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1 **Organotropism and biomarker response in oyster *Crassostrea gigas***
2 **exposed to platinum in seawater**

3 Melina Abdou^{1*}, Beñat Zaldibar^{2,3}, Rebeca Medrano³, Jörg Schäfer¹, Urtzi Izagirre^{2,3}, Lionel
4 Dutruch¹, Alexandra Coynel¹, Gérard Blanc¹, Manu Soto^{2,3}

5 ¹*University of Bordeaux, UMR CNRS 5805 EPOC, 33615 Pessac, France,*

6 ²*CBET Res. Grp., Dept. Zoology & Animal Cell Biology, Univ. Basque Country (UPV/EHU),*
7 *Leioa, Basque Country (Spain)*

8 ³*Research Centre for Experimental Marine Biology and Biotechnology (Plentzia Marine*
9 *Station; PiE-UPV/EHU), Univ. Basque Country, Plentzia, Basque Country (Spain)*

10 *Corresponding author: melina.abdou@u-bordeaux.fr

11
12 **Abstract**

13 Platinum (Pt) is a Technology Critical Element (TCE) for which biogeochemical cycles are still
14 poorly understood. This lack of knowledge includes Pt effects on marine organisms, which
15 proved to be able to bioconcentrate this trace element. Oysters *Crassostrea gigas* were exposed
16 to stable Pt isotope spiked daily in seawater for 35 days. Seawater was renewed daily and spiked
17 (with Pt(IV)) to three nominal Pt concentrations (50, 100, and 10,000 ng.L⁻¹) for two replicate
18 series. Organotropism study revealed that gills, and to a lesser extent mantle, are the key organs
19 regarding Pt accumulation, although a time- and concentration-dependent linear increase in Pt
20 levels occurred in all the organs investigated (i.e. digestive gland, gonads, gills, mantle, and
21 muscle). In oysters exposed to Pt concentrations of 10,000 ng.L⁻¹, significant biomarker
22 impairments occurred, especially at cellular levels. They reflect altered lipofuscin and neutral
23 lipid contents, as well as intralysosomal metal accumulation. These observations were
24 attributed to activation of excretion / detoxification mechanisms, including Pt elimination
25 through faeces and clearly support the importance of the digestive gland in the response to
26 direct Pt exposure. Despite relatively constant condition index, the Integrative Biological
27 Response Index (IBR) suggests a generally decreasing health status of oysters.

28
29 **Keywords:** Platinum; Emerging Contaminant; Isotopes; Marine Bivalve; Biomarker;
30 Ecotoxicology

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37 assistance.

38 1. Introduction

39 The Platinum Group Elements, including platinum (Pt) and five other rare metals, belong to the
40 Technology Critical Elements (TCEs), considered as elements of great relevance in the
41 development of emerging key technologies (Cobelo-García et al., 2015). The extensive use of
42 TCEs has led to a global disturbance of their natural geochemical cycles with anthropogenic
43 fluxes being dominant over natural fluxes (Sen and Peucker-Ehrenbrink, 2012). Increasing Pt
44 concentrations in the environment have been attributed to various anthropogenic sources such
45 as industries, anti-cancer drugs and, more importantly, car catalytic converters (Moldovan et
46 al., 2002). Catalytic car exhaust transformation is an efficient green key technology, but goes
47 along with the emission of Pt along the roadsides, resulting in elevated Pt concentrations in
48 many environmental compartments. Maximum Pt contamination occurs in highly populated
49 and urbanized areas, but anthropogenic Pt anomalies are also recorded in very remote sites (e.g.
50 polar ice, Soyol-Erdene et al., 2011). Only little information on Pt concentrations in natural
51 water bodies is available, but field observations and laboratory exposure studies have shown Pt
52 uptake and bioaccumulation by different aquatic organisms such as annelids, crustaceans,
53 bivalves, snails and various fish species (summarized in Ruchter et al., 2015 and Zimmermann
54 and Sures, 2018). The small number of field studies include data on Pt concentrations in
55 bivalves, such as freshwater clams, as well as seawater mussels and oysters (Ruchter and Sures,
56 2015; Neira et al., 2015; Abdou et al., 2016, respectively). Those widely distributed organisms
57 are sedentary, easy to sample and continuously available throughout the year. Considering their
58 high potential for metal bioconcentration, bivalves are preferentially used in biomonitoring
59 programs reflecting trace metal contamination over decades (e.g. Mussel Watch Program;
60 Goldberg et al., 1978). Ideal sentinel organism accumulate contaminant without suffering
61 mortality and showing high accumulation capacity (Ruchter, 2012). Literature reports that a
62 couple of adverse effects including deleterious effects at molecular, organelle, and tissue/organ
63 levels occur in aquatic organisms following Pt exposure under laboratory conditions.
64 Physiological and morphological adverse effects include the induction of heat shock proteins
65 (hsp70, stress conditions; Singer et al. 2005) or tissue alteration (e.g. cytoplasm vacuolization;
66 Osterauer et al., 2010). Although such exposure studies provide valuable knowledge, test
67 organisms are generally exposed to relatively high Pt concentrations exceeding by far
68 environmental levels (Sures et al., 2015). Previous work has reported on short-term Pt
69 bioaccumulation kinetics in oysters (*Crassostrea gigas*) exposed to isotopically-labelled Pt
70 (^{194}Pt) in seawater covering a wide concentrations range, as an approach to environmentally

71 relevant levels (Abdou et al., 2018). The present work introduces results from the same
72 experiment, focusing on Pt organotropism and toxicological effects in oysters using a battery
73 of cell and tissue level biomarkers and their integration into the Integrative Biological Response
74 Index (IBR Index) for environmental health assessment (Beliaeff and Burgeot, 2002; Broeg and
75 Lehtonen, 2006).

76 The digestive gland of mollusks is an organ of major interest in ecotoxicological studies, since
77 it is involved in pollutant accumulation and detoxification processes, including heavy metals
78 (Marigómez et al., 2002), and it is a plastic organ that reacts under stress situations. Biomarkers
79 measured at the molecular or cellular level have been proposed as sensitive ‘early warning’
80 tools for biological effect measurement in environmental quality assessment (McCarthy and
81 Shugart, 1990). Biomarkers indicate that an organism has been exposed to a determined
82 pollutant or family of pollutants (exposure biomarkers) and/or the magnitude of the organism’s
83 response to the pollutant (effect biomarkers or biomarkers of stress; Cajaraville et al., 2000).
84 For this study, the selected metal exposure biomarker was the quantification of the volume
85 density of Black Silver Deposits (BSD) revealed after autometallographical staining, (Soto and
86 Marigómez, 1997). This histochemical technique is based on the principle that silver ions react
87 with a catalyst present in the tissue (metals) and are reduced to metallic silver that precipitates
88 surrounding the metal in the core (Danscher, 1984). In mollusks, non-essential metals, such as
89 Pt, are strongly bound to metallothioneins and then transferred to the digestive lysosomes,
90 where due to the low pH metallothioneins are degraded and metals are accumulated (Soto et
91 al., 2002). Therefore, BSD in the cells are normally found in cytoplasm or within the digestive
92 lysosomes. The connective tissue surrounding the digestive tubules of oysters is known to play
93 an important role as energy reservoir (Thompson et al., 1996). The depletion and relocation of
94 neutral lipids has been described as an alteration in oysters exposed to heavy metals
95 (Rementeria et al., 2016; Séguin et al., 2016) and therefore this endpoint was investigated as an
96 effect biomarker. Other endpoints include lipofuscins which are one of the main cellular
97 products accumulating in lysosomes as a result of enhanced oxidative damage. This increased
98 oxidative damage has been previously reported in mollusks exposed to metals (Marigómez et
99 al., 2013; Rementeria et al., 2016). The presence of metals and other stress situations have been
100 described to provoke alterations at tissue level in the digestive gland of bivalves including the
101 atrophy of the digestive epithelium (Izagirre et al., 2014) and the shrink, reduction in size and
102 finally loss of digestive tubules, with a concomitant increase the relative proportion of the
103 interstitial connective tissue (Brooks et al., 2011).

104 The objectives are to assess Pt effects at (i) cellular level, with the measurement of lipofuscin
105 and lipid contents and (ii) tissue level, including quantification of histopathological alterations

106 and digestive gland integrity; and Pt toxicokinetics at organism level, in the light of Pt
107 distribution in the different organs and possible excretion through faeces. Accordingly, this
108 work aims at characterizing Pt accumulation/distribution and the related physiological and
109 morphological effects in marine organisms such as oysters.

111 2. Material and Methods

112 Experimental setup as well as sampling protocols and analyses for Pt quantification in soft
113 tissues were already described in a previous paper (Abdou et al., 2018). They are briefly
114 summarized in the following sections, together with the description of protocols for histological
115 and biomarker studies.

117 2.1. Experimental setup

118 Stock isotopic solution was prepared using solid metal shavings of ^{194}Pt (116.5 mg;
119 Cortecnet®) which were acid digested using 4 mL of concentrated HCl and 2 mL of
120 concentrated HNO_3 (both Suprapur®, Merck) heated up at 110 °C for 4 h. The stock solution
121 was diluted using ultrapure water (MilliQ®) and ^{194}Pt final concentration ($532 \pm 1 \text{ mg.L}^{-1}$) was
122 quantified through Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo® X
123 Series II; n = 5). The dissolution protocol (Gammons et al., 1996) and the probable dominance
124 of Pt(IV) in seawater (Cobelo-García et al., 2013) suggest that oysters were exposed to Pt(IV).

125 Clean oysters (*Crassostrea gigas*, n= 545) obtained from a commercial seller (“OSTRANOR
126 S.L.”, San Vicente de la Barquera, Cantabria, Spain), with similar shell length ($\sim 90 \pm 5 \text{ mm}$)
127 were transported to the Plentzia Marine Station of the University of the Basque Country
128 (PiE-UPV/EHU) to perform exposure experiments under controlled conditions. Prior the
129 beginning of exposure, oysters were maintained in naturally filtered seawater in an open flow
130 system (12 days). Exposure tanks were equilibrated spiking the respective nominal total Pt
131 concentrations in seawater (12 days, daily renewal). The different tank conditions namely A,
132 B, C, and D consisted in: control tanks where no Pt was spiked, and exposure tanks with Pt
133 spikes of 50 ng.L^{-1} Pt, 100 ng.L^{-1} Pt, and $10,000 \text{ ng.L}^{-1}$ Pt respectively. Literature reports that
134 environmental concentrations range from $\sim 0.1 \text{ ng.L}^{-1}$ in relatively clean coastal environments
135 (Cobelo-García et al., 2014a) to $\sim 7 \text{ ng.L}^{-1}$ Pt in contaminated areas such as the Tokyo Bay
136 (Obata et al., 2006). Therefore, as explained in Abdou et al. (2018), the different concentrations
137 were chosen in order to cover a range of concentrations, from environmentally relevant (B and
138 C conditions are only 10 and 20 times higher than Pt concentrations measured in urban polluted

139 coastal areas) to relatively high exposure levels (D conditions). Each condition was carried out
140 in replicate, as reflected by the tank numbers 1 and 2 (e.g. A1 and A2).

141 Before starting Pt exposure, we isolated and dissected twenty-five oysters which constitute the
142 T = 0 (initial conditions) for the targeted parameters. Thereafter, we performed an even
143 distribution of the 520 individuals in eight 45 L polypropylene (PP) tanks (65 individuals per
144 tank) which were at first filled with 40 L aerated seawater. The experimental room was
145 temperature-controlled (17 °C) and equipped with an artificial photoperiod set up on a
146 12 h : 12 h light : dark cycle. Chemical and physical parameters namely temperature, pH,
147 salinity, and dissolved O₂ concentrations were measured daily during the experiment using a
148 TetraCon 96® and a Sentix ® 41 probes (PROFILINE, WTW®). On a daily basis and after
149 mortality checking, oysters were rinsed, fed and transferred in clean separate tanks while
150 exposure tanks were rinsed, filled up with clean seawater and spiked to nominal Pt
151 concentrations using ¹⁹⁴Pt stock solution. After 4 h feeding and spike equilibration, oysters were
152 transferred again in the control and exposure tanks. Seawater, spike, and food volumes were
153 adapted according to the number of oyster organisms left after each dissection in order to
154 maintain exposure and feeding conditions constant by individual. Respective proportions per
155 individual per day are reported in Abdou et al., 2018.

157 2.2. Sampling protocols

158 Organotropism analyses were performed on oysters from tanks D, sampled at T = 3, T = 7,
159 T = 28, and T = 35. Histological and cellular studies were performed on oysters from each
160 exposure condition at T = 3, T = 7, and T = 28. At time of sampling, five individuals per tank
161 (pseudo-replicates) were sampled for both types of analysis. Oysters were opened, water
162 remaining inside the shell was removed, and soft body rinsing was performed using
163 uncontaminated seawater.

164 For organotropism analyses, organs (gills, digestive gland, gonads, muscle, and mantle) were
165 dissected and placed separately in acid-cleaned (10 % HNO₃; 65 % Honeywell), PP tubes
166 (DigiTUBEs®, SCP SCIENCE). Gonads could not be systematically isolated from digestive
167 glands. Therefore, pieces of pure digestive gland and the remaining digestive gland and gonads
168 were mineralized and analyzed separately. Wet weight of each organ was quantified
169 (approximate weight for gonads and digestive gland). In addition, each valve was weighted and
170 measured (thickness, width, and length), allowing the calculation of the Condition Index (CI)
171 calculated for each oyster according to the equation (1) from Strady et al., (2011):

172 $CI = \text{Flesh weight (wet weight; g)} / \text{Shell weight (wet weight; g)} \times 100.$ (1)

173 As CI has been already discussed in Abdou et al. (2018), no results are presented herein but the
174 CI data have been used in the integrative biological response index (IBR, see section 2.4.). After
175 dissection, soft tissues were deep-frozen (- 80 °C). Tissues were thereafter freeze-dried, crushed
176 in an agate mortar, and stored at room temperature, in the dark, until Pt quantification.

177 For histological analyses, a ~ 5 mm thick cross-section of the soft body was performed using a
178 sharp blade. This section included main organs and tissues (digestive gland, gills, and gonad)
179 of the oysters. Sections were immersed in seawater buffered with formalin and kept at 4 °C for
180 24 h. Samples were rinsed with ethanol (70 %), processed in the tissue processor (Leica
181 ASP300®), finally embedded in paraffin (Leica Surgipath® Paraplast) and sectioned in a
182 microtome (Leica® RM2125 RTS). Sections of 5 µm were placed in a microscope slide and
183 were stained for histopathology and histochemical analyses (section 2.3.2 and 2.3.3).
184 Histochemical analyses were obtained from the same tissue samples: digestive glands were
185 excised, put in cryovials, and frozen in liquid nitrogen rapidly. We kept the frozen samples at
186 – 80 °C pending sectioning and staining as described in section 2.3.3.

187 In addition to dissections, faeces were collected four times (T = 7, 21, 28, and 35) during the
188 experiment in each feeding tank (uncontaminated seawater in order to avoid Pt adsorption on
189 faeces). Sampling was performed using a Pasteur syringe. All visible faeces at time of collection
190 were sampled but this sampling cannot be considered as fully quantitative since we might miss
191 some material excreted later on. Samples were collected in a Teflon bottle, filtered on pre-
192 weighted Teflon filters (47 mm, 0.45 µm FHLC filter, Merck Millipore), deep-frozen (- 80 °C),
193 then oven-dried at 50 °C, and stored in sealed containers pending analysis.

194

195 2.3. Analytical procedure

196 2.3.1. Quantification of Pt concentrations

197 Organ tissues were prepared for Pt quantification by ICP-MS as described in Abdou et al., 2018.
198 The steps consisted in ashing (800 °C) followed by acid digestion using *aqua regia* (3 h at
199 110°C). Same procedure (without the ashing step) was applied to faeces collected on Teflon
200 filters. We applied the standard addition method (mono-elementary Pt standard solution
201 1,000 µg.mL⁻¹ PLASMACAL, SCP Science). Spectral interference (hafnium-oxygen species)
202 corrections were applied on the ¹⁹⁴Pt and ¹⁹⁵Pt isotopes as detailed in Abdou et al. (2018).
203 According to an inter-method comparison between ICP-MS and Adsorptive Cathodic Stripping
204 Voltammetry (AdCSV) methods, sufficient sample mass (i.e. > 0.25 g dry weight, 0.13 ng Pt)
205 is necessary to reliably measure Pt concentrations in natural oysters (not exposed to Pt spikes)
206 by quadrupole ICP-MS (Abdou et al., 2018). As for organotropism measurements, organ
207 masses were generally too low (e.g. ~ 0.1 g for digestive gland) to allow for ICP-MS analyses
208 in non-exposed oysters, these samples were analyzed by AdCSV as described in Abdou et al.,
209 (2018). Briefly, after ashing, samples were acid-digested using *aqua regia* (1 h at 195 °C),
210 followed by an evaporation step and dissolution with sulfuric acid (H₂SO₄). Samples are
211 thereafter evaporated again and diluted with hydrochloric acid (HCl). Platinum voltammetric
212 quantification was performed using a µAutolab Type III potentiostat (Metrohm® Autolab B.V.)
213 connected to a polarographic stand (Metrohm® 663 V.A.) and controlled using the NOVA 2.1
214 software. Aliquots of acid-digested sample were added to the voltammetric cell, together with
215 formaldehyde and hydrazine sulfate as well as H₂SO₄ and concentrations of Pt were quantified
216 applying the standard addition method and considering the second derivative of the
217 voltammogram (Cobelo-García et al., 2014b). “Reconstituted” whole organism Pt
218 concentrations have been estimated from Pt values in the different organs and considering the
219 respective organ masses.

220
221 2.3.2. Histopathological alterations

222 In order to assess histopathological alterations, paraffin embedded tissue sections were stained
223 with haematoxylin-eosin (Gamble and Wilson, 2002) using a stainer integrated workstation
224 (Leica® ST5010 Autostainer XL; Leica® CV5030). Microscopic slide observations were
225 performed under a light microscope (Olympus BX-61).

226 Connective-to-diverticula (CTD) ratio was quantified in order to assess the integrity of the
227 digestive gland tissue. Five randomly selected fields of digestive gland were acquired per

228 individual using a digital camera coupled to the microscope at 20x magnification. Images were
229 processed through Image J program (Image Processing and Analysis in Java, Maryland, USA)
230 as described in Rementeria et al. (2016). The CTD ratio is the area (extent) occupied by the
231 interstitial connective tissue relative to the area occupied by the digestive diverticula. This ratio
232 was obtained using the following equation (2) from Brooks et al. (2011):

$$233 \text{ CTD ratio} = c / (b + d + l) \quad (2)$$

234 Where (c) hits on interstitial connective tissue, (b) hits on basophilic cells, (d) hits on digestive
235 cells and (l) hits on diverticular lumen.

236 Atrophy of the digestive tubules was calculated using a semi-quantitative scale according to
237 Kim et al. (2006), with values in a range from 0 (no atrophy) to 4 (severe atrophy in most
238 tubules).

239 240 2.3.3. Histochemical analyses

241 In order to evaluate the lipofuscin content in the digestive gland, sections of 8 µm thickness
242 corresponding to the digestive gland were obtained using a cryostat with a – 26 °C chamber
243 temperature. Frozen sections were stained according to the Schmorl method (Pearse, 1985) in
244 order to detect reducing substances, such as lipofuscins, within the cells when ferricyanide is
245 converted into ferrocyanide that is converted to insoluble Prussian blue in the presence of ferric
246 ions. Five randomly selected pictures of each sample were acquired using a digital camera
247 coupled to a microscope (Leitz Laborlux S, Leica Microsystems, Wetzlar, Germany). Using the
248 BMS (Biological Measurement System) program, lipofuscins were optically segmented. Using
249 the SPSS/PC program, the relative proportion between lipofuscin surface and the remaining
250 extent of the digestive tissue was quantified (Rementeria et al., 2016; Zorita et al., 2006).

251 For neutral lipid content measurements, frozen 8 µm sections of digestive gland were similarly
252 obtained using the cryostat at – 26 °C. Samples were thereafter processed following the ORO
253 (Oil Red O) staining method (Culling, 1974). Image analysis was performed following the same
254 method as aforementioned for lipofuscin content but applied to neutral lipid (Marigómez et al.,
255 2013, Rementeria et al., 2016).

256 For autometallographical staining, a 5 µm thick sections of paraffin embedded samples were
257 dewaxed in xylene, re-hydrated and dried at 37 °C for 24 h. Samples were thereafter covered
258 using the commercial silver enhancement kit (Silver Enhancement Kit, BBI Solutions) at room
259 temperature (in the dark) and reaction was stopped after 20 min. Slides were washed using
260 distilled water (Elix®) and mounted in Kaiser's gelatin-glycerin (Merck®). Black silver

261 deposits (BSD), which reveal the presence of metals when AMG is applied, were mainly
262 localized in the digestive cell lysosomes (Soto et al. 2002). Thus, the volume density of BSD
263 (VvBSD) was considered as a measure for intralysosomal metal accumulation.

264 265 2.4. Integrative Biological Response (IBR/n) index

266 Integrative Biological Response (IBR) index was calculated according to Devin et al. (2014),
267 based on the method by Beliaeff and Burgeot (2002). Five biomarkers, at different biological
268 organization levels, were used for the index calculation (lipofuscin content, neutral lipid
269 accumulation, atrophy, CTD ratio and Condition Index). The selection of biomarkers has been
270 based on effect biomarkers for the determination of the response level of oysters and correspond
271 to different levels of biological complexity (from cell to organism). Since the IBR value
272 depends on the number of applied biomarkers, the IBR/n value presented in the results section
273 was obtained dividing the IBR by 5 (Brooks et al., 2011; Marigómez et al., 2013).

274 275 2.5. Quality control and statistics

276 We analyzed the only available Certified Reference Materials (CRMs) for Pt: the Jsd-2
277 sedimentary rocks (indicative value from Geological Survey of Japan) and the BCR®-723 road
278 dust (Institute for Reference Materials and Measurements). The ICP-MS analysis of these
279 CRMs provided satisfactory recoveries of 101 % and 87 %, respectively (n = 3). They were
280 also quantified through AdCSV providing satisfactory recovery of 98 % for Jsd-2 and 89 % for
281 BCR®-723 (n = 3). The detection limit for Pt measured in biological samples by ICP-MS (3 x
282 blank standard deviation, n = 10) ranged between 0.06 and 0.006 ng.g⁻¹ for typical sample mass
283 comprised between 0.1 and 1 g, while it is of 0.01 ng.g⁻¹ for sample mass of 0.1 g analyzed
284 through AdCSV.

285 To evaluate significant differences (p-values < 0.05) in (i) physical-chemical parameters and
286 (ii) Pt concentrations between the different sampling times and the different organs, we
287 performed one-way ANOVA tests for parametric data and we applied Kruskal-Wallis tests for
288 non-parametric data. In addition, we checked for the homoscedasticity of the data applying
289 Bartlett test. Pairwise post-hoc tests were carried out (Mann-Whitney or Holm-Bonferroni).

290 For histological measurements, statistical analyses were performed using the SPSS v 23.0
291 (IBM®). Data normality and homogeneity (Kolmogorov Smirnov and Levene's tests) were
292 checked and analyses of variance and Duncan's post hoc test were carried out in order to assess
293 significant differences between conditions (p < 0.05). Kruskal-Wallis tests and Dunns post hoc

294 test were applied to non-parametric data. Pearson's correlations between Pt concentration and
295 biomarkers in tank D were also carried out.

3. Results

3.1. Platinum accumulation kinetics in the different organs of oyster exposed to
10,000 ng.L⁻¹ Pt"

Temporal aspects

Platinum concentrations quantified separately in each organ from non-exposed organisms
(T = 0) and from exposed oysters (T = 3 to T = 35), covered several orders of magnitude
(Fig. 1; Table 1). For oysters exposed to isotopically-labelled Pt, both replicate series gave
similar Pt concentration evolution through time for each organ studied, showing a steep increase
in concentrations throughout the experiment (Fig. 1). Determination coefficients (R²) revealed
a significant linear accumulation of Pt over time with values > 0.9 in each organ (Table 1). All
Pt concentrations in oysters exposed to Pt spikes throughout the experiment (from T = 3) were
statistically different (p < 0.002) from those in non-exposed oysters. In fact, soft tissues showed
Pt concentrations at least 100 times higher than initial conditions after only three days of
exposure (T = 3; Table 1). At the end of the experiment (T = 35), Pt concentrations in mantle
and gills were ~ 10-fold higher than at T = 3 (Table 1). Final muscle and gonads Pt values were
~ 15-fold higher than at T = 3, ~ 20-fold for digestive gland + gonads, and ~ 60-fold for
digestive gland (Table 1). For most exposure times and organs, results were significantly
different (p < 0.05) from the previous sampling time (values labelled * in Fig. 1). Note that, for
all the organs studied, no significant differences were observed between T = 28 and T = 35 for
both replicate series.

Organ comparison

At initial conditions, differences of Pt concentrations between muscle and digestive gland and
between muscle and mantle were non-significant although digestive gland + gonads and gonads
alone tended to show the lowest Pt concentrations (Table 1). Platinum distribution between
organs expressed as average percentage (n = 10) with respect to total organism Pt content
(Fig. 2) showed that at initial conditions gills contained ~ 40 % of the total Pt content, whereas
muscle and mantle had similar percentages of ~ 20 %. The other organs, digestive
gland + gonads, and gonads and digestive gland alone showed similar values of ~ 7 %.

326 After exposure, gills generally showed the highest Pt concentrations followed by the mantle.
1 327 At all exposure times, significant differences occurred between gills and the other organs as
2
3 328 well as between mantle and the other organs. Platinum concentrations in exposed oysters
4
5 329 followed the general trend: gills > mantle > digestive gland ~ digestive
6
7 330 gland + gonad > gonads > muscle (Table 1). Compared to estimated whole organism
8
9 331 concentrations, gills reached the highest percentage of Pt of up to 70 % at all times (Fig. 2).
10
11 332 Platinum percentages in muscle dropped from ~ 20 % to ~ 3 % and remained stable. The
12
13 333 percentages of all other organs showed only minor variations compared to initial conditions and
14
15 334 throughout the exposure period. The lowest Pt contribution occurred in digestive gland at T = 3
16
17 335 with only ~ 0.5 %. The comparison of “reconstituted” whole organism Pt concentrations with
18
19 336 those measured in whole individuals from the same experiment, analyzed without organ
20
21 337 dissection (Abdou et al., 2018) showed overall good fit of both datasets (less than 20 %
22
23 338 difference), confirming the previously observed linear trend in Pt accumulation with high
24
25 339 coefficients of determination for whole oysters from tanks D1 ($R^2 = 0.94$) and D2 ($R^2 = 0.97$).

26 340

28 341 3.2. Platinum concentrations in faeces

31 342 Platinum concentrations in faeces were monitored through the exposure period. Control tanks
32
33 343 showed very low, similar Pt concentrations along the experiment (from < 0.285 to 3.59 ng.g⁻¹;
34
35 344 Table 1). Faeces from oysters exposed to Pt showed clearly higher Pt concentrations, reflecting
36
37 345 the order of exposure levels in the different tanks and generally the proportions of exposure
38
39 346 concentrations. Values between replicate series were similar. Faeces did not display a clear
40
41 347 trend over time. In fact, at T = 7 oysters faeces showed mean Pt concentrations of ~ 40 ng.g⁻¹
42
43 348 in tanks B, ~ 70 ng.g⁻¹ in tanks C, and ~ 7,000 ng.g⁻¹ in tanks D. After 21 days of exposure,
44
45 349 similar Pt concentrations are measured for tanks B while slightly higher Pt concentrations
46
47 350 occurred in oyster faeces from tank C. Yet, at this sampling time, faeces mean Pt concentrations
48
49 351 in tanks D reached ~ 40,000 ng.g⁻¹. At the following sampling times, there was a slight decrease
50
51 352 in Pt concentrations in faeces from tanks B, no major change for tanks C, and a clear decrease
52
53 353 for those from tanks D. Accordingly, at the final sampling time (T = 35), faeces showed Pt
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55 354 concentrations of ~ 30 and 80 ng.g⁻¹ in tanks B and C respectively, and ~ 15,000 in tanks D
56
57 355 (Table 1).

58 356

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3.3. Histopathological alterations

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359 Histological observation of the digestive gland indicated the presence of some minor
360 histopathological alterations such as the presence of ciliated organisms, *Mytilicola intestinalis*
361 or the presence of necrotic tubules and haemocytic infiltrations. However, prevalence and
362 intensities were in general low and did not present a clear pattern regarding differences between
363 control and exposed groups. These results are in agreement with tissue level biomarkers (CTD
364 ratio and atrophy levels; Fig. S1a, b) for which no significant differences between experimental
365 groups occurred, but rather modest trends were observed. Overall, oysters exposed to higher Pt
366 concentrations and with longer periods of time presented slightly enhanced lumen of the
367 digestive tubules and bigger amount of connective tissue between the tubules (Fig. 3a, b). The
368 atrophy level at T = 0 was 1.93 ± 0.78 , while the highest level was recorded in group D after
369 28 days of exposure with a value of 2.68 ± 0.51 (Fig. S1a). Similarly, for CTD ratio no
370 significant differences occurred between groups and time but dose-response trends occurred
371 after 7 days of exposure with values ranging from 0.158 ± 0.05 in oysters from tanks A, to
372 0.181 ± 0.04 in the oysters from tanks D. At T = 21, CTD ratio varied from 0.175 ± 0.05 in
373 control individuals while it was of 0.199 ± 0.04 in tanks D (Fig. S1b).

374 Concerning histochemical measurements, lipofuscins were generally detected in the digestive
375 epithelium as dark spots (Fig. 3c, d). An increase in the lipofuscin content was observed in Pt
376 exposed oysters after 3 days of exposure while this increase is more important in oysters from
377 tanks C and D from T = 7 onwards (Fig. 4a) with values higher than control (tanks A) and tanks
378 B oysters. It should also be noted that even in control oysters, a modest increase of lipofuscin
379 content was measured after 28 days of exposure (Fig. 4a).

380 Neutral lipids in the digestive gland were observed as red deposits mainly located in the
381 interstitial connective tissue of the digestive gland of oysters (Fig. 3e, f). Quantification of
382 neutral lipid accumulation showed a significant decrease of lipids through time in oysters from
383 tanks C and D (Fig. 4b), with more marked differences at longest exposure periods.

384 Metals were detected as Black Silver Deposits (BSD) and observed in the gills, stomach,
385 intestine, haemocytes and in the digestive epithelium (Fig. 3g, h). Due to the lack of relevant
386 amount of gill, intestine and stomach tissue in many samples and the scattered distribution of
387 haemocytes, BSD volume density quantification was limited to the digestive epithelium. The
388 quantification of the volume density of BSD could only be performed in oysters from tanks C
389 and D but not in tanks A and T = 0, nor in tanks B due to very low accumulation (Fig. 4c). A
390 significant increase in the BSD was observed in oysters from tanks D compared to those from
391 tanks C and these differences increased through time. Differences mainly occurred in the basal

392 lamina at the beginning of the experiment and increased in digestive tubules with exposure
393 time.

3.4. IBR/n Index and correlation study

395 The IBR/n index showed different response patterns after 3, 7 and 28 days of exposure. After
396 3 days of exposure no clear trends were observed, with all groups presenting similar values in
397 studied responses (Fig. 5a, b). However, after 7 and 28 days of exposure, oysters from tanks C
398 and D showed higher IBR/n levels, this higher levels in the index are mainly related with
399 alterations at the cellular level (lipofuscin and neutral lipids contents) indicating some stress
400 situation in those oysters exposed to higher Pt concentrations (Fig. 5c - f).

401 Regarding correlations between chemical and biological parameters, since Pt organotropism
402 was measured in tanks D correlations were limited to this exposure condition. Similarly to the
403 IBR/n Index, clear association is observed between Pt accumulation in the digestive gland and
404 alterations at cellular level (Lipofuscin and Neutral Lipids) while lower correlation coefficients
405 were detected between Pt concentrations and tissue (CTD, Atrophy) and organism level (CI)
406 measurements (Table S1).

4. Discussion

4.1. Platinum accumulation and organ distribution in oysters

410 During the whole exposure period abiotic factors were closely monitored, showing: (i) constant
411 physical/chemical master variables (temperature, pH, salinity, dissolved oxygen concentration),
412 (ii) constant Condition Index, (iii) “natural”, constant dissolved Pt concentrations in control
413 tanks ($\sim 0.25 \text{ ng.L}^{-1}$), (iv) $\sim 100 \%$ of nominal spiked dissolved Pt concentrations retrieved in
414 exposure media after 1 h of exposure and $\sim 70 \%$ after 20 h (ensuring limited abiotic adsorption
415 or precipitation; Abdou et al., 2018). This setup supports the idea that the observed Pt
416 accumulation and related biomarker responses were due to direct Pt exposure of the test
417 organisms.

418 Initially, gonads had the lowest Pt load ($\sim 5 \%$), followed by digestive gland and digestive
419 gland + gonads ($\sim 8 - 10 \%$ each), muscle and mantle accounting each for $\sim 20 \%$ and gills with
420 the highest percentage ($\sim 40 \%$; Fig. 2). This distribution pattern in oysters from the Cantabrian
421 coast is somewhat different from that observed in an organotropism study in wild oysters from
422 the Gironde Estuary. In fact, in the Gironde oysters, Pt was evenly distributed between mantle,
423 gills and the visceral mass (including the digestive gland) accounting for $\sim 30 \%$ each. The

424 lowest contribution was reported for muscle (~ 10 %; Abdou et al., unpublished data). Although
1 425 Pt quantification in whole oyster individuals from the Cantabrian coast showed similar
2 426 concentrations ($0.24 \pm 0.07 \text{ ng.g}^{-1}$, $n = 10$) than those from the Gironde Estuary
3 427 ($0.33 \pm 0.03 \text{ ng.g}^{-1}$, pool of 5 individuals; Abdou et al., unpublished data), an apparent shift
4 428 towards relatively higher Pt content occur in the digestive gland of the Gironde oysters. This
5 429 observation is in line with major accumulation of Ag and Cu in wild oysters from the same site,
6 430 highlighting the importance of this organ in environmental metal exposure (Mikolaczyk et al.,
7 431 2016). This shift could be explained by differences between both sites in terms of Pt sources,
8 432 speciation and/or uptake pathway including a possible greater importance of the trophic
9 433 pathway in the Gironde Estuary.

17 434 An increasing trend of Pt concentrations in all organs, both replicate series giving similar
18 435 values, suggests continuous Pt accumulation in the test-organisms (Fig. 1). Interestingly, both
19 436 replicate series displayed higher variability in Pt concentrations between the pseudo-replicate
20 437 individuals from the same tank ($n = 5$) after longer exposure time. This result suggests that
21 438 inter-individual variability in Pt uptake and distribution may increase with exposure time. This
22 439 observation is in line with increasing inter-individual variability of several parameters at all
23 440 biological organization levels following exposure to pollutants including trace metals (e.g. Cd
24 441 exposure, Depledge and Lundebye, 1996). Such changes in variability of physiological
25 442 parameters may provide additional useful insights into chemical contamination impact, i.e.
26 443 serve as a tool to assess the severity of pollution effects on biota (Depledge and Lundebye,
27 444 1996), suggesting increasingly adverse effects of Pt exposure during the experiment. However,
28 445 these variabilities do not change the trend of linear accumulation of Pt in each of the different
29 446 organs studied (average determination coefficient $\sim 0.97 \pm 0.02$; Table 1). This reflects the
30 447 accumulation dynamics observed at the whole organism level in a separate set of oyster
31 448 individuals under the same experimental conditions (Abdou et al., 2018). Accordingly, the
32 449 obtained results suggest very efficient Pt uptake and accumulation by direct exposure pathway.
33 450 Accumulation trends close to linearity following Cd exposure were reported for *Corbicula*
34 451 *fluminea*, at both the whole organism level and at the organ level (gills, mantle, foot, and
35 452 visceral mass; Baudrimont et al., 1997). Linear accumulation over time occurs when excretion
36 453 of metal is negligible or slow compared to the uptake, leading to continuously increasing body
37 454 burdens during exposure (Langston and Bebianno, 1998). It is assumed that metal-binding
38 455 ligands do not become saturated or that the growth rate does not exceed the metal assimilation
39 456 rate, therefore steady-state may take a considerable time to be reached (Langston and Bebianno,
40 457 1998). In addition it should be considered that the present exposure design corresponds to a
41 458 semi-static experiment for which accumulation kinetics differ from static experiments (plateau
42 459 generally reached after a shorter period; Zimmermann and Sures, 2018).

460 The Pt distribution between organs, expressed as % Pt content with respect to total organism Pt
1 461 load, showed a clear shift between the initial conditions and after 3 days of exposure (Fig. 2).
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3 462 This shift is particularly important for gills, containing ~ 40 % of whole soft body Pt at T = 0
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5 463 and ~ 70 % from T = 3 to T = 35. Relative Pt content in mantle remains constant (~ 20 %)
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7 464 throughout the experiment. Both organs were constantly exposed to ambient Pt in seawater
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9 465 during the exposure periods and may have served as the tissues allowing Pt to enter into the
10 466 organism. Those results were compared to accumulation kinetics observed in freshwater
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12 467 organisms despite the significant differences with marine organisms regarding the osmotic
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14 468 situation which may affect the route of metal uptake and excretion. They appear consistent with
15 469 the observation that all tissues in fish *Barbus barbus*, exposed to ground automobile catalytic
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17 470 converter material (i.e. particles), showed a clear metal uptake with gills revealing the highest
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19 471 Pt content, although these authors could not exclude a possible artefact due to superficially
20 472 adsorbed catalyst particles on the gill filaments (Sures et al., 2005). Platinum organotropism in
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22 473 eels *Anguilla anguilla* exposed to PtCl₂ in tap water, did not indicate major accumulation in
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24 474 gills, but the following pattern of Pt distribution:
25 475 muscle = spleen < liver < gill < kidney < bile < intestine (Zimmermann et al., 2004). These
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27 476 results obtained in fish are not in line with findings in mollusks, such as the marine gastropod
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29 477 *Littorina littorea*, exposed to Pt through the aqueous phase, suggesting Pt ions uptake through
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31 478 gill epithelial cells by passive diffusion and subsequent transport to other tissues via
32 479 haemoplasm (Mulholland and Turner, 2011). In fact, in mollusks bivalves exposed to other
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34 480 elements through direct pathway, trace metal uptake generally occurs mainly via the gills, and
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36 481 to a lesser extent, via the mantle (e.g. *Mytilus edulis* exposed to Cd Marigómez et al., 2002).
37 482 Accordingly, Pt distribution between oyster organs as observed in the present work is in
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39 483 agreement with previous studies reporting that, in aquatic mollusks, gills constitute a key
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41 484 interface for dissolved metal uptake (Marigómez et al., 2002). This implies that Pt may be re-
42 485 distributed between organs, suggesting the possible active response of the organism following
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44 486 Pt exposure.

4.2. Platinum effects on oysters at different levels of biological organization

489 Tracing direct contamination in digestive gland from bivalves is possible since metal
490 redistribution by hemolymph occurs from the most exposed organs (gills) to less affected
491 tissues (Strady et al., 2011). Furthermore, the digestive gland is the main site for metabolic
492 activity since it is involved in immune defense, detoxification, and in homeostatic regulation of
493 the internal medium (Marigómez et al., 2002). This key organ is also strongly involved in
494 processes of detoxification and elimination of xenobiotics. Digestive gland consists of many

495 blind-ending tubules which are lined by a digestive epithelium constituted of digestive and
1 496 basophilic cells (Aarab et al., 2011). Histopathological alteration study was therefore focused
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3 497 on the digestive gland, taking in consideration comparison purposes with literature.
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5 498 In general, few differences were observed for tissue level parameters such as integrity of the
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7 499 digestive gland and digestive tubule atrophy. Yet, higher digestive tubule atrophy was recorded
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9 500 in oysters exposed to highest Pt levels after 28 days (Fig. 3a, b). This tissue alteration has
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11 501 previously been related to general stress conditions including food deprivation (Couch, 1984),
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13 502 and also exposure to pollutants including trace metals (e.g. exposure to Cd; Zaldibar et al.,
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15 503 2007). Although the obtained results did not indicate a clear tissue-level response, they suggest
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17 504 the onset of tissue impairments. In oysters *C.gigas* exposed to trace metals (Ag and Cu), the
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19 505 ratio between digestive and connective tissues (CTD) is a sensitive biomarker increasing
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21 506 together with atrophy of digestive tubules (Rementeria et al., 2016). Other histopathological
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23 507 alterations such as haemocytic infiltration occurred after Ag and Cu exposure and were clearly
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25 508 related to trace metal accumulation. Non-significant results obtained after 28 days of Pt
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27 509 exposure suggest that longer exposure time may be required in order to assess the effects of Pt
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29 510 at the tissue-level.

30 511 In contrast, cellular level responses indicated more marked alterations. Lipofuscins are mainly
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32 512 produced due to lipid peroxidation; originating from poly-saturated fatty acids oxidation by
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34 513 Reactive Oxygen Species (ROS); and are accumulated in lysosomes as insoluble lipoprotein
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36 514 granules (Viarengo and Nott, 1993). Oysters exposed to 100 ng.L⁻¹ and 10,000 ng.L⁻¹ Pt (tanks
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38 515 C and D) showed significantly higher lipofuscin contents in the digestive epithelium, compared
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40 516 to control conditions after only 7 days of exposure (Fig. 4a) and this trend was maintained
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42 517 through exposure time (Fig. 3d). Lipofuscin accumulation throughout the experiment can be
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44 518 attributed to Pt exposure that may increase the presence of ROS in the digestive gland cells.
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46 519 Similar alterations were already described in bivalves exposed to metals (e.g. Marigómez et al.,
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48 520 2013; Rementeria et al., 2016).

49 521 In marine bivalves, neutral lipids contribute to energy resources and help in the gamete
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51 522 development during maturation. Previous studies have indicated that exposure to pollutants
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53 523 (including metals) can lead to a depletion of such reserves in freshwater mussels (e.g. Guerlet
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55 524 et al., 2007; Zorita et al., 2006), and more specifically in oyster *C. gigas* (Séguin et al., 2016).
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57 525 Results of the present study are in agreement with previous works showing a significantly
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59 526 decreasing trend in the accumulation of neutral lipids through time and with increasing
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61 527 exposure concentration. Relatively constant levels of neutral lipids along with conserved tissue
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63 528 integrity in oysters from control tanks reveal that food supply was sufficient for maintenance
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65 529 throughout the exposure period (Rementeria et al., 2016). However, significant differences

530 between tanks C and D oysters, and oysters from tanks B and control occurred only from T = 28
531 (Fig. 3e, f; Fig. 4b). Accordingly, we can state that similarly to lipofuscin content, Pt has an
532 effect on neutral lipid content for oysters exposed to 100 ng.L⁻¹ and 10,000 ng.L⁻¹ Pt, while no
533 significant changes occurred for lowest exposure conditions. Consequently, one may assume
534 that Pt contamination as observed in these oysters could deplete energy reserves that were
535 initially intended to growth and which should be allocated to defense mechanisms under
536 stressful conditions.

537 Black Silver Deposits reveal softly bound metals localized within cellular compartments
538 (Danscher, 1991; Soto et al., 2002). The presence of BSD in the basal layer of the digestive
539 epithelium, a typical zone of metal accumulation (Rodriguez-Iruretagoiena et al., 2016) and in
540 the digestive cells of the epithelium increased with exposure time and -concentration.
541 Furthermore, lysosomes in the digestive cells have been previously identified as target sites for
542 many contaminants (e.g. Cajaraville et al., 1995; Izagirre and Marigómez, 2009). Quantification
543 of BSD in the digestive cells revealed increased intracellular metal levels in Pt exposed groups
544 with concentrations and -time, with significant differences. Although specific works have not
545 been performed in order to identify the presence of Pt bound to the BSD in the digestive
546 lysosomes, it is conceivable that the accumulation of BSD in the digestive cells is related to the
547 presence of metallothioneins that have sequestered Pt ions in the cell cytoplasm and further
548 transferred them to the lysosomes, in many cases together with lipofuscins (Soto et al., 2002).

549 Overall, the observed responses at the cellular levels reveal a clear toxicological impact of Pt
550 exposure under the present experimental conditions, especially showing significant effects in
551 oysters from tanks D. In these oysters increasing Pt accumulation occurred with a linear pattern,
552 suggesting that oysters are continuously accumulating Pt together with the activation of
553 detoxification mechanisms (intralysosomal metal accumulation, faeces excretion). Platinum
554 exposure induced oxidation reactions as revealed by the increase in lipofuscin content. These
555 organisms also show accumulation of Pt in lysosomes of digestive cells and supposedly
556 intracellular digestion and detoxification (Izagirre and Marigómez, 2009). Activation of these
557 mechanisms is also supported by relatively high Pt contents in faeces of exposed oysters at the
558 different sampling times showing a similar trend in oysters from both, tanks B and C. In fact,
559 average Pt concentration in faeces from oysters exposed to 50 ng.L⁻¹ Pt are of ~ 40 ng.g⁻¹, while
560 it is about twice this value in oysters exposed to 100 ng.L⁻¹ at T = 7. Those proportions stay
561 approximately constant over the other sampling times, except from a small increase at T = 21.
562 Proportions are also kept in faeces content from oysters exposed to 10,000 ng.L⁻¹ at T = 3 while
563 a clear increase occurred at T = 21, which might suggest intensified excretion mechanism
564 facing the high exposure level. No adsorption of Pt from seawater on faeces can occur outside

565 the oyster body, since faeces recovery was performed during/after feeding in uncontaminated
1 566 seawater. This observation clearly suggests that the contamination of faeces by Pt occurs inside
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3 567 the oyster body, either through adsorption of dissolved Pt from seawater in the digestive tract
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5 568 or through active excretion of Pt by digestive gland organelles (e.g. residual bodies) as a
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7 569 detoxification mechanism (similar to Cd exposure, Strady et al., 2011). Mulholland and Turner
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9 570 (2011) studied Pt concentrations in *L. littorea* exposed to Pt-contaminated food source: *Ulva*
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11 571 *lactuca*. Platinum concentrations in faeces of the marine gastropod were higher than those
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13 572 observed in the macroalgae. These authors suggest that either (i) components of ingested food
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15 573 with relatively low Pt concentration are preferentially digested and/or assimilated by *L. littorea*,
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17 574 or (ii) aqueous Pt adsorbs to faecal material transiting the gut or following egestion (Mulholland
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19 575 and Turner, 2011). Considering our results on cellular Pt toxicology, demonstrating the
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21 576 activation of digestive gland detoxification mechanisms, one may hypothesize that Pt in faeces
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23 577 rather originates from active metal excretion than from passive adsorption, at least in oysters.
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25 578 Decreasing Pt values in faeces of oysters from tanks D at the end of the experiment could
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27 579 suggest the exhaustion of excretion mechanism, although other detoxification mechanisms are
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29 580 still active at least until T = 28. This is supported by the fact that oyster tissue accumulation did
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31 581 not reach a steady state at the end of the experiment, contrary to oysters exposed to lower,
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33 582 environmentally relevant concentrations (Abdou et al., 2018).

31 583 The Index of Biological Response (IBR) has been successfully applied to pollution sentinel
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33 584 bivalve species in order to assess their health status (Brooks et al., 2011; Cravo et al., 2012;
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35 585 Garmendia et al., 2011; Rementeria et al., 2016). Presently, the overall response pattern
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37 586 suggests clear cell level alterations (lipofuscin and neutral lipid accumulation) after only 7 days
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39 587 of exposure. However, no clear increase in tissue-level biomarkers occurred at T = 28, except
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41 588 for the atrophy in oysters from tanks D. These results suggest that the overall health status of
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43 589 oysters is not affected at low levels of exposure (tanks B, 50 ng.L⁻¹ Pt), but that some alterations
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45 590 occur at higher Pt exposure concentrations. These alterations seem to be limited to the cellular
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47 591 level, without significant deleterious effects at tissue (no significant histopathological
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49 592 alterations) or organism (constant Condition Index) levels. However, the decrease of tissue
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51 593 integrity and increased atrophy observed for higher Pt exposure concentration indicate that Pt
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53 594 accumulation may impact the health status of oysters.

52 595 In marine organisms, previous studies on Pt contamination mainly focused on aquatic plants
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54 596 such as the marine macroalgae *Ulva lactuca* or microalgae *Chlorella stigmatophora*
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56 597 investigating Pt internalization and extra-cellular accumulation or seawater removal kinetics
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58 598 (Cosden et al., 2003; Shams et al., 2014; Turner et al., 2007). To the best of our knowledge,
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60 599 only two laboratory studies report on inorganic Pt accumulation by marine animals, one being
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600 the polychaete *Arenicola marina* exposed to Pt in a series of microcosms (French and Turner,
601 2008), and the second being the study of Mulholland and Turner (2011) exposing Pt to the
602 marine gastropod *Littorina littorea* through direct and trophic pathways. There is therefore a
603 crucial need for more studies on marine organisms, yet, taking into account several
604 recommendations to apply in exposure studies as summarized in Zimmermann and Sures
605 (2018).

606 In nature, Pt sources may be diverse and may occur together at the same time. Accordingly,
607 regarding autocatalyst-derived Pt emissions, a recent study proved that the contact with
608 stormwater causes the release of a relatively small fraction (0.2 – 18 %, with 3.3 % on average)
609 of the total Pt present in the road dust under the form of nanoparticles (Folens et al., 2018).
610 These authors estimate that, although representing only a minor fraction of the total content in
611 road dust, the nanoparticulate Pt leachate is most susceptible to biological uptake and hence
612 most relevant in terms of bioavailability. Additionally, apart from inorganic Pt sources, Pt
613 contamination may occur under organic forms due to the presence of Pt-based anticancer drugs,
614 originating especially from hospital effluents (e.g. Kümmerer et al., 1999; Vyas et al., 2014).
615 This Pt source may pose adverse effects on aquatic organisms, including polychaete *Nereis*
616 *diversicolor* (Fonseca et al., 2017) or marine mussels *Mytilus galloprovincialis* (Trombini et
617 al., 2016). Furthermore, environmental Pt contamination may co-occur with other
618 contamination including other Platinum Group Elements (PGE) for which a synergistic toxicity
619 is observed following exposure to the model aquatic organism *Daphnia magna* (binary metal
620 exposure to Pt and Pd; Zimmermann et al., 2017). Further work considering exposure to
621 different Pt species, to interacting PGE and related responses is therefore required.

623 5. Conclusions

624 Overall, this exposure study provides more knowledge on Pt toxicology in marine organisms.
625 Regarding metal organ distribution, similar features compared to freshwater organism studies
626 were observed. Effects of Pt exposure occurred from individual down to molecular levels. The
627 interpretation of biomarkers at different biological organization levels (IBR) suggests the
628 occurrence of a cascade of responses (oxidative stress, intralysosomal metal accumulation, and
629 lipid depletion) and enhanced tissue alterations. Increasing Pt contents in faeces along with Pt
630 exposure levels suggests effective excretion of accumulated Pt but dose-related linear
631 accumulation in tissues and whole organisms over time suggests that excretion of metal is
632 relatively slow compared to the uptake. Although the present work provides clear evidences of
633 adverse effects in oysters exposed to elevated Pt levels, non-significant histopathological
634 observations for the lowest exposure concentration i.e. for the most environmentally relevant

635 level is a valuable information. In fact, generally lower Pt levels are recorded in coastal
1 636 seawater. However, it should be considered that Pt inputs may occur from several sources and
2 637 under different forms (dissolved, ground catalyst particles, nanoparticles, organic forms...),
3 638 which may reach organisms through direct or trophic pathways.
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7 639 Biomarkers, reflecting the impact of all stressors to which organisms are exposed, provide
8 640 direct evidence of pollution in surrounding environment (Capela et al., 2016), and may be used
9 641 as “early” warning signals to anticipate potential impacts at higher levels of biological
10 642 organization (Allan et al., 2006). Although biomarkers are already part of biological and
11 643 chemical monitoring requirements in the scope of the Marine Strategy Framework Directive
12 644 2008/56/EC (MSFD), their potential responses to emerging metallic contaminants, such as Pt
13 645 and other TCEs are widely unknown. The present work contributes to improving our
14 646 understanding of biomarker responses to Pt exposure as integrative tools between chemicals
15 647 monitoring and their impacts on aquatic organisms.
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911 List of figures:

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4 913 **Fig. 1** Platinum concentrations evolution in the different organs of oyster exposed to
5 914 10,000 ng.L⁻¹. Organ average Pt concentrations (n = 5, dry weight) for each replicate series of
6 915 the tanks D (replicate 1: full symbols, replicate 2: empty symbols). Cross symbols represent
7 916 initial Pt concentrations in organs from oysters at T = 0 (i.e. non-exposed individuals; n = 5)
8 917 for each organ. Note the discontinued scale of the time axis. Error bars represent standard
9 918 deviation (SD) and asterisks (*) significant difference from the previous sampling time.

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14 920 **Fig. 2** Evolution of Pt distribution between the different organs. Average Pt percentage per
15 921 organ (from both replicates; n = 10) with respect to total organism Pt content. Error bars
16 922 represent standard deviation (SD).

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20 924 **Fig. 3** Microscopic observations of the digestive gland of oysters from initial condition T = 0
21 925 (A), and control tanks A (G), exposed organisms at T = 28 for tanks B (C and E) and tanks D
22 926 (B, D, F and H). Hematoxylin-Eosin staining (A and B), Lipofuscin Schmorl staining (C and
23 927 D), ORO staining (E and F) and autometallographical staining (G and H), (LF) Lipofuscin;
24 928 (NL) Neutral Lipids, (BSD) Black Silver Deposits in the Basal Layer (BL), epithelium of the
25 929 digestive tract (E) and hemocytes (H). Arrows indicate the presence of neutral lipids as red
26 930 precipitates (E and F) and metals as Black Silver Deposits (G and H).

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31 932 **Fig. 4** Lipofuscin contents in digestive cells (A), Neutral lipid accumulation (B) and
32 933 Intralysosomal metal accumulation (VvBSD) (C) in the digestive gland of oysters from the
33 934 different tanks after 3, 7 and 28 days of exposure. The horizontal line indicates values at T = 0
34 935 and dashed lines indicate the standard deviations T = 0. Letters on top of the bars indicate
35 936 significant differences between groups at the same exposure-time (p < 0.05), BDL: below
36 937 detection limit.

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41 939 **Fig. 5** Integrative Biological Response Index (IBR) star plots and IBR/n values assessed at
42 940 T = 3 (a and b respectively) T = 7 (c and d respectively) and T = 28 (e and f respectively); CI:
43 941 Condition Index, ORO: Oil Red O, CTD: Connective-to-diverticula ratio.

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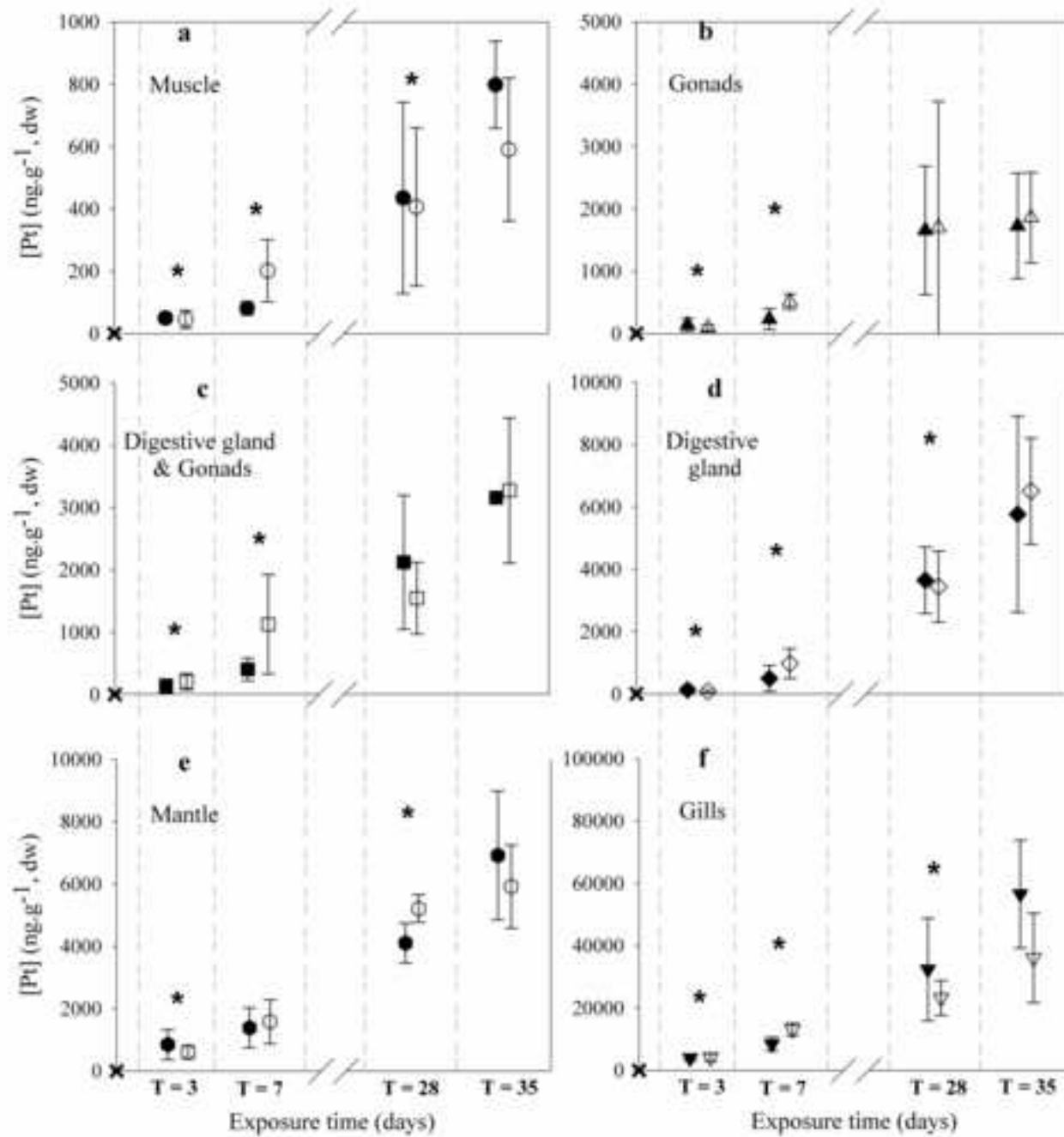
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943 List of tables:

944 **Table 1** Platinum concentrations in the different oyster organs (mean \pm SD) exposed to
945 10,000 ng.L⁻¹ Pt and in faeces from all conditions. Mean Pt concentrations (ng.g⁻¹, dry weight)
946 in organs of oysters from both replicate series of tanks D (n = 10); platinum concentrations
947 (ng.g⁻¹, dry weight) quantified in oyster faeces from each replicate series of tanks D (replicate
948 1 – replicate 2). *n.d.*: not determined; R²: determination coefficients determined from linear
949 regressions of time-dependent Pt concentration increase in each organ.

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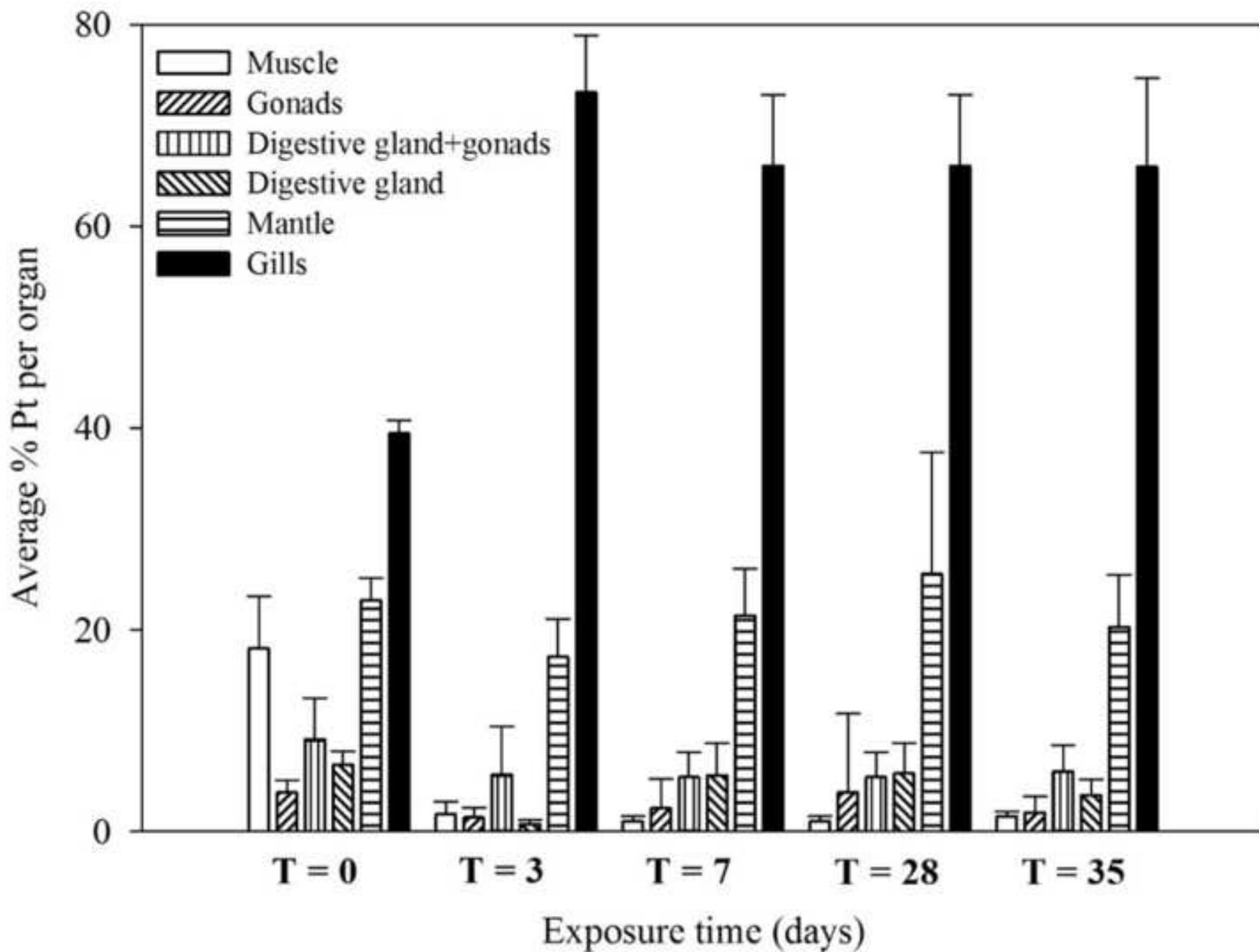
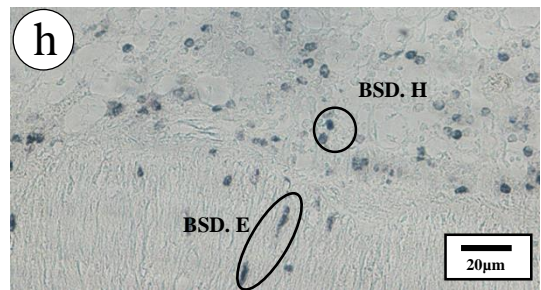
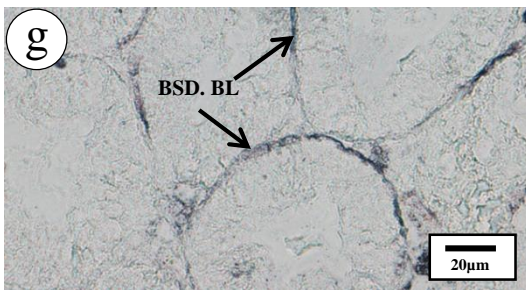
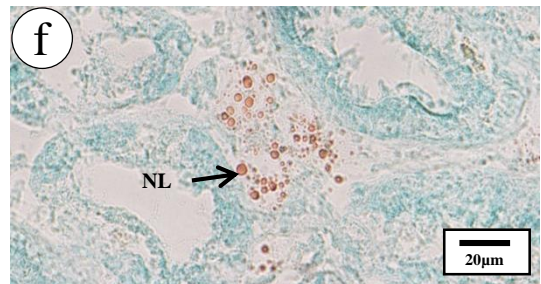
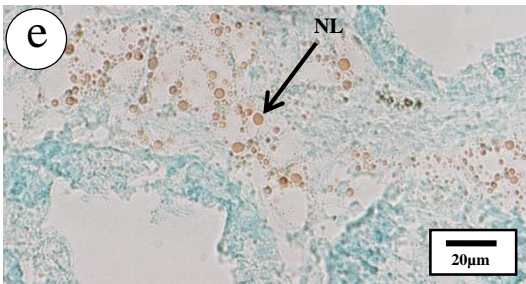
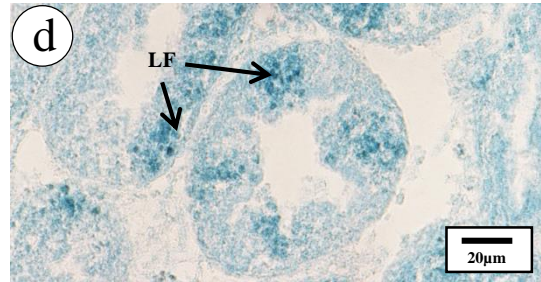
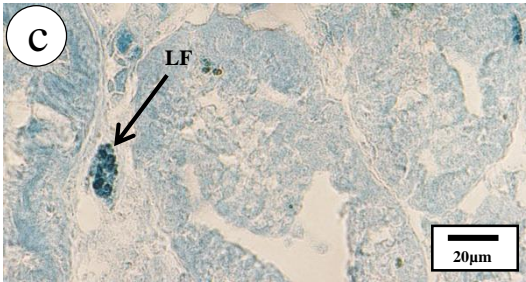
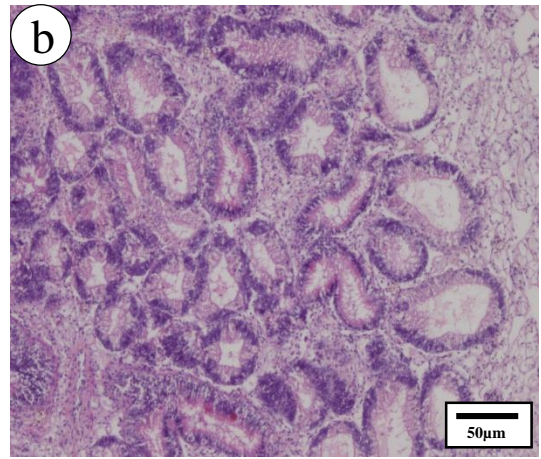
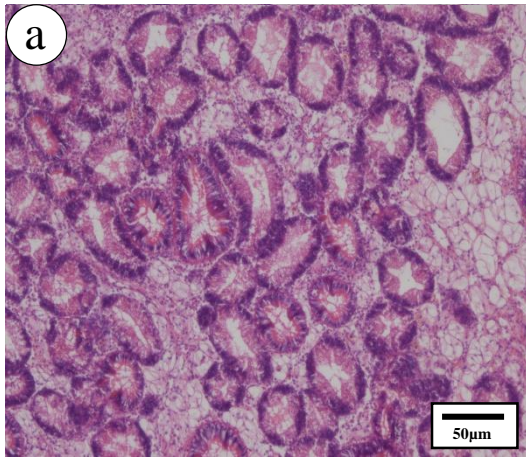
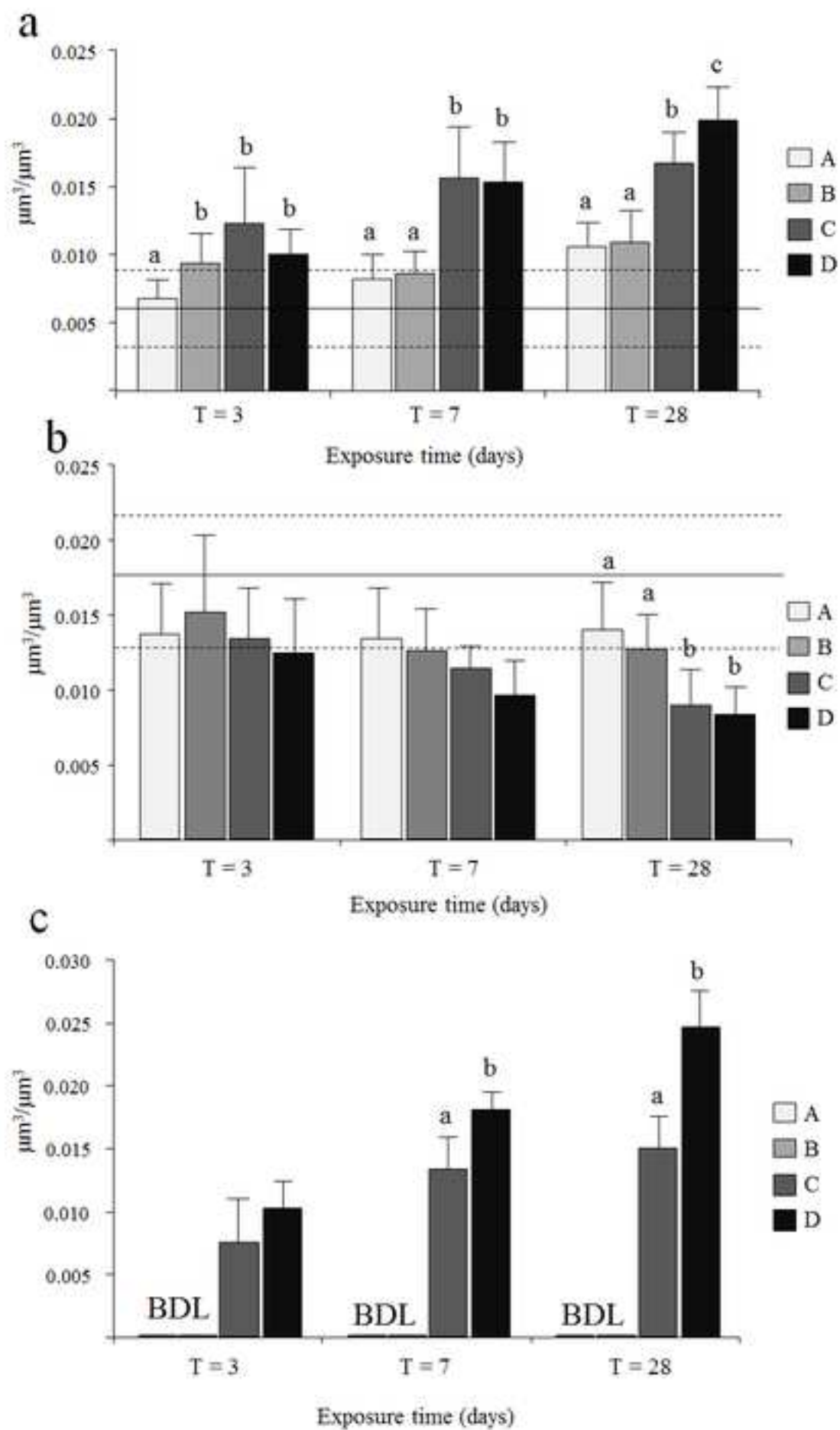


Fig. 3 Abdou et al.





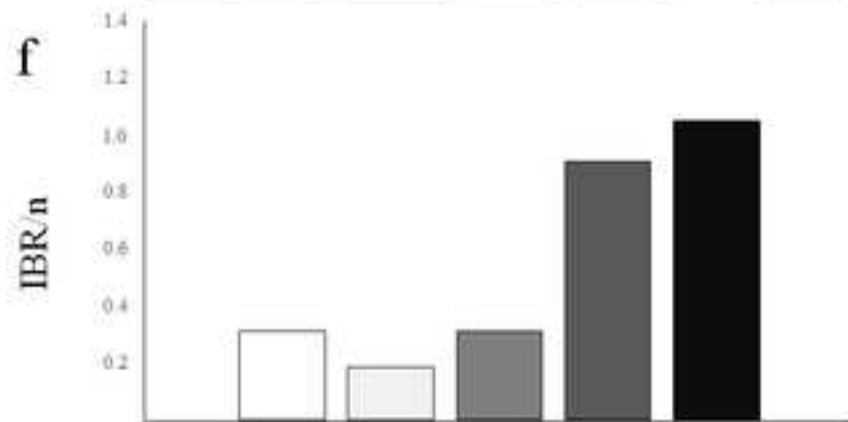
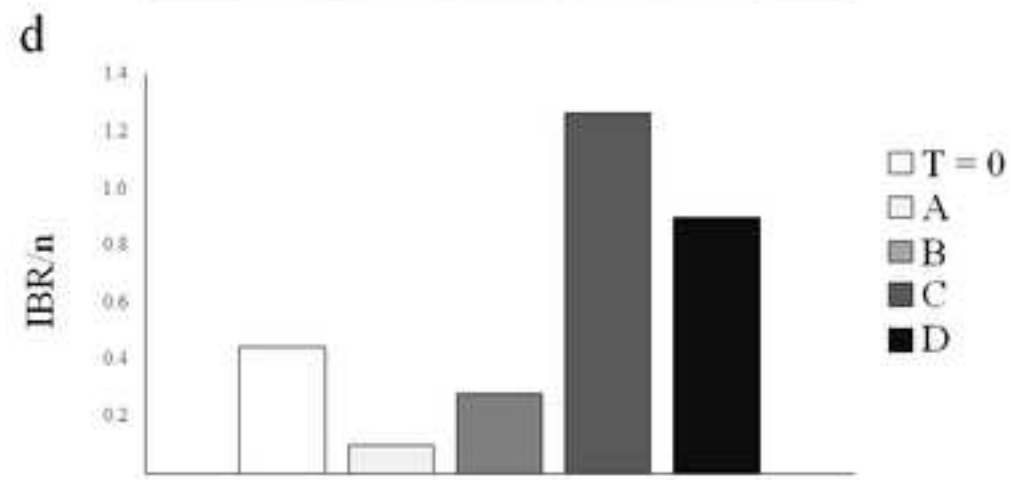
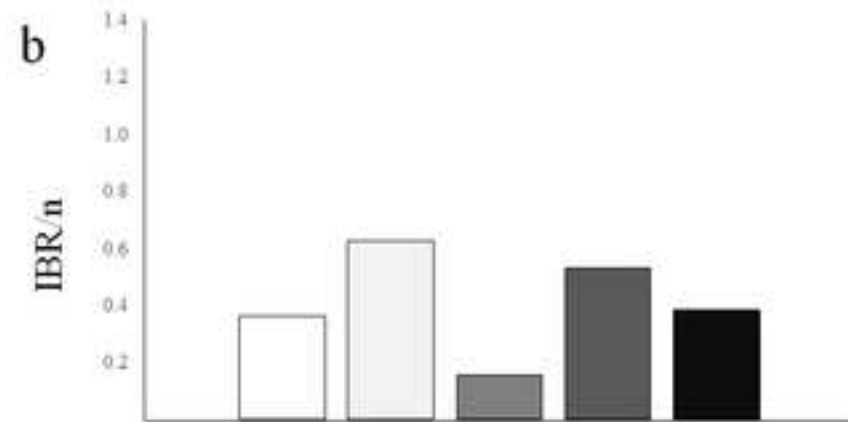
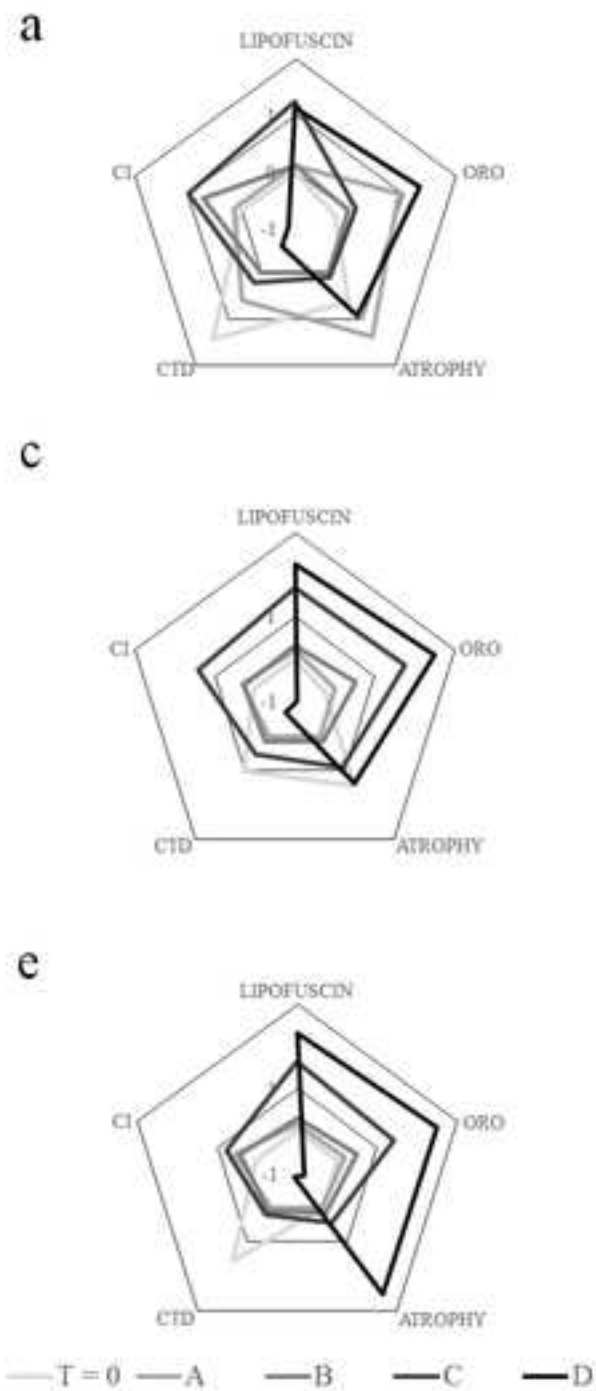


Table 1 Platinum concentrations in the different oyster organs (mean \pm SD) exposed to 10,000 ng.L⁻¹ Pt and in faeces from all conditions. Mean Pt concentrations (ng.g⁻¹, dry weight) in organs of oysters from both replicate series of tanks D (n = 10); platinum concentrations (ng.g⁻¹, dry weight) quantified in oyster faeces from each replicate series of tanks D (replicate 1 – replicate 2). *n.d.*: not determined; R²: determination coefficients determined from linear regressions of time-dependent Pt concentration increase in each organ.

	T = 0	T = 3	T = 7	T = 21	T = 28	T = 35	R²
Muscle	0.289 \pm 0.127	48.1 \pm 23.2	141 \pm 93.9	<i>n.d.</i>	421 \pm 282	683 \pm 220	0.967
Gonads	0.090 \pm 0.030	117 \pm 82	372 \pm 204	<i>n.d.</i>	1,680 \pm 1,610	1,800 \pm 781	0.988
Digestive gland + gonads	0.083 \pm 0.027	184 \pm 128	766 \pm 681	<i>n.d.</i>	1,870 \pm 933	4,090 \pm 2,280	0.948
Digestive gland	0.341 \pm 0.053	113 \pm 100	712 \pm 503	<i>n.d.</i>	3,540 \pm 1,110	6,460 \pm 2,500	0.960
Mantle	0.313 \pm 0.027	731 \pm 388	1,490 \pm 681	<i>n.d.</i>	4,660 \pm 781	6,350 \pm 1,760	0.993
Gills	0.677 \pm 0.006	4,090 \pm 1,120	10,800 \pm 3,140	<i>n.d.</i>	28,300 \pm 13,600	45,200 \pm 18,700	0.961
Faeces A1- A2	<i>n.d.</i>	<i>n.d.</i>	< 0.285 - 3.25	0.498 - 3.59	<i>n.d.</i>	1.63 - <i>n.d.</i>	
Faeces B1- B2	<i>n.d.</i>	<i>n.d.</i>	27.4 – 51.4	33.2 - 55.7	23.4 – 34.5	14.3 - 44.0	
Faeces C1- C2	<i>n.d.</i>	<i>n.d.</i>	77.1 - 58.6	82.0 - 140	49.3 – 91.3	61.7 - 90.9	
Faeces D1- D2	<i>n.d.</i>	<i>n.d.</i>	7,080 – 7,850	44,000 - 38,400	<i>n.d.</i>	10,000 – 20,400	



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Supplementary Material

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