \pm 0.38$  nmol MDA mg. protein<sup>-1</sup> for,  
319 respectively, the control, C1, C2 and C3. In contrast, MDA levels in the gills revealed stable even after  
320 84 days; values ranged from 2.38 to 3.13 nmol MDA mg. protein<sup>-1</sup>.

### 321 **3.6) Glycogen and Brix index**

322 The brix index measured in the bivalve hemolymph did not show any variation either as a function of  
323 time or exposure conditions (Kruskal-Wallis,  $P > 0.05$ ). Values ranged from 4.20 to 5.05 °brix (*data*  
324 *not shown*). Figure 6 shows the kinetics of oyster glycogen content during the 3 months of exposure.  
325 At the beginning of the experiment, the average glycogen content was of  $117.1 \pm 5.75$  mg. g<sup>-1</sup> of  
326 flesh. Whether after 29 days, 47 days or 84 days of exposure we did not measure any significant  
327 differences between the oysters exposed to the 4 conditions. Overall, the glycogen content  
328 decreased during the experiment to reach values around the order of 38.4 to 64 mg. g<sup>-1</sup> of flesh. At  
329 84 days, a trend could be noted because glycogen content was slightly lower for the 2 highest  
330 concentrations.

### 331 **3.7) Principal components analysis and correlation matrix**

332 A correlation matrix (supplementary data 2) performed with the Pearson test between  
333 bioaccumulated aluminum and zinc in relation to the biomarkers after 84 days reveals that Al in  
334 oysters is negatively correlated with MDA levels in the digestive gland. Such a result can also be  
335 illustrated when looking at the principal component analysis (Figure 7) where Al and Zn  
336 concentrations present inverse relationship to both MDA and glycogen levels along F1 (Figure 7A). All  
337 immune parameters go in the same direction along F2. Furthermore, several groups emerged  
338 corresponding to the 4 conditions on the F1F2 plane (explained variance: F1: 23.08% and F2: 19.39%)  
339 (Figure 7B).

## 340 **4) Discussion**

341 Over the last several decades, the evaluation of biological effects of anthropogenic contaminants has  
342 become a crucial importance to maintain a good ecological status of coastal environment as  
343 recommended by European directives such as the Marine Strategy Framework Directive (MSFD)  
344 (2008/56/EC). To our knowledge, the present study is one of the first to focus on the chronic impact  
345 of aluminum galvanic anodes on a marine organism. We conducted an experimental integrative  
346 approach, designed to complement the previously published results on the effects of galvanic anodes  
347 (Caplat et al., 2020, 2012, 2010; Deborde et al., 2015; Mao et al., 2011; Mottin et al., 2012).

348 The concentrations of contaminants tested during the experiment did not induce significant  
349 mortalities of oysters and sublethal effects had thus been to be researched. The results of  
350 bioaccumulation analyses confirmed that oysters have bioaccumulated the various constituents of  
351 the galvanic anode. At the beginning of the experiment, metals concentrations in oysters were close  
352 to the natural levels given for coastal seawaters in Normandy (RIN program, *personal data*). BCF  
353 measures the ability of an organism to bioconcentrate an element in its tissue considering the

354 concentration of that element in the water. The BCF values measured for Al and Zn in oysters  
355 depended on the concentration of these two elements in the chronic exposure medium. At the  
356 concentration measured under C3 condition, reverse relationships were observed between metallic  
357 BCF values and total concentrations in water. In their review about metals, McGeer et al. (2003) have  
358 already reported similar relationships, that they linked to the ability of organisms to regulate uptake  
359 of chronically Zn elevated concentrations. In any case, BCFs for Al are low (around 50) indicating that  
360 *C. gigas* is not a hyper accumulator of aluminium in contrast to Zn for which BCFs are particularly  
361 high (around 10 000). Furthermore, it is observed that the BCFs of Zn decrease when the  
362 concentration in water increases. Indeed, the bioaccumulation of metals results from different  
363 mechanisms compared to organic substances. Metals in the environments are mostly in hydrophilic  
364 and hydrated forms that are not able to cross biological membranes by simple diffusion. Their uptake  
365 thus requires facilitated transport involving protein transporters or transmembrane channels which  
366 can reach saturation at high metal concentrations (Pelletier and Campbell, 2008).

367 The filter feeding of bivalves contributes to the high bioavailability of aquatic contaminants. For an Al  
368 concentration of 296  $\mu\text{g L}^{-1}$  (C3 condition) after 24 hours of exposure, the Al content in oysters  
369 reaches 109  $\text{mg kg}^{-1}$ . The detoxification process of Al starts after 7 days of exposure when the  
370 concentration drops to 71  $\text{mg kg}^{-1}$ , and it is necessary to wait 47 days to measure again a  
371 concentration above 100  $\text{mg kg}^{-1}$ . Concerning C2 condition, such an increase in Al content is not  
372 observed after 47 days of exposure, the oyster physiological processes being sufficiently efficient to  
373 detoxify at this Al concentration. One of the few marine studies on Al released from a galvanic anode  
374 concerns the bioaccumulation of Al in the mussel *Mytilus edulis* (Mao et al., 2011). Mussels were  
375 exposed to 530  $\mu\text{g L}^{-1}$  Al for 8 weeks which is higher than the C3 concentration in our study (i.e. 296  
376  $\mu\text{g L}^{-1}$ ). Mao et al. (2011) showed that the Al concentration reached its maximum after 13 days of  
377 exposure in the digestive gland (1 700  $\text{mg kg}^{-1}$ ) and after 8 days of exposure in total tissues (308  $\text{mg}$   
378  $\text{kg}^{-1}$ ). In our study we did not focus on any specific organ but aluminum levels in tissues are about the  
379 same order of importance between the two species (71  $\text{mg kg}^{-1}$  after 7 days for oyster *versus* 308  $\text{mg}$   
380  $\text{kg}^{-1}$  after 8 days for mussels).

381 The zinc profile is totally different because the enrichment in oysters' tissues was gradual over the  
382 three months of exposure and no equilibrium level has been reached at the end of the  
383 experimentation. At 0 day, the Zn concentration is 672  $\text{mg kg}^{-1}$  and it takes 29 days to observe  
384 significant differences between conditions. After 84 days, the Zn content in oysters reaches 2753  $\text{mg}$   
385  $\text{kg}^{-1}$  for C3 condition. This value is comparable to those found in the natural environment for polluted  
386 sites on the southern Spanish coast, such as Punta Umbria and Mazagon, where the Zn content in  
387 *Crassostrea angulata* oysters were, respectively, 2 240 and 3 016  $\text{mg kg}^{-1}$  (Funes et al., 2006). Finally,

388 in the present study, metallic results do not indicate significant enrichment of all other quantified  
389 elements (Cu, Cd, Pb...) in oysters chronically exposed to products of the dissolution of the  
390 aluminum-based galvanic anode suggesting that Al and Zn are alone to be of concern.

391 In natural environments, contaminants adsorb more onto suspended organic matter, but low  
392 amount of dissolved or suspended organic matter occur in controlled conditions, which increases the  
393 bioavailability of contaminants to organisms. Therefore, the experimental BCF can be higher than  
394 that measured *in situ*. For filter-feeding bivalves such as *C. gigas*, a metal can become available by  
395 two major ways: dissolved uptake, which is mainly through the gills and probably requires no energy  
396 investment; and the ingestion of particulates associated with food particles which becomes available  
397 to bivalves. It has been already shown that aluminium dissolved in water can be incorporated into  
398 the frustules of diatoms as a detoxification mechanism (Dixit et al., 2001; Gehlen et al., 2002).  
399 Aluminium can therefore be ingested by oysters through the trophic pathway. Our results show that  
400 the oyster is not a good bioaccumulator of Al, whereas Zn is more bioaccumulated in tissues. In the  
401 future, it would be interesting to distinguish the two uptake mechanisms by performing an  
402 experiment in which oysters would be placed in non-contaminated water but fed with microalgae  
403 previously cultivated in water contaminated with aluminium.

404 Although Al has no biological function, it can substitute for essential metals in some biochemical  
405 processes (Exley and House, 2011). By contrast, zinc is an essential trace element for the functioning  
406 of many cellular processes, but in excess it is toxic and generates ROS that damage the organism  
407 (Calabrese et al., 1973; Mottin et al., 2012). At the same concentration, Al is known to be less toxic  
408 than Zn in marine organisms. To our knowledge, no studies have investigated the biological effects  
409 of aluminum-based galvanic anode on bivalves. Bell et al (2020) investigated the effect of galvanic  
410 anodes during acute exposure on an amphipod species, *Corophium volutator*, a diatom species,  
411 *Phaeodactylum tricornutum*, and a bacterial species, *Aliivibrio fischeri*. They found no acute toxicity  
412 for the bacteria tested and only slight effects on algal growth. Regarding amphipods, significant  
413 increases in mortality were observed at the highest concentration of 100 mg L<sup>-1</sup>. Sublethal effects  
414 would have probably occurred at lower concentrations but all tested concentrations were very far  
415 from the environmental maximums. It is interesting to note that in the study by Caplat et al. (2010),  
416 it is shown that the dissolution of Al and Zn from a galvanic anode was less toxic for the urchin  
417 *Paracentrotus lividus* than sulphate salts. The dissolution of the anode could result in hydroxide  
418 precipitation due to a local increase in pH or associations of metallorganic complexes that could  
419 reduce the levels of free ionic Al (III) and Zn (II) species and therefore their availability. As a  
420 consequence, this could result in a lower toxicity of the degradation products of galvanic anodes  
421 compared to Al and Zn in the form of sulphate salts for which there is a higher proportion of free

422 metal forms due to the sulphate anion association. Using aluminum salts is consequently not the  
423 right solution for ecotoxicological studies of galvanic anodes. Considering Al in all its forms in  
424 literature, various LC<sub>50</sub> (lethal concentration) values have been reported: 3 000 µg L<sup>-1</sup> in the coral  
425 *Acropora tenuis* (Negri et al., 2011); 10 000 µg L<sup>-1</sup> in the copepod *Nitocra spinipes* (Bengtsson, 1978),  
426 while no effect is observed in annelids *Ctenodrilus serratus* and *Neanthes arenaceodentata* at 2 000  
427 µg. L<sup>-1</sup> (Petrich and Reish, 1979), and at 28 000 µg L<sup>-1</sup> in the sea urchin *Heliocidaris turberculata*  
428 (Golding et al., 2015). Nevertheless, at these elevated total concentrations of Al, it can be assumed  
429 that the element was principally under a non-bioavailable particulate form (Al(OH)<sub>3</sub>) since the Al  
430 solubility limit is 500 µg L<sup>-1</sup> (Angel et al., 2016; Golding et al., 2015).

431 The quantification of ROS and the number of circulating hemocytes remained stable throughout the  
432 experiment. However, after 84 days, phagocytic efficiency and the NRRRA tended to, respectively,  
433 decrease and increase. Regarding NRRRA, a change in DO can be interpreted as a change in the  
434 stability of lysosomal membranes or a change in the number or size of lysosomes. The decrease of  
435 almost 40% in phagocytic efficiency associated with a modulation of the lysosomal system may be  
436 indicative of a weakened immune system. A study on the effect zinc-based galvanic anode in *C. gigas*  
437 have reported a stimulation of the immune system during a 10 weeks chronic exposure ( $0.53 \pm 0.04$   
438 mg L<sup>-1</sup>) while immune parameters were inhibited during the one week acute exposure ( $10.2 \pm 1.2$  mg  
439 L<sup>-1</sup>) (Mottin et al., 2012). In the present study, the absence of significant effects on the immune  
440 system can be explained by the low doses of contaminant in the water and probably the adaptation  
441 of the molluscs to chronically exposure concentrations until elevated value. Indeed, Mottin et al  
442 (2012) investigated concentrations in Zn approximately 30 times higher than in our C3 condition.

443 In bivalves, energy reserves and the reproductive cycle are described as being closely linked (Heude-  
444 Berthelin, 2000). Glycogen represents an important part of energy reserves; it is particularly used as  
445 an energy source for gametogenesis. The measurement of glycogen levels in oysters gives us  
446 information about the metabolic state of individuals. The overall decrease in glycogen levels after 29  
447 days can be explained by active gametogenesis, with a majority of oysters being in stages 2 (active  
448 gametogenesis) and 3a (beginning of sexual maturity). There was a slight trend of decrease for  
449 conditions C2 and C3 after 84 days but this result was not related to the MGI. It is possible that  
450 oysters spend more energy to detoxify. Actually, the observation of histological sections showed no  
451 differences in the progress of gametogenesis between the 4 conditions. Concerning the BRIX index  
452 measured on hemolymph, it indicated no significant variation according to time or condition. To our  
453 knowledge, our study is the first one to use this type of index on bivalves during exposure to  
454 contaminants.

455 Reactive oxygen species (ROS) have the capacity to damage tissues and cellular components of  
456 organisms, which is known as oxidative stress. It is well established that marine organisms and  
457 particularly bivalves show a large panel of antioxidant defenses to protect themselves from exposure  
458 to contaminants with oxidative potential (Pipe and Coles, 1995). The role of these antioxidant  
459 systems and their sensitivity may be of great importance in ecotoxicological studies. MDA  
460 accumulation is one of the manifestations of oxidative stress, an indicator of lipid peroxidation  
461 corresponding to the oxidation of polyunsaturated fatty acids by ROS or enzymes. In our study,  
462 individuals exposed to C3 concentration showed significantly lower levels of MDA in the digestive  
463 gland after 84 days, while no variation in MDA levels in the gills was observed. The MDA values  
464 recorded are in agreement with those found in other studies in which *C. gigas* was exposed to  
465 pesticides (Mottier et al., 2015; Séguin et al., 2016). Various laboratory studies showed an increase in  
466 MDA levels in several bivalve species following metal exposures (Khebbeb et al., 2010; Moore et al.,  
467 2020). However, it has also been shown that a 21-day exposure to cadmium, silver, and mercury  
468 induced a decrease in MDA levels in the gills of *C. gigas* (Géret, 2002). A clear decrease of MDA in *P.*  
469 *perna* gills was recorded during the cycle of contamination by *Staphylococcus aureus* (Bendjoudi et  
470 al., 2013). It has also been shown a significant positive correlation between zinc and copper levels in  
471 *C. angulata* oysters and biomarkers involved in defense against oxidative stress such as catalase  
472 (CAT), superoxide dismutase, glutathione peroxidase, glutathione-S-transferases as well as  
473 metallothioneins (Funes et al., 2006). In chronic exposures to contaminants released from the  
474 estuaries of Huelva and Guadalquivir, low levels of MDA were associated with high activity of  
475 metallothioneins in the clams *Chamaelea gallina* (Rodríguez-Ortega et al., 2002). Therefore, the  
476 decrease in MDA levels in the digestive gland may be explained by antioxidant defense provided by  
477 sensitive enzymes of which activity increases in target organs depending on contaminant  
478 concentration (Damiens et al., 2006). CATs, a family of key enzymes in the regulation of oxidative  
479 stress, as well as metallothioneins, proteins particularly important in metal sequestration and  
480 detoxification, are examples of defense mechanisms against oxidative stress that were able to  
481 overtake lipid peroxidation. We did not have measure these enzymatic activities but in further  
482 studies, it would be interesting to evaluate these biomarkers in the digestive gland of oysters  
483 exposed to the C3 concentration for which MDA levels are the lowest. In addition, the absence of  
484 variation in ROS production measured in hemocytes by flow cytometry suggests that the digestive  
485 gland is impacted before the circulating system.

486 The principal component analysis confirms this antagonist relationship between MDA levels in the  
487 digestive gland with aluminum and zinc concentrations in water. The emergence of 4 distinct clusters  
488 on the PCA along dimension 1 corresponds to the 4 concentrations tested in our experiment. These



489 results demonstrate a sensitivity of oysters even if only the highest concentration induced  
490 pronounced biological effects.

#### 491 *Conclusion*

492 On the basis of the concentrations tested and experimental conditions, the assessment of the  
493 chronic toxicity of the degradation products of an aluminum-based galvanic anode on the Pacific  
494 oyster *C. gigas*, does not seem to indicate a direct environmental risk given the absence of significant  
495 mortality. Nevertheless, results demonstrate a sensitivity of oysters even if only the highest  
496 concentration induced pronounced biological effects. It is important to relate these results to the  
497 context of climate change, with the ocean acidification that has been shown to amplify metal  
498 bioaccumulation and may exacerbate their toxicity in *C. gigas* (Cao et al., 2018). As a non-essential  
499 element, Al shows low bioconcentration factors but galvanic anodes may facilitate the entry of Al  
500 into the food chain and its biomagnification in higher trophic level organisms considering that  
501 invertebrates are food sources of many species. It is known that Al in freshwater can be transferred  
502 from a primary to a secondary consumer at a pH of 7.1 (Walton et al., 2010). Zinc is also important  
503 to monitor because it represents 5% of an aluminum-based galvanic anode. Furthermore, in view of  
504 the bioaccumulation results, the Zn level equilibrium has not been achieved in oysters even after 84  
505 days and it would be interesting to extend the exposure period. The sustainability of marine  
506 organisms sensitive to contaminants is essential in terms of ecosystem services as well as to limit the  
507 health risks associated with the consumption of organisms grown near areas rich in galvanic anodes.  
508 As the biological effects of aluminum-based galvanic anode are poorly studied in the literature,  
509 further investigations on larval stages and other taxonomic groups remain necessary.

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### Tables and figures captions

666 Table I: Average metal concentrations in water during the exposure period for the 4  
667 conditions. Metals levels are expressed as  $\mu\text{g L}^{-1}$  (mean values  $\pm$  SEM, n=15).

668 Table II: Bioaccumulation kinetics of different elements for 0 day and after 84 days of  
669 exposure for the 4 experimental conditions. Elements levels are expressed as  $\text{mg kg}^{-1}$  of flesh  
670 (mean values  $\pm$  SEM, n=10). These results were obtained by High Resolution ICP-MS.

671

672 Figure 1: Scheme of the electrochemical device consisting of a galvanostat and an aluminium  
673 galvanic anode simulating its degradation in the marine environment modified according to  
674 Caplat et al. (2010) (A). Scheme of the installation of the different tanks for the oysters'  
675 exposure to the products resulting from the dissolution of the aluminium galvanic anode.  
676 The setup is replicated 4 times for the control and the three aluminum concentrations tested  
677 ( $65$ ,  $125$  and  $296 \mu\text{g L}^{-1}$ ) (B).

678 Figure 2: Bioaccumulation kinetics of aluminium and zinc for the 84 days of exposure under  
679 the 4 experimental conditions. Metals levels are expressed as  $\text{mg kg}^{-1}$  of flesh (mean values  $\pm$   
680 SEM, n=10). The significance of data is presented for each date compared to the control. A  
681 letter common to two conditions indicates that there is no significant difference between  
682 them.

683 Figure 3: Bioconcentration factors of aluminium and zinc for the 84 days of exposure under  
684 the 4 experimental conditions (mean values  $\pm$  SEM, n=10).

685 Figure 4: Immune parameters of oyster hemocytes after 84 days of exposure to metals  
686 released from the dissolution of an aluminum galvanic anode at different concentrations.  
687 Parameters studied are the number of hemocytes  $\text{mL}^{-1}$ , phagocytic activity, ROS production  
688 and lysosomal system in oyster hemocytes. Results were given compared to the 100%  
689 control (mean values  $\pm$  SEM, n=13). A letter common to two conditions indicates that there  
690 is no significant difference between them.

691 Figure 5: MDA (malondialdehyde) levels in the digestive gland and gills after 84 days of  
692 exposure to metals released from the dissolution of an aluminum galvanic anode at different  
693 concentrations. MDA levels are expressed as  $\text{nmol MDA mg proteins}^{-1}$  (mean values  $\pm$  SEM,  
694 n=8). A letter common to two conditions indicates that there is no significant difference  
695 between them

696 Figure 6: Glycogen content for 84 days of exposure to metals released from the dissolution  
697 of an aluminum galvanic anode for the four conditions. Glycogen levels are expressed as  $\text{mg}$   
698  $\text{g}^{-1}$  of flesh (mean values  $\pm$  SEM, n=8). A letter common to two conditions indicates that there  
699 is no significant difference between them

700 Figure 7: The principal components analysis (PCA) of the different variables (A) and  
701 individuals (B) of the study after 84 days of exposure. Al = aluminium bioaccumulated. Zn =  
702 zinc bioaccumulated. MDA GD = MDA levels in the digestive gland. MDA Br = MDA levels in  
703 the gills. NB = number of hemocytes. Lys = lysosomal system. Phago = phagocytosis  
704 efficiency, ROS = ROS production; Glyco = Glycogene content. BRIX = Brix index.

Figure 1:

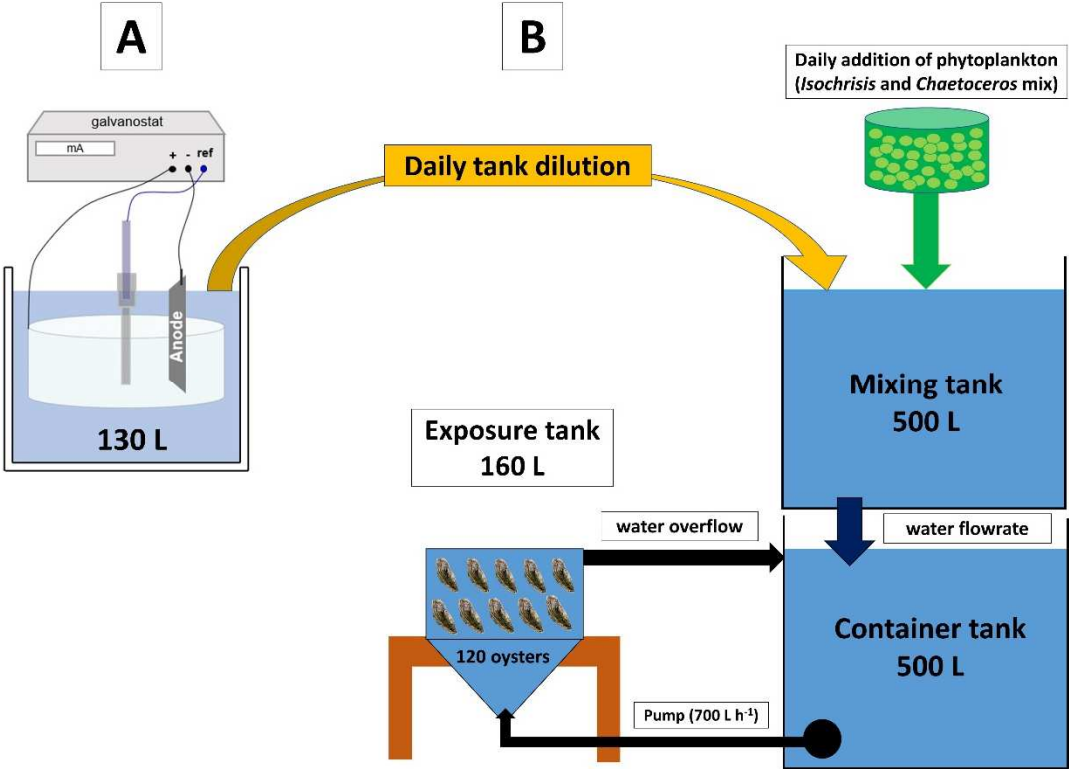


Figure 2:

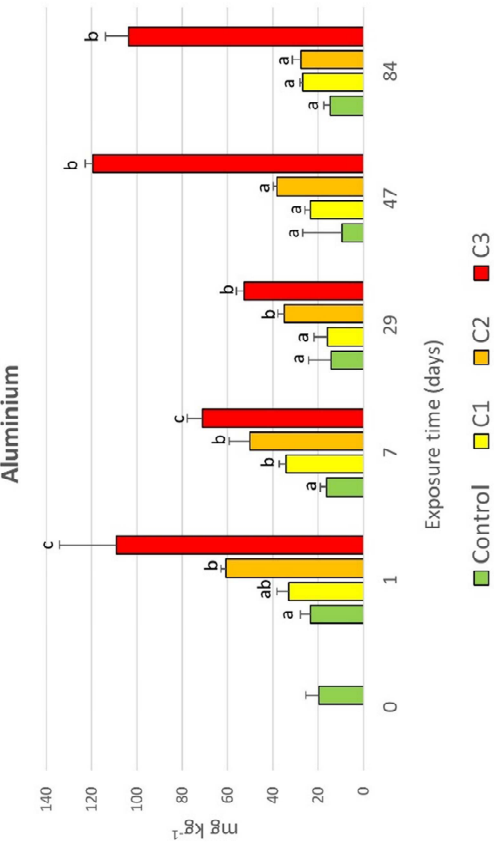
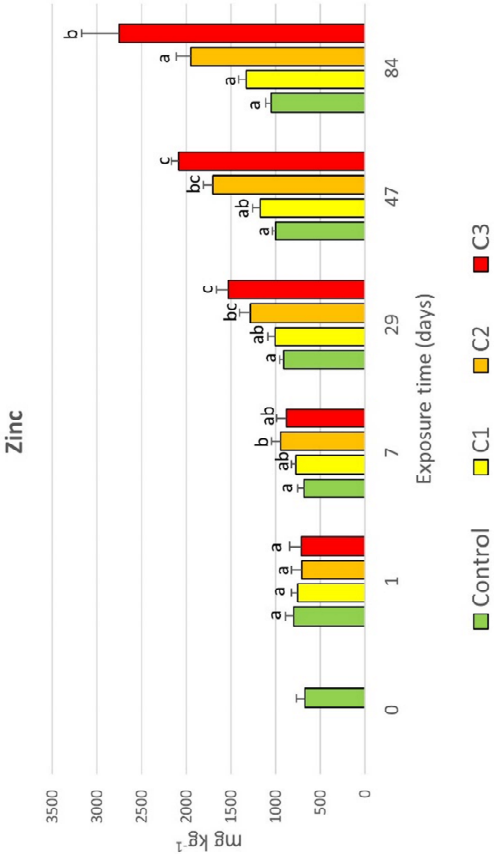


Figure 3:

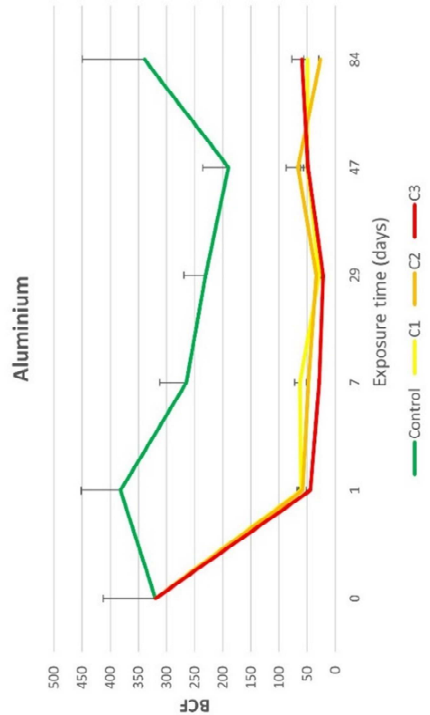
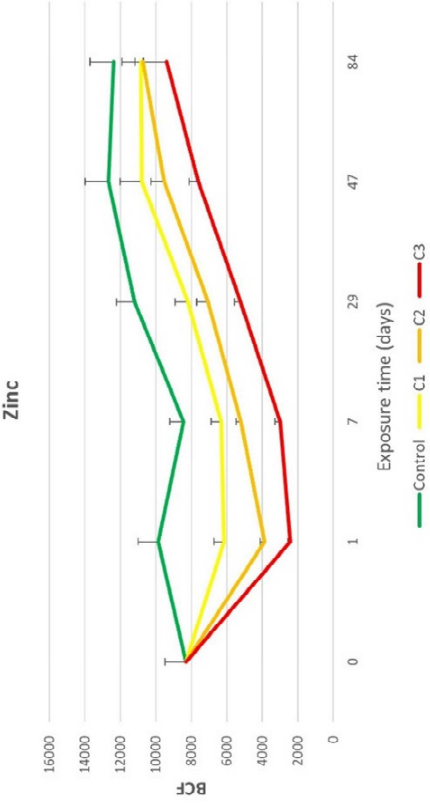




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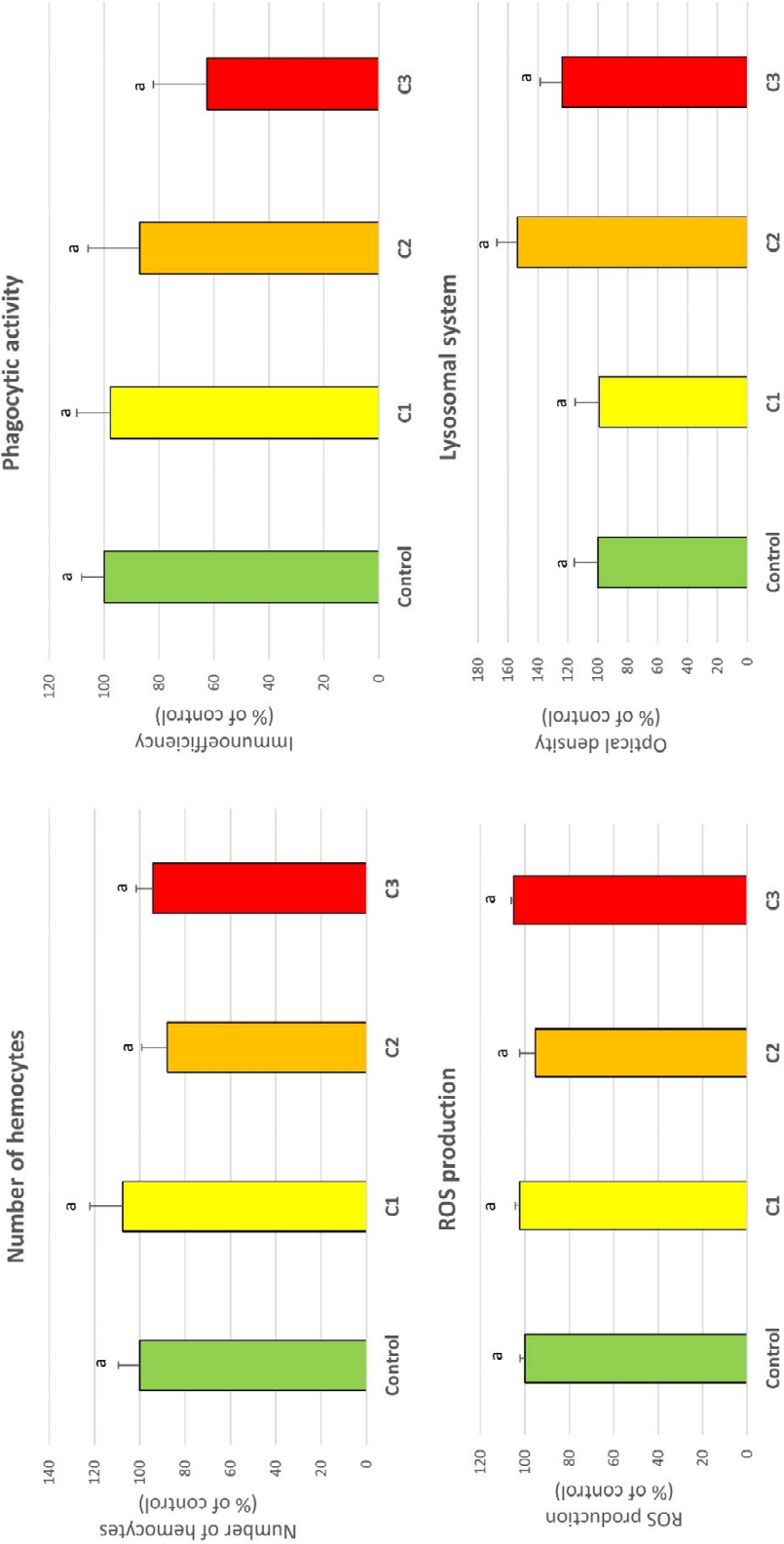


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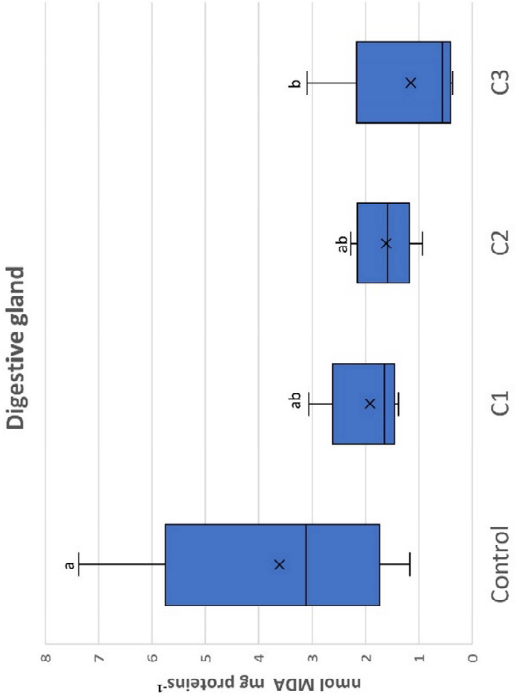
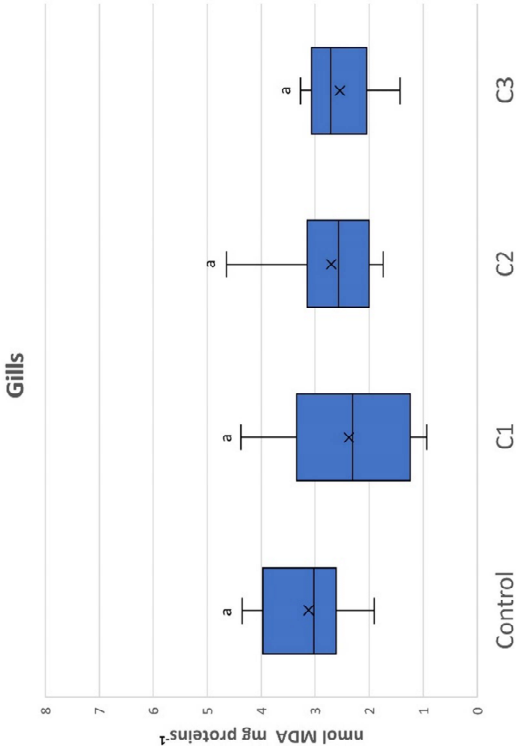


Figure 6:

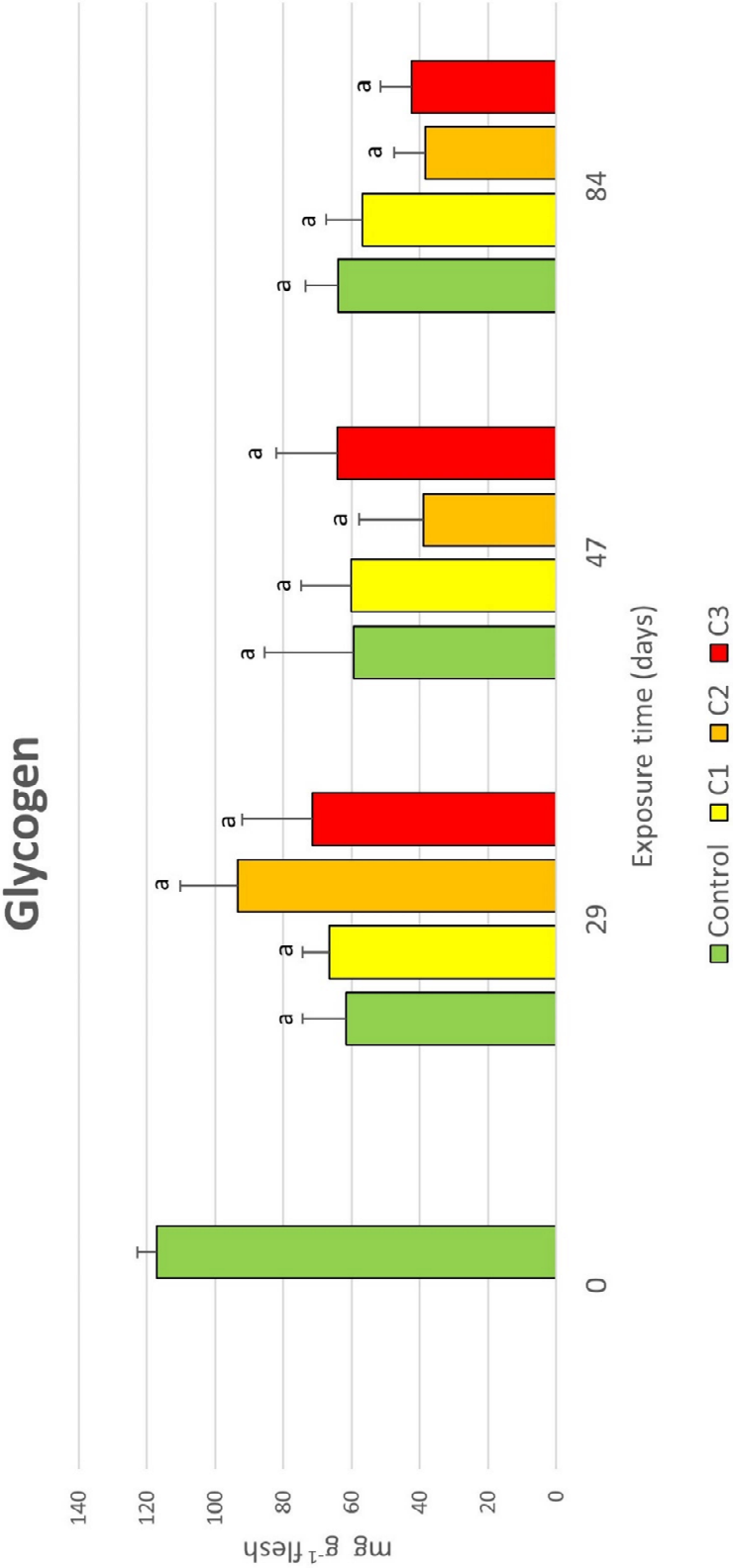
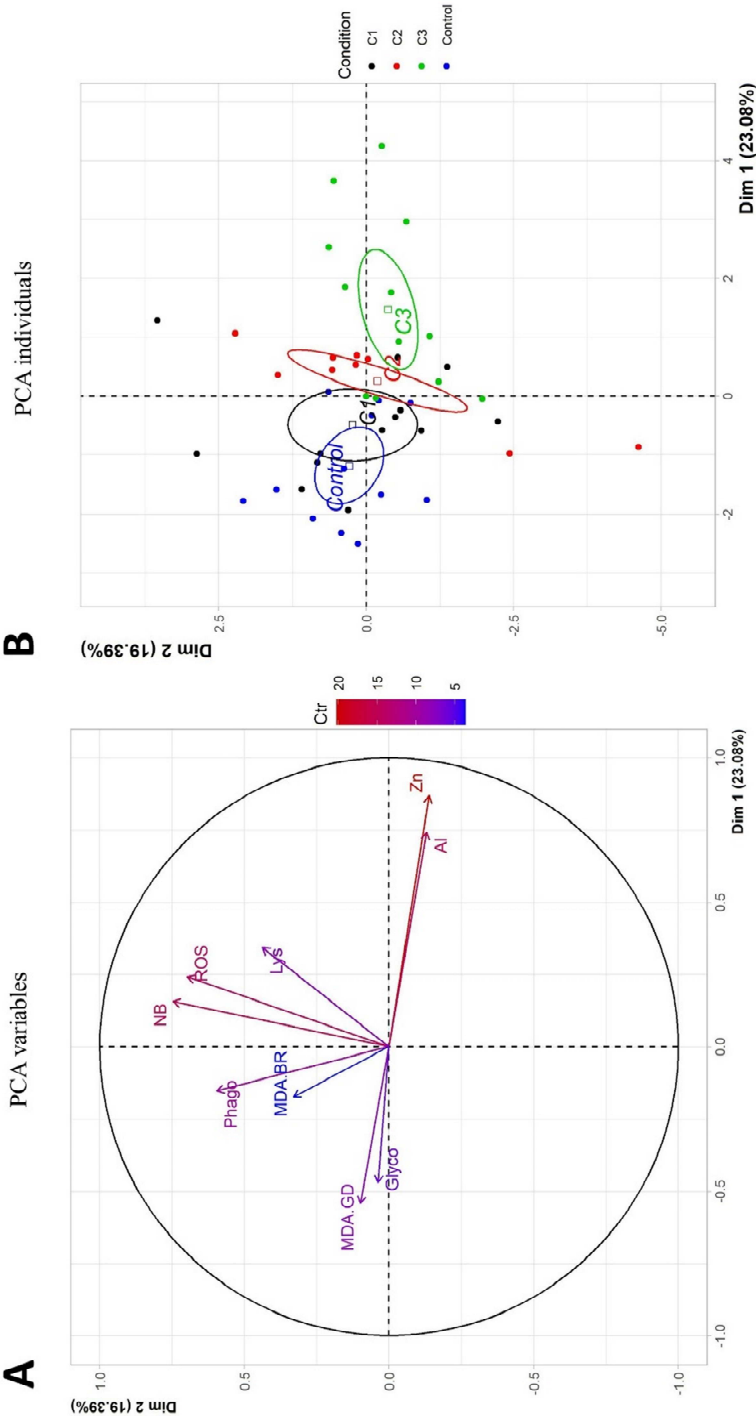


Figure 7:



**Table I**

<b>Concentration (<math>\mu\text{g L}^{-1}</math>)</b>	<b>Control</b>	<b>C1</b>	<b>C2</b>	<b>C3</b>
<b>Nominal concentrations of aluminium</b>	0	50	100	300
<b>Aluminium</b>	$7.4 \pm 1.3$	$65 \pm 6.7$	$125 \pm 11.2$	$296 \pm 29$
<b>Zinc</b>	$9.7 \pm 0.8$	$15 \pm 0.5$	$22 \pm 0.6$	$35 \pm 22.6$

**Table II**

<b>Concentration (mg kg<sup>-1</sup>)</b>	<b>Aluminium</b>	<b>Zinc</b>	<b>Copper</b>	<b>Manganese</b>	<b>Iron</b>	<b>Arsenic</b>	<b>Silver</b>	<b>Cadmium</b>	<b>Nickel</b>	<b>Lead</b>	<b>Potassium</b>	<b>Calcium</b>	<b>Magnesium</b>
Control (0 day)	19.7 ± 5.7	672.5 ± 96.4	44.2 ± 5.4	33.5 ± 4.7	100.8 ± 4.8	18.15 ± 0.6	2.5 ± 0.4	1.5 ± 0.2	0.4 ± 0.03	0.6 ± 0.02	12112 ± 383.4	4415 ± 809.5	2656 ± 123.7
Control (84 days)	14.6 ± 6.8	1051.7 ± 108.3	53.7 ± 7.7	47.0 ± 9.6	125.5 ± 5.1	11.5 ± 0.9	3.0 ± 0.4	1.8 ± 0.1	0.5 ± 0.1	0.4 ± 0.04	12418 ± 393.8	8636.3 ± 1737.7	4397.9 ± 558.1
C1 (84 days)	26.8 ± 3.5	1327.8 ± 131.9	65.9 ± 8.1	30.2 ± 6.7	107.5 ± 5.7	11.2 ± 0.5	3.2 ± 0.4	1.7 ± 0.1	0.5 ± 0.04	0.4 ± 0.02	13159 ± 436.3	6213.7 ± 1035	4409.6 ± 283.5
C2 (84 days)	27.6 ± 3.3	1952 ± 81.8	79.9 ± 5.5	31.3 ± 9.4	91.9 ± 2.5	13.2 ± 1.6	4 ± 0.3	1.5 ± 0.1	0.3 ± 0.05	0.3 ± 0.02	11960.4 ± 332.2	6245.5 ± 3966.1	3751.1 ± 195
C3 (84 days)	103.7 ± 10.3	2753.6 ± 418.7	63.1 ± 9.8	29.7 ± 4.8	109.5 ± 5.1	13.3 ± 1.3	3.7 ± 0.6	1.6 ± 0.1	0.3 ± 0.12	0.4 ± 0.02	11660.3 ± 314.2	9481.5 ± 2820	3777.4 ± 216