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Abstract

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

Keywords: Platinum; Emerging Contaminant; Isotopes; Marine Bivalve; Biomarker; Ecotoxicology

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1. Introduction

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

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

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

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

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

2. Material and Methods

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

2.1. Experimental setup

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

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

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

Before starting Pt exposure, we isolated and dissected twenty-five oysters which constitute the T = 0 (initial conditions) for the targeted parameters. Thereafter, we performed an even distribution of the 520 individuals in eight 45 L polypropylene (PP) tanks (65 individuals per tank) which were at first filled with 40 L aerated seawater. The experimental room was temperature-controlled (17 °C) and equipped with an artificial photoperiod set up on a 12 h : 12 h light : dark cycle. Chemical and physical parameters namely temperature, pH, salinity, and dissolved O_2 concentrations were measured daily during the experiment using a TetraCon 96® and a Sentix ® 41 probes (PROFILINE, WTW®). On a daily basis and after mortality checking, oysters were rinsed, fed and transferred in clean separate tanks while exposure tanks were rinsed, filled up with clean seawater and spiked to nominal Pt concentrations using ^{194}Pt stock solution. After 4 h feeding and spike equilibration, oysters were transferred again in the control and exposure tanks. Seawater, spike, and food volumes were adapted according to the number of oyster organisms left after each dissection in order to maintain exposure and feeding conditions constant by individual. Respective proportions per individual per day are reported in Abdou et al., 2018.

2.2. Sampling protocols

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

For organotropism analyses, organs (gills, digestive gland, gonads, muscle, and mantle) were dissected and placed separately in acid-cleaned (10 % HNO_3; 65 % Honeywell), PP tubes (DigiTUBEs®, SCP SCIENCE). Gonads could not be systematically isolated from digestive glands. Therefore, pieces of pure digestive gland and the remaining digestive gland and gonads were mineralized and analyzed separately. Wet weight of each organ was quantified (approximate weight for gonads and digestive gland). In addition, each valve was weighted and measured (thickness, width, and length), allowing the calculation of the Condition Index (CI) calculated for each oyster according to the equation (1) from Strady et al., (2011):

CI = Flesh weight (wet weight; g) / Shell weight (wet weight; g) x 100. (1)

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

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

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

2.3. Analytical procedure

2.3.1. Quantification of Pt concentrations

Organ tissues were prepared for Pt quantification by ICP-MS as described in Abdou et al., 2018. The steps consisted in ashing (800 °C) followed by acid digestion using *aqua regia* (3 h at 110°C). Same procedure (without the ashing step) was applied to faeces collected on Teflon filters. We applied the standard addition method (mono-elementary Pt standard solution 1,000 $\mu g.mL^{-1}$ PLASMACAL, SCP Science). Spectral interference (hafnium-oxygen species) corrections were applied on the ^{194}Pt and ^{195}Pt isotopes as detailed in Abdou et al. (2018). According to an inter-method comparison between ICP-MS and Adsorptive Cathodic Stripping Voltammetry (AdCSV) methods, sufficient sample mass (i.e. > 0.25 g dry weight, 0.13 ng Pt) is necessary to reliably measure Pt concentrations in natural oysters (not exposed to Pt spikes) by quadrupole ICP-MS (Abdou et al., 2018). As for organotropism measurements, organ masses were generally too low (e.g. ~ 0.1 g for digestive gland) to allow for ICP-MS analyses in non-exposed oysters, these samples were analyzed by AdCSV as described in Abdou et al., (2018). Briefly, after ashing, samples were acid-digested using *aqua regia* (1 h at 195 °C), followed by an evaporation step and dissolution with sulfuric acid (H_2SO_4). Samples are thereafter evaporated again and diluted with hydrochloric acid (HCl). Platinum voltammetric quantification was performed using a μAutolab Type III potentiostat (Metrohm® Autolab B.V.) connected to a polarographic stand (Metrohm® 663 V.A.) and controlled using the NOVA 2.1 software. Aliquots of acid-digested sample were added to the voltammetric cell, together with formaldehyde and hydrazine sulfate as well as H_2SO_4 and concentrations of Pt were quantified applying the standard addition method and considering the second derivative of the voltammogram (Cobelo-García et al., 2014b). "Reconstituted" whole organism Pt concentrations have been estimated from Pt values in the different organs and considering the respective organ masses.

2.3.2. Histopathological alterations

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

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

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

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

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

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

2.3.3. Histochemical analyses

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

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

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

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

2.4. Integrative Biological Response (IBR/n) index

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

2.5. Quality control and statistics

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

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

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

test were applied to non-parametric data. Pearson´s correlations between Pt concentration and 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 (R^2) 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 %.

After exposure, gills generally showed the highest Pt concentrations followed by the mantle. At all exposure times, significant differences occurred between gills and the other organs as well as between mantle and the other organs. Platinum concentrations in exposed oysters followed the general trend: gills > mantle > digestive gland ~ digestive gland + gonad > gonads > muscle (Table 1). Compared to estimated whole organism concentrations, gills reached the highest percentage of Pt of up to 70 % at all times (Fig. 2). Platinum percentages in muscle dropped from ~ 20 % to ~ 3 % and remained stable. The percentages of all other organs showed only minor variations compared to initial conditions and throughout the exposure period. The lowest Pt contribution occurred in digestive gland at T = 3 with only ~ 0.5 %. The comparison of "reconstituted" whole organism Pt concentrations with those measured in whole individuals from the same experiment, analyzed without organ dissection (Abdou et al., 2018) showed overall good fit of both datasets (less than 20 % difference), confirming the previously observed linear trend in Pt accumulation with high coefficients of determination for whole oysters from tanks D1 ($R^2 = 0.94$) and D2 ($R^2 = 0.97$).

3.2. Platinum concentrations in faeces

Platinum concentrations in faeces were monitored through the exposure period. Control tanks showed very low, similar Pt concentrations along the experiment (from < 0.285 to 3.59 $ng.g^{-1}$; Table 1). Faeces from oysters exposed to Pt showed clearly higher Pt concentrations, reflecting the order of exposure levels in the different tanks and generally the proportions of exposure concentrations. Values between replicate series were similar. Faeces did not display a clear trend over time. In fact, at T = 7 oysters faeces showed mean Pt concentrations of ~ 40 $ng.g^{-1}$ in tanks B, ~ 70 $ng.g^{-1}$ in tanks C, and ~ 7,000 $ng.g^{-1}$ in tanks D. After 21 days of exposure, similar Pt concentrations are measured for tanks B while slightly higher Pt concentrations occurred in oyster faeces from tank C. Yet, at this sampling time, faeces mean Pt concentrations in tanks D reached ~ 40,000 $ng.g^{-1}$. At the following sampling times, there was a slight decrease in Pt concentrations in faeces from tanks B, no major change for tanks C, and a clear decrease for those from tanks D. Accordingly, at the final sampling time (T = 35), faeces showed Pt concentrations of ~ 30 and 80 $ng.g^{-1}$ in tanks B and C respectively, and ~ 15,000 in tanks D (Table 1).

3.3. Histopathological alterations

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

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

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

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

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

3.4. IBR/n Index and correlation study

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

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

4. Discussion

4.1. Platinum accumulation and organ distribution in oysters

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

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

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

An increasing trend of Pt concentrations in all organs, both replicate series giving similar values, suggests continuous Pt accumulation in the test-organisms (Fig. 1). Interestingly, both replicate series displayed higher variability in Pt concentrations between the pseudo-replicate individuals from the same tank (n = 5) after longer exposure time. This result suggests that inter-individual variability in Pt uptake and distribution may increase with exposure time. This observation is in line with increasing inter-individual variability of several parameters at all biological organization levels following exposure to pollutants including trace metals (e.g. Cd exposure, Depledge and Lundebye, 1996). Such changes in variability of physiological parameters may provide additional useful insights into chemical contamination impact, i.e. serve as a tool to assess the severity of pollution effects on biota (Depledge and Lundebye, 1996), suggesting increasingly adverse effects of Pt exposure during the experiment. However, these variabilities do not change the trend of linear accumulation of Pt in each of the different organs studied (average determination coefficient ~ 0.97 ± 0.02; Table 1). This reflects the accumulation dynamics observed at the whole organism level in a separate set of oyster individuals under the same experimental conditions (Abdou et al., 2018). Accordingly, the obtained results suggest very efficient Pt uptake and accumulation by direct exposure pathway. Accumulation trends close to linearity following Cd exposure were reported for *Corbicula fluminea,* at both the whole organism level and at the organ level (gills, mantle, foot, and visceral mass; Baudrimont et al., 1997). Linear accumulation over time occurs when excretion of metal is negligible or slow compared to the uptake, leading to continuously increasing body burdens during exposure (Langston and Bebianno, 1998). It is assumed that metal-binding ligands do not become saturated or that the growth rate does not exceed the metal assimilation rate, therefore steady-state may take a considerable time to be reached (Langston and Bebianno, 1998). In addition it should be considered that the present exposure design corresponds to a semi-static experiment for which accumulation kinetics differ from static experiments (plateau generally reached after a shorter period; Zimmermann and Sures, 2018).

The Pt distribution between organs, expressed as % Pt content with respect to total organism Pt load, showed a clear shift between the initial conditions and after 3 days of exposure (Fig. 2). This shift is particularly important for gills, containing ~ 40 % of whole soft body Pt at T = 0 and ~ 70 % from T = 3 to T = 35. Relative Pt content in mantle remains constant (~ 20 %) throughout the experiment. Both organs were constantly exposed to ambient Pt in seawater during the exposure periods and may have served as the tissues allowing Pt to enter into the organism. Those results were compared to accumulation kinetics observed in freshwater organisms despite the significant differences with marine organisms regarding the osmotic situation which may affect the route of metal uptake and excretion. They appear consistent with the observation that all tissues in fish *Barbus barbus,* exposed to ground automobile catalytic converter material (i.e. particles), showed a clear metal uptake with gills revealing the highest Pt content, although these authors could not exclude a possible artefact due to superficially adsorbed catalyst particles on the gill filaments (Sures et al., 2005). Platinum organotropism in eels *Anguilla anguilla* exposed to $PtCl_2$ in tap water, did not indicate major accumulation in gills, but the following pattern of Pt distribution: muscle = spleen < liver < gill < kidney < bile < intestine (Zimmermann et al., 2004). These results obtained in fish are not in line with findings in mollusks, such as the marine gastropod *Littorina littorea,* exposed to Pt through the aqueous phase, suggesting Pt ions uptake through gill epithelial cells by passive diffusion and subsequent transport to other tissues via haemoplymph (Mulholland and Turner, 2011). In fact, in mollusks bivalves exposed to other elements through direct pathway, trace metal uptake generally occurs mainly via the gills, and to a lesser extent, via the mantle (e.g. *Mytilus edulis* exposed to Cd Marigómez et al., 2002). Accordingly, Pt distribution between oyster organs as observed in the present work is in agreement with previous studies reporting that, in aquatic mollusks, gills constitute a key interface for dissolved metal uptake (Marigómez et al., 2002). This implies that Pt may be re-distributed between organs, suggesting the possible active response of the organism following Pt exposure.

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

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

blind-ending tubules which are lined by a digestive epithelium constituted of digestive and basophilic cells (Aarab et al., 2011). Histopathological alteration study was therefore focused on the digestive gland, taking in consideration comparison purposes with literature.

In general, few differences were observed for tissue level parameters such as integrity of the digestive gland and digestive tubule atrophy. Yet, higher digestive tubule atrophy was recorded in oysters exposed to highest Pt levels after 28 days (Fig. 3a, b). This tissue alteration has previously been related to general stress conditions including food deprivation (Couch, 1984), and also exposure to pollutants including trace metals (e.g. exposure to Cd; Zaldibar et al., 2007). Although the obtained results did not indicate a clear tissue-level response, they suggest the onset of tissue impairments. In oysters *C.gigas* exposed to trace metals (Ag and Cu), the ratio between digestive and connective tissues (CTD) is a sensitive biomarker increasing together with atrophy of digestive tubules (Rementeria et al., 2016). Other histopathological alterations such as haemocytic infiltration occurred after Ag and Cu exposure and were clearly related to trace metal accumulation. Non-significant results obtained after 28 days of Pt exposure suggest that longer exposure time may be required in order to assess the effects of Pt at the tissue-level.

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

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

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

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

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

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

The Index of Biological Response (IBR) has been successfully applied to pollution sentinel bivalve species in order to assess their health status (Brooks et al., 2011; Cravo et al., 2012; Garmendia et al., 2011; Rementeria et al., 2016). Presently, the overall response pattern suggests clear cell level alterations (lipofuscin and neutral lipid accumulation) after only 7 days of exposure. However, no clear increase in tissue-level biomarkers occurred at T = 28, except for the atrophy in oysters from tanks D. These results suggest that the overall health status of oysters is not affected at low levels of exposure (tanks B, 50 $ng.L^{-1}$ Pt), but that some alterations occur at higher Pt exposure concentrations. These alterations seem to be limited to the cellular level, without significant deleterious effects at tissue (no significant histopathological alterations) or organism (constant Condition Index) levels. However, the decrease of tissue integrity and increased atrophy observed for higher Pt exposure concentration indicate that Pt accumulation may impact the health status of oysters.

In marine organisms, previous studies on Pt contamination mainly focused on aquatic plants such as the marine macroalgae *Ulva lactuca* or microalgae *Chlorella stigmatophora* investigating Pt internalization and extra-cellular accumulation or seawater removal kinetics (Cosden et al., 2003; Shams et al., 2014; Turner et al., 2007). To the best of our knowledge, only two laboratory studies report on inorganic Pt accumulation by marine animals, one being

the polychaete *Arenicola marina* exposed to Pt in a series of microcosms (French and Turner, 2008), and the second being the study of Mulholland and Turner (2011) exposing Pt to the marine gastropod *Littorina littorea* through direct and trophic pathways. There is therefore a crucial need for more studies on marine organisms, yet, taking into account several recommendations to apply in exposure studies as summarized in Zimmermann and Sures (2018).

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

5. Conclusions

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

level is a valuable information. In fact, generally lower Pt levels are recorded in coastal seawater. However, it should be considered that Pt inputs may occur from several sources and under different forms (dissolved, ground catalyst particles, nanoparticles, organic forms…), which may reach organisms through direct or trophic pathways.

Biomarkers, reflecting the impact of all stressors to which organisms are exposed, provide direct evidence of pollution in surrounding environment (Capela et al., 2016), and may be used as "early" warning signals to anticipate potential impacts at higher levels of biological organization (Allan et al., 2006). Although biomarkers are already part of biological and chemical monitoring requirements in the scope of the Marine Strategy Framework Directive 2008/56/EC (MSFD), their potential responses to emerging metallic contaminants, such as Pt and other TCEs are widely unknown. The present work contributes to improving our understanding of biomarker responses to Pt exposure as integrative tools between chemicals monitoring and their impacts on aquatic organisms.

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

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Fig. 3 Microscopic observations of the digestive gland of oysters from initial condition T = 0 (A), and control tanks A (G), exposed organisms at T = 28 for tanks B (C and E) and tanks D (B, D, F and H). Hematoxilin-Eosin staining (A and B), Lipofuscin Schmorl staining (C and D), ORO staining (E and F) and autometallographical staining (G and H), (LF) Lipofuscin; (NL) Neutral Lipids, (BSD) Black Silver Deposits in the Basal Layer (BL), epithelium of the digestive tract (E) and hemocytes (H). Arrows indicate the presence of neutral lipids as red precipitates (E and F) and metals as Black Silver Deposits (G and H).

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

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

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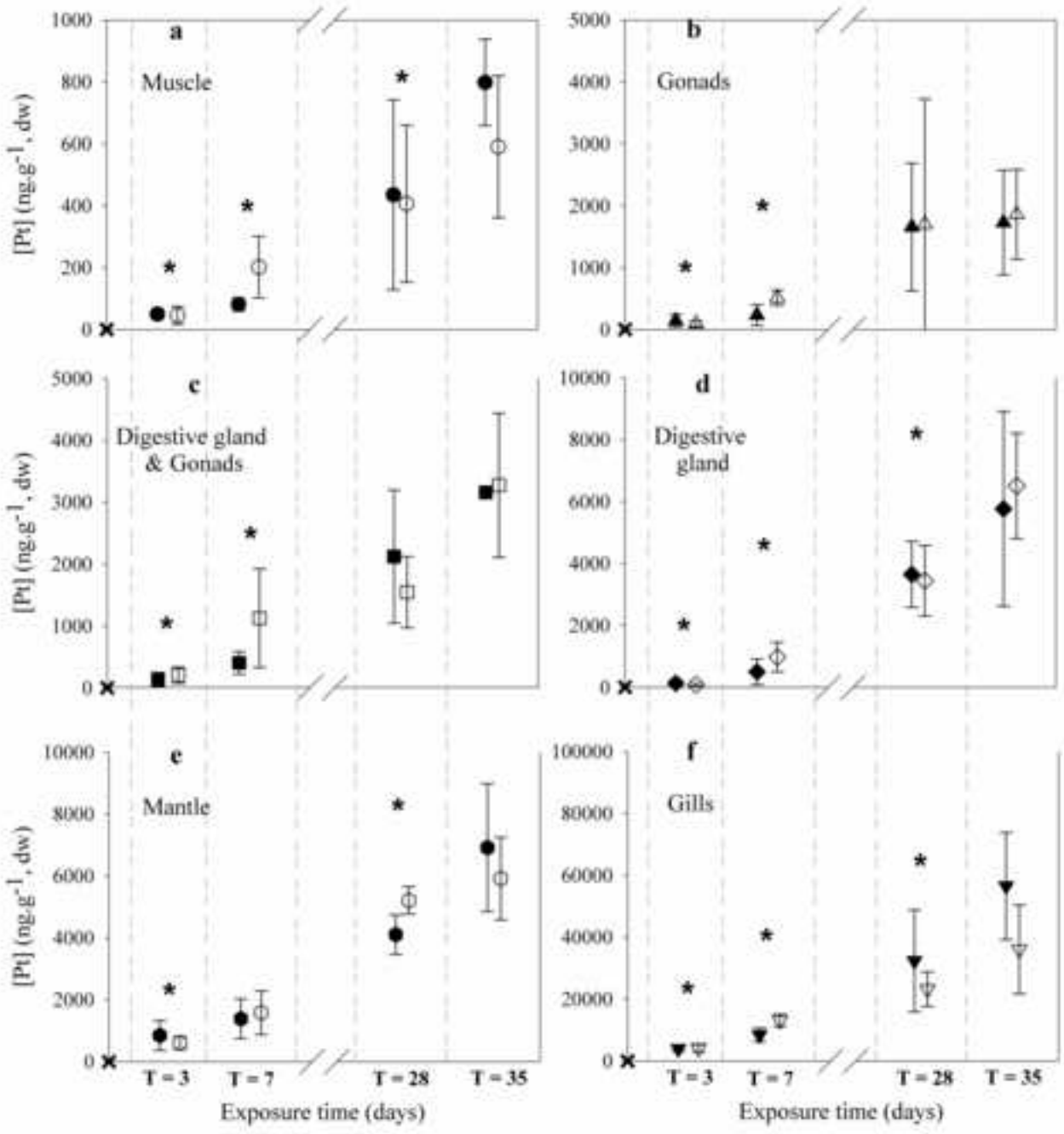

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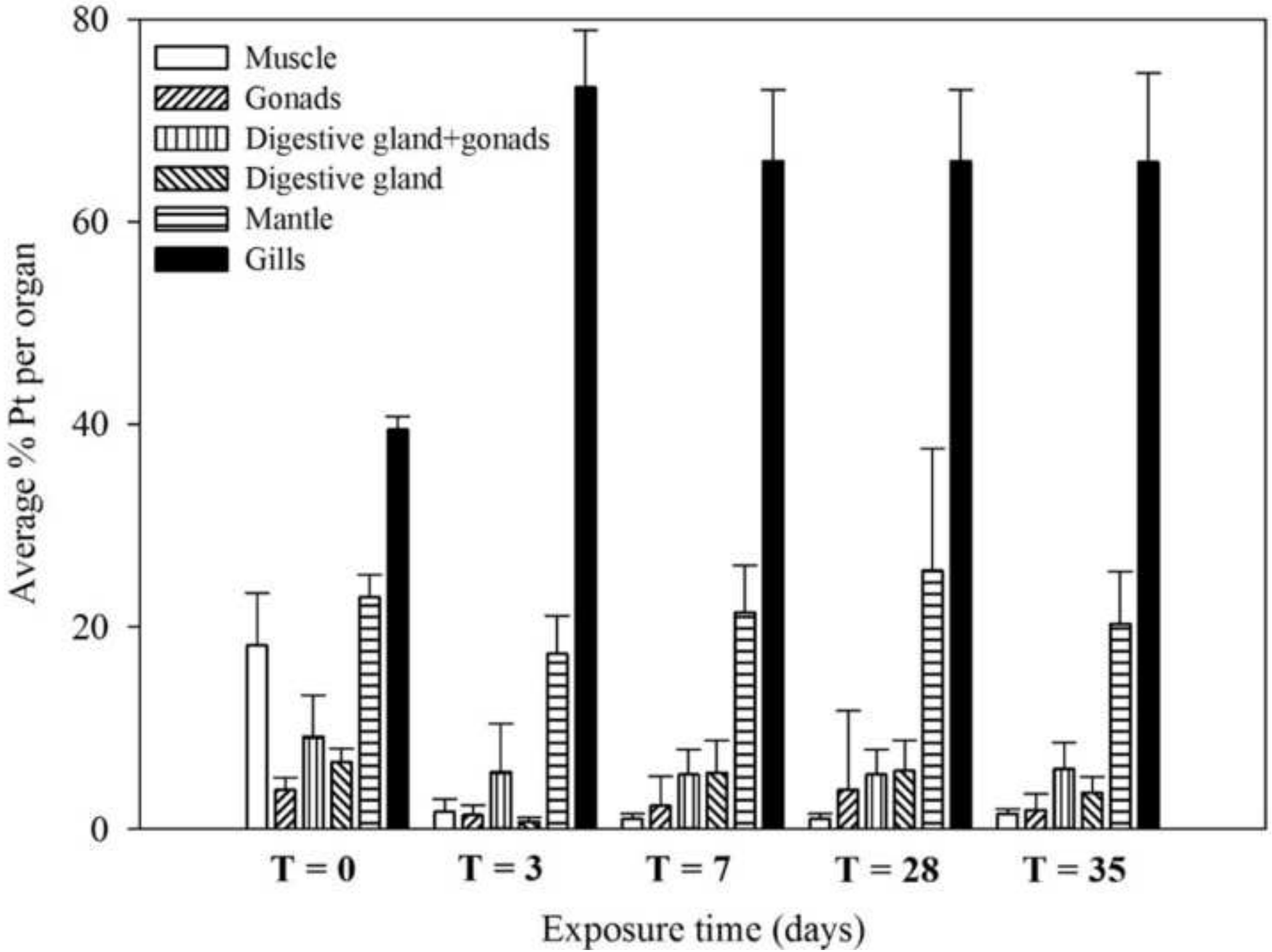

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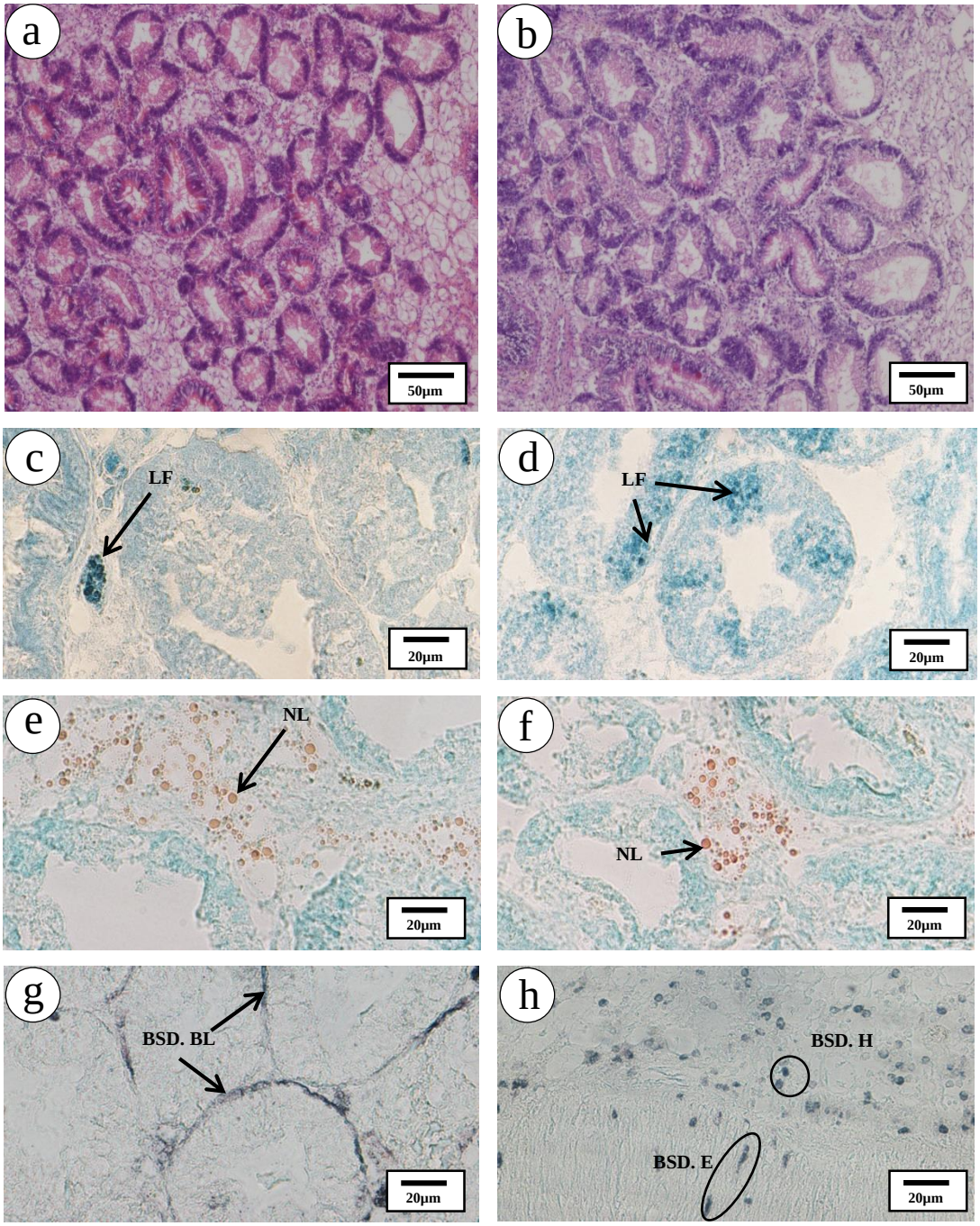

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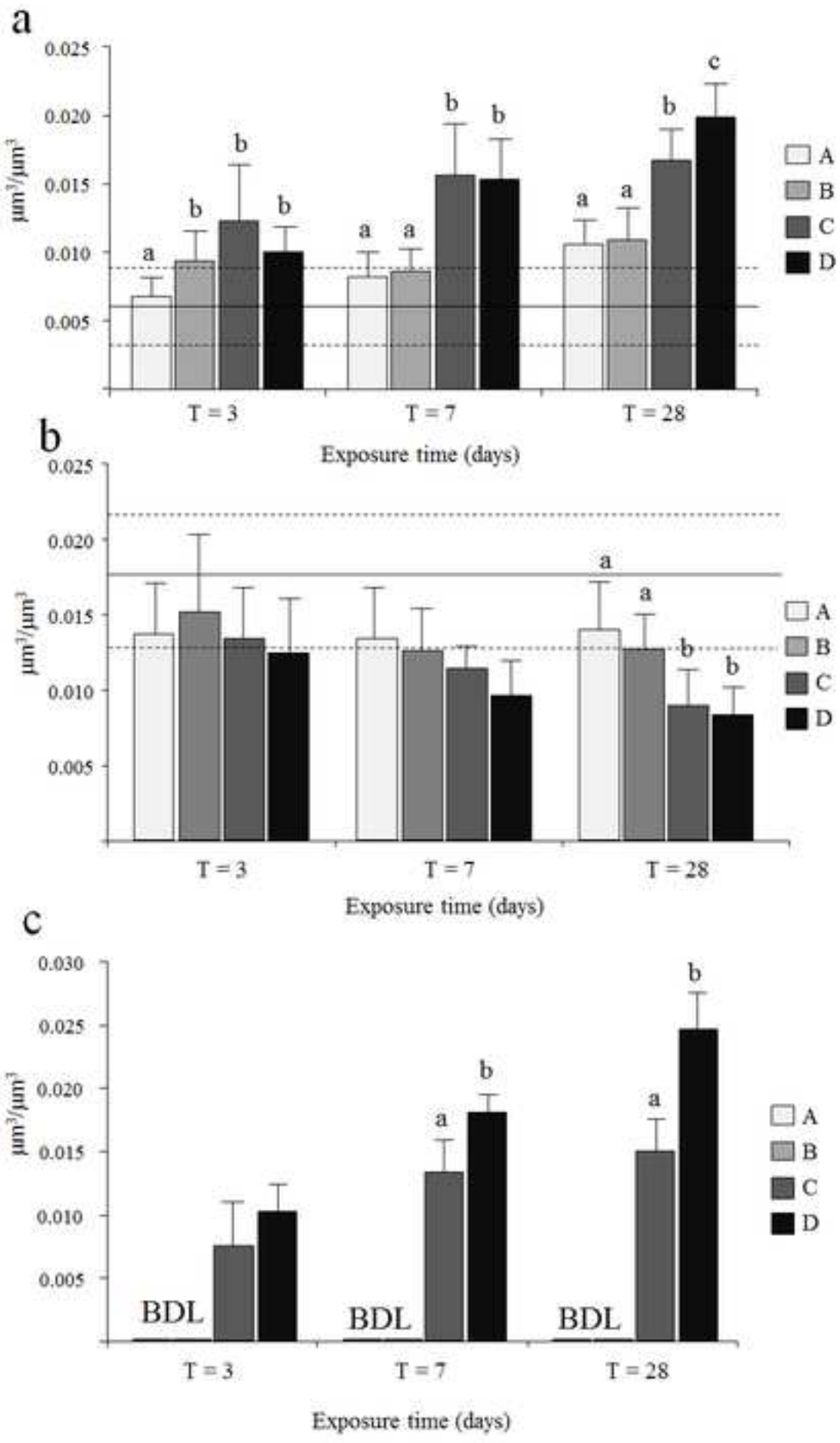

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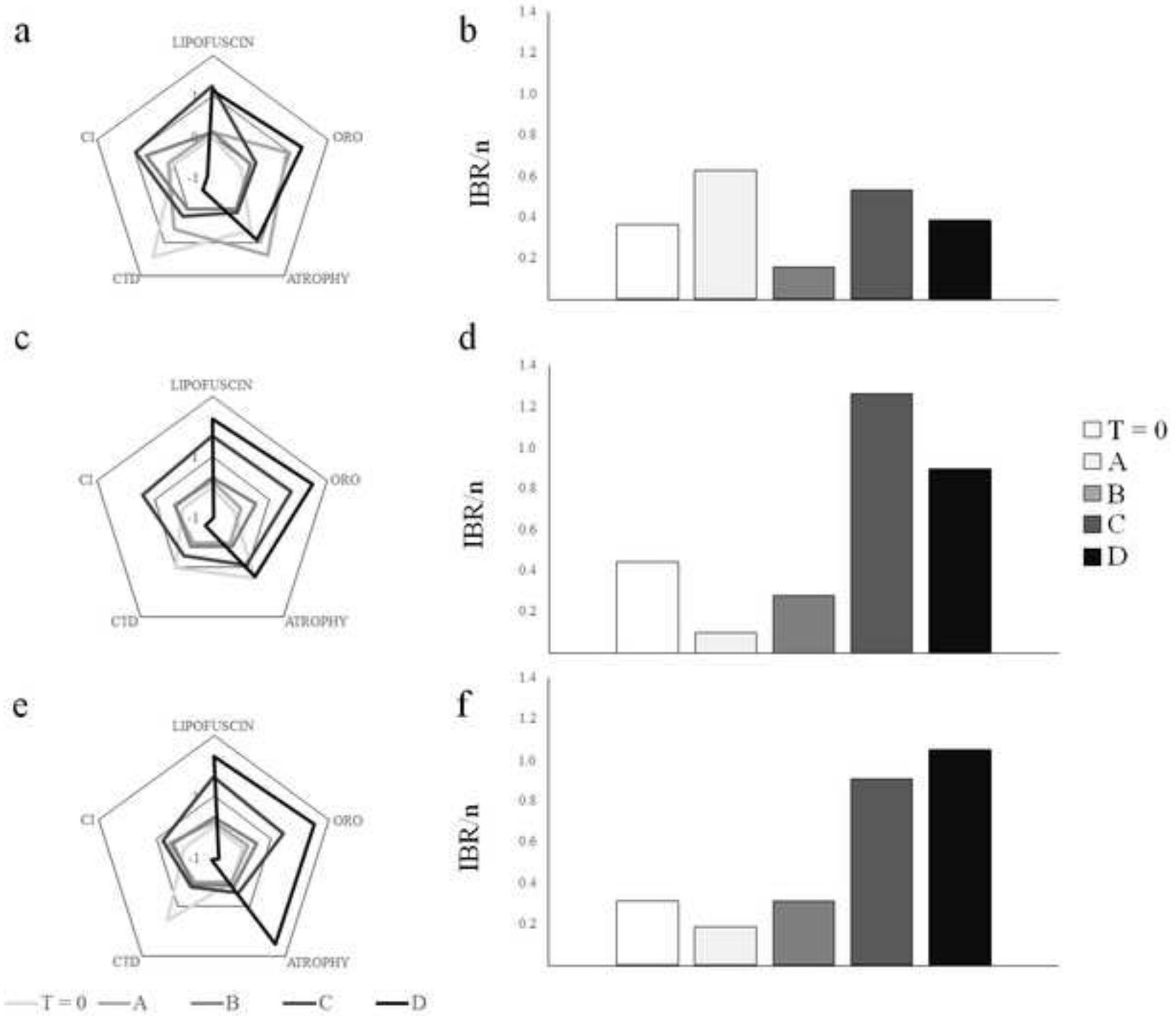

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	T = 0	**T = 3**	**T = 7**	**T = 21**	**T = 28**	**T = 35**	**R^2**
Muscle	0.289 ± 0.127	48.1 ± 23.2	141 ± 93.9	*n.d.*	421 ± 282	683 ± 220	0.967
Gonads	0.090 ± 0.030	117 ± 82	372 ± 204	*n.d.*	1,680 ± 1,610	1,800 ± 781	0.988
Digestive gland + gonads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