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1 **Enhanced immunological and detoxification responses in Pacific oysters, *Crassostrea gigas*, exposed**
2 **to chemically dispersed oil**

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20 **Abstract:** The aim of this study was to evaluate the effects of chemically dispersed oil on an
21 economically and ecologically important species inhabiting coasts and estuaries, the Pacific oyster
22 *Crassostrea gigas*. Studies were carried out with juveniles, known to generally be more sensitive to
23 environmental stress than adults. A set of enzyme activities involved in immune defence mechanisms and
24 detoxification processes, i.e. superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx),
25 catecholase-type phenoloxidase (PO), laccase-type PO and lysozyme were analysed in different oyster
26 tissues, i.e. the gills, digestive gland and mantle, and in the plasma and the haemocyte lysate supernatant
27 (HLS) of the haemolymph. Results indicated that total PAH body burdens were 2.7 times higher in the
28 presence than in the absence of the chemical dispersant. After 2 days of exposure to chemically dispersed
29 oil, alkylated naphthalenes accounted for 55% of the total PAH body burden, whereas alkylated fluorenes
30 and alkylated dibenzothiophenes accounted for 80% when the chemical dispersant was absent.
31 Importantly, a higher number of enzyme activities were modified when oil was chemically dispersed,
32 especially in the plasma and gills. Moreover, independently of the presence or absence of chemical
33 dispersant, oil exposure generally inhibited enzyme activities in the gills and plasma, while they were
34 generally activated in the mantle and haemocytes. These results suggest that the gills and plasma
35 constitute sensitive compartments in *C. gigas*, and that mantle and haemocytes may play an important
36 role in protection against xenobiotics. Among the six enzyme activities that were analysed in these body
37 compartments, five were modulated in the chemical dispersion (CD) treatment while only half of the
38 enzyme activities were modulated in the mechanical dispersion treatment. Furthermore, CD treatment
39 effects were often observed following exposure, but also during depuration periods. These results suggest
40 that immune and/or detoxification responses are likely to be affected when dispersants are used to treat oil
41 spills in shallow waters.

42

43 **Key words:** chemical dispersion, oil spill, bivalve, defence mechanisms, tissue-dependent response

44 **1. Introduction**

45 The biological and economical consequences of numerous accidental oil spills that have occurred during
46 the last 40 years are concrete proof of the need to develop reliable oil spill countermeasures. Large oil
47 spills (> 100 tonnes) represent only 6% of the total introduction of oil into the environment (NCR, 2003).
48 Indeed, most oil spills are relatively small (~1 m³). They occur close to the shore and oil slicks hit
49 shorelines relatively quickly. As a result of the Torrey Canyon incident, where large amounts of toxic
50 dispersants were used and caused severe long-term biological impacts, research efforts have focused on
51 the mechanical clean-up and containment of floating oil. However, these types of techniques in special
52 areas, such as estuaries and closed bays, can also cause additional harm to oil-impacted zones (Marchand,
53 1998). During the same period, chemical dispersants evolved from first generation products, effective but
54 highly toxic, to third generation products, with low toxicity and high biodegradability. Chemical
55 dispersants are complex mixtures, primarily containing surfactants and solvents, which reduce the
56 interfacial tension at the oil-water interface, and therefore facilitate the downward mixing of oil into the
57 water (Canevari, 1973; Li and Garrett, 1998). In this way, oil can be dispersed to concentrations below
58 toxicity threshold limits (Lessard and DeMarco, 2000; Page et al., 2000), become more accessible to
59 hydrocarbon-degrading bacteria (Venosa and Zhu, 2003), and have a lower impact on shorelines.
60 Moreover, third generation dispersants are considered non-toxic and biodegradable. Unfortunately, little
61 is known about the behavior and effects of dispersed oil in the nearshore environment (ASTM
62 Committee, 1984). Indeed, because of the higher exposure of organisms to petroleum compounds that
63 may be triggered by the use of chemical dispersants in zones with low water-column depth, e.g. coastal
64 areas and estuaries, most countries that allow the use of dispersants have strict rules: minimum water
65 depth (normally 20 m), minimum current speed (normally 1 meter per second), and minimum distance
66 from the shore or from offshore islands (normally 2 km) (Ramachandran et al., 2004). Recently, a Net
67 Environmental Benefit Analysis (NEBA) carried out by Baca et al. (2006) on a 20-year field study,
68 revealed the lack of long-term environmental impact of dispersed oil in nearshore tropical areas.
69 However, dispersants are a wide-ranging group of chemicals with varying chemical properties and related
70 toxicities. Therefore, many questions remain unanswered about the possible direct or indirect effects of

71 their use in other nearshore zones, in response to oil spills, where human activities are heavily
72 concentrated, such as in the case of the Transocean Deepwater Horizon oil rig explosion.

73 Coasts and estuaries are considered to be sensitive areas since they provide habitats for a large number of
74 organisms, constitute nursery grounds for juveniles of several commercially important species, and bear
75 very high productivity. Sessile and filter-feeder organisms inhabiting these zones, such as the Pacific
76 oyster *Crassostrea gigas* (Thunberg, 1753), are constantly in contact with various chemical molecules.
77 Environmental stress from pollutants is likely to be an important determining factor in weakening defence
78 mechanisms in these organisms and therefore promoting the occurrence or increase in diseases,
79 particularly at early life stages, i.e. larvae and juveniles (Perdue et al., 1981; Lacoste et al., 2001). Among
80 immune defence mechanisms in bivalves, antioxidant enzyme activities, e.g. superoxide dismutase
81 (SOD), catalase, glutathione peroxidase (GPx), and enzyme activities involved in humoral innate
82 defences, e.g. phenoloxidase (PO) and lysozyme, have been shown to be modulated by the presence of
83 several types of pollutants (Verlecar et al., 2007; Bado-Nilles et al., 2008; Stabili and Pagliara, 2009). In
84 this respect, these responses have been shown to be tissue-dependent (Cheung et al., 2001; Luna-Acosta
85 et al., 2010a).

86 In this general context, the aim of this study was to experimentally assess 1) the bioaccumulation and 2)
87 the effects of chemically-dispersed oil on immune defence and/or detoxification mechanisms, i.e. SOD,
88 catalase, GPx, catecholase- and laccase-type PO, and lysozyme activities of Pacific oyster *C. gigas*
89 juveniles. For this purpose, enzyme activities were determined in different tissues, i.e. gills, digestive
90 gland, mantle, and in the haemocytes and plasma, or acellular fraction, of the haemolymph. To this end,
91 comparisons were made between oysters exposed to oil subjected to chemical dispersion (CD) or
92 mechanical dispersion (MD), to the water soluble fraction of the oil (WSF) and to the dispersant alone
93 (D).

94

95 **2. Material and methods**

96 **2.1. Chemicals**

97 *Oil* • A Brut Arabian Light crude oil (BAL 110) was used for this study. The crude oil was topped at
98 110°C to remove the most volatile components, in order to simulate the natural weathering of the oil after

99 its release at sea (evaporation of most volatile components), before it reaches coastal zones. BAL 110
100 possesses the following physico-chemical characteristics, similar to the oil spilled by the Amoco Cadiz in
101 1978: density of 0.860 at 20°C, viscosity of 60 mPa s at 15°C, 12% polar compounds, 34% aromatic
102 hydrocarbons and 54% saturated hydrocarbons.

103 *Dispersant* • The chemical dispersant used in this study was selected following an evaluation carried out
104 by the Centre of Documentation, Research and Experimentation on Accidental Water Pollution (Cedre),
105 which defines it as 1) effective for use in the marine environment, 2) non-toxic at the concentration
106 recommended by the manufacturer Total Fluides (i.e. 5% v/v) and (3) biodegradable. Its physico-
107 chemical characteristics were not available for reasons of confidentiality.

108

109 **2.2. Biological material**

110 Pacific oyster *C. gigas* juveniles (3-4 cm in height, less than 1 year old) were purchased from the hatchery
111 France Naissain, located in Bouin (France). The oysters were acclimatised in the laboratory at $15 \pm 1^\circ\text{C}$
112 for two weeks before starting the experiments. They were fed daily with an algal diet (5×10^4 cell ml^{-1})
113 composed of *Heteroskeletonema* sp. (Bacillariophyceae) purchased from the hatchery SATMAR
114 (Normandie, France).

115

116 **2.3. Experimental design**

117 The experiment, consisting of an exposure period followed by a depuration period, was carried out three
118 times to provide analysis replicates.

119 *Experimental system* • The experimental system consisted of 300 L static water tanks. Because of the
120 hydrophobic character of the oil, a funnel (at the surface of each tank) was connected to a submersed
121 Johnson L450 water pump (at the bottom of the tank), in order to maintain the mixture of oil and
122 dispersant as a homogenous solution. Preliminary tests confirmed that total petroleum hydrocarbon
123 concentrations in the water column were depth-independent, suggesting that small petroleum droplets
124 were homogeneously dispersed in the water column (data not shown). The oxygen saturation of water in
125 each tank was maintained at around 96% by a compressor that injected air *via* an air stone. The

126 experimental system was placed in a thermoregulated greenhouse, in order to maintain the temperature of
127 the exposure studies at 15 ± 1 °C.

128 *Exposure* • One experimental system was used per condition, making a total of five experimental systems.
129 Thirty oysters were transferred into each experimental system. The different exposure conditions are
130 summarized in Table 1. The exposure period lasted 2 days. The seawater used for this study (pH: $7.95 \pm$
131 0.05 , salinity: 35.2 ± 0.1 p.s.u.) was provided by Oceanopolis, Brest (France). This seawater was treated
132 by UV-light and filtrated at 0.45 μm before use. In the first tank, the control exposure condition was set
133 up with clean seawater. For the amount of BAL selected, the aim of this study was to obtain a
134 concentration of dispersed oil in the same rank than those reported *in situ*, following an oil spill, such as
135 reported by Lunel (1995) following the Braer oil spill. Thus, in the second tank, the chemical dispersion
136 (CD) condition was set up by pouring 20 g of BAL 110 and the volume of dispersant recommended by
137 the manufacturer, i.e. 1.2 g of dispersant, into the funnel of the experimental system. In the third tank, the
138 mechanical dispersion (MD) condition was set up by pouring 20 g of BAL 110 into the funnel. In the
139 fourth tank, the toxicity of molecules that naturally dissolve in seawater was tested, by exposing oysters
140 to a water-soluble fraction (WSF) of BAL 110. To obtain the WSF, a plastic circle was set on the surface
141 of the seawater in the experimental system. The BAL 110 (20 g) was then poured into the plastic circle, in
142 order to contain the oil slick at the surface, without mixing. The oysters were therefore only exposed to
143 the soluble fraction of the oil, i.e. free of particles of bulk material, and, contrary to the water-
144 accommodated fraction (WAF), free of soluble and volatile compounds that can naturally evaporate
145 (Anderson et al., 1974; Singer et al., 2000). In the fifth tank, an internal control for the CD condition was
146 set up by pouring 1.2 g of chemical dispersant into the funnel (i.e., dispersant condition, D). All
147 experimental systems contained a funnel connected to a submersed water pump. All exposure media were
148 added to the tanks 13h before adding the oysters, the time needed to obtain a relatively stable oil
149 concentration in the water column. The oysters were not fed during the exposure period.

150 *Depuration* • Ten oysters per treatment condition were placed in a decontamination tank, located in the
151 thermoregulated greenhouse ($T = 15 \pm 1$ °C) and containing clean seawater, for a recovery period of 15
152 days. The oysters were fed daily with an algal diet (5×10^4 cell ml^{-1}) composed of *Heteroskeletonema* sp.
153 (Bacillariophyceae).

154 **2.4. Sampling procedure**

155 Pooled gills, digestive glands, mantles, haemocyte fraction and plasma of ten oysters were used for each
156 replicate sample, and three replicates were prepared per treatment. After opening the oyster shells by
157 cutting off the adductor muscle, approx. 0.3-0.5 ml of haemolymph was withdrawn from the pericardial
158 cavity using a 1-ml syringe equipped with a needle (0.9 x 25 mm). Haemolymph samples were
159 centrifuged at 260 x g for 10 min at 4°C in order to separate the cellular (haemocytes) fraction from the
160 plasma. The gills, digestive gland and mantle were removed from the soft tissues and homogenized at 4°C
161 in 0.1 M Tris HCl buffer pH 7.0 (0.45 M NaCl, 26 mM MgCl₂, 10 mM CaCl₂; 0.5 ml of buffer.g⁻¹ of
162 fresh weight for the gills and the mantle, and 1 ml.g⁻¹ of fresh weight for the digestive gland), using an
163 Ultra Turrax (T25 basic, IKA-WERKE) and a Thomas-Potter homogenizer (IKA-Labortechnik RW 20.n,
164 size 0.13-0.18 mm, BB). The homogenates were centrifuged at 10 000 x g for 10 min at 4°C. The
165 resulting supernatant was collected for enzymatic studies.

166

167 **2.5. Biochemical analysis**

168 *Superoxide dismutase assay* • SOD was determined as described previously (Luna-Acosta et al., 2010a)
169 based on competition of SOD with iodinitrotetrazolium (INT) for dismutation of superoxide anion (O₂⁻).
170 In the presence of O₂⁻, INT is reduced into a red formazan dye that can be measured at 505 nm at 25°C
171 (Kit Ransod SD 125, Randox, France). One unit of SOD is defined as the amount of enzyme that
172 promotes a 50% decrease in the rate of INT reduction.

173 *Glutathione peroxidase assay* • GPx activity was determined as described previously (Luna-Acosta et al.,
174 2010a). In the presence of glutathione reductase and substrates (i.e. reduced glutathione and cumene
175 hydroperoxide), the decrease of absorbance at 340 nm was proportional to the reduction of the oxidised
176 glutathione by NADPH, H⁺ (Kit Ransel RS 504, Randox, France). One unit of GPx oxidises 1 µmol of
177 NADPH ($\epsilon_{\text{NADPH}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) per minute.

178 *Catalase assay* • Catalase activity was determined according to the method of Fossati et al. (1980). This
179 method is based on the measurement of the hydrogen peroxide substrate remaining after the action of
180 catalase. First, catalase converts hydrogen peroxide into water and oxygen and then this enzymatic

181 reaction is stopped with sodium azide. An aliquot of the reaction mix was then assayed for the amount of
182 hydrogen peroxide remaining using a colorimetric method (Kit Catalase CAT-100, Sigma).

183 *Catecholase-type and laccase-type phenoloxidase assay* • Catecholase- and laccase-type phenoloxidase
184 (PO) activities were determined according to the method described previously (Luna-Acosta et al.,
185 2010b). For PO assays, 100 mM of dopamine or 50 mM of PPD were used as substrates and the increase
186 of absorbance at 490 and 420 nm was monitored for 4 and 2h for catecholase- and laccase-type PO
187 activity, respectively. Nonenzymatic oxidation by the substrate was monitored in wells without oyster
188 sample and subtracted from oxidation of the substrate with oyster sample. One unit of catecholase- and
189 laccase-type PO activities corresponds to the amount of enzyme that catalyzes the production of 1 μ mole
190 of product ($\epsilon=3300 \text{ M}^{-1} \text{ cm}^{-1}$ and $43\ 160 \text{ M}^{-1} \text{ cm}^{-1}$, respectively), per minute.

191 *Lysozyme assay* • Lysozyme was determined as described previously (Luna-Acosta et al., 2010a). The
192 lysozyme assay was done in triplicate for each sample and compared to hen egg white lysozyme
193 standards (2.5-20 $\mu\text{g ml}^{-1}$), in the presence of *Micrococcus lysodeikticus* (Sigma-Aldrich, France). One
194 unit of lysozyme corresponds to the amount of enzyme that diminishes absorbance at 450 nm by 0.001
195 per minute at pH 7.0, at 25°C.

196 *Protein assay* • All activities were expressed in relation to the protein concentration measured according
197 to the Lowry method with slight modifications, using bicinchoninic acid and copper sulphate 4% (Smith
198 et al., 1985). Serum albumin was used as protein standard (Sigma-Aldrich, France).

199 All enzyme activities were measured in the gills, digestive gland, mantle, plasma and HLS, except for
200 lysozyme that was not measured in the HLS, making a total of 29 enzymatic analyses.

201

202 **2.6. Chemical analysis in oysters**

203 The levels of polycyclic aromatic hydrocarbons (PAHs) in oysters were determined with a Varian Saturn
204 2100 T GC-MS device, using the procedure of Baumard et al. (1997) with some modifications. Three
205 pools of five oysters were analysed per treatment. Prior to extraction, each oyster sample was
206 homogenized using an Ultra Turrax (Janke and Kunkel, IKA[®]-Labortechnik). 150 μl of perdeuterated
207 internal standards (CUS-7249, Ultra Scientific, Analytical solutions) were added to 3 g of homogenized
208 oyster samples and the mixtures obtained were digested for 4 h under reflux in 50 ml of an ethanolic

209 solution of potassium hydroxide (2 M, Fisher Chemicals). After cooling, settling and addition of 20 ml of
210 demineralised water, the digest was extracted in a 250 ml funnel twice with 20 ml of pentane (Carlo Erba
211 Reactifs, SDS). The extract was evaporated with a TurboVap 500 concentrator (Zyman, Hopkinton, MA,
212 USA, at 880 mbar and 50 °C) to obtain 1 ml of concentrated extract. The purification of the extract was
213 performed by transfer to a silica column (5 g of silica). Hydrocarbons were eluted with 50 ml of pentane:
214 dichloromethane (80:20, v:v, SDS) and concentrated to 200 µl by means of a TurboVap 500 concentrator
215 (Zyman, 880 mbar, 50°C). Aromatic compounds were analysed by GC-MS, with a detection limit of
216 0.005 µg g⁻¹ of dry weight, and PAHs were quantified relative to the perdeuterated internal standards
217 introduced at the beginning of the sample preparation (Roy et al., 2005). Five perdeuterated standard
218 compounds, i.e. Naphthalene d8, Biphenyl d10, Phenanthrene d10, Chrysene d12, and Benzo[a]pyrene
219 d12, representative of all the PAHs analysed, were used as internal standards. A total of 20 parent PAHs
220 and 25 alkylated compounds were quantified. The PAH recovery was > 60% and the relative standard
221 deviation (RSD, i.e. (standard error / mean x 100) was < 15%.

222

223 **2.7. Statistical analysis**

224 All values are reported as mean ± standard deviation (SD). Statistical analysis was carried out with
225 STATISTICA 7.0. Values were tested for normality (Shapiro test) and homogeneity of variances (Bartlett
226 test). In some cases, logarithmic transformations (Log₁₀) were used to meet the underlying assumptions of
227 normality and homogeneity of variances. Two-way nested MANOVA were used to analyse results, with
228 treatment and period as fixed factors, and pool as a random factor. The period factor corresponds to the
229 exposure period and the depuration period. Pool was nested within each combination of treatment and
230 time (Zar, 1984). When the null hypothesis (H₀: no difference between treatments or within treatment at
231 different time intervals) was rejected, significant differences were tested using Tukey's HSD test. For non
232 normal values, i.e. to compare oysters' PAH content between different treatments, a Kruskal-Wallis test
233 was used, followed by a Dunn's multiple comparisons test. Statistical significance was determined as
234 being at the level of $p < 0.05$. The relationships between body burdens for the different PAH categories
235 (HMW PAHs, LMW PAHs, parent PAHs and alkylated PAHs) and the responses of the enzyme activities
236 were analysed using principal component analysis (PCA) and redundancy analysis (RDA) (Leps and

237 Smilauer, 1999). Separate analyses were performed for the different organs. Data for enzyme activities
238 were centered and standardized before analysis. PCA and RDA were performed using CANOCO for
239 Windows® software package, Version 4.5 (Center for Biometry, Wageningen, The Netherlands). For
240 RDA analyses, the significance of the relationship between enzyme activities and PAH body burdens
241 were tested using a global Monte Carlo permutation test.

242

243 3. Results

244 3.1. Chemical analyses

245 After 2 days of exposure, PAHs had been efficiently bioaccumulated in the soft tissues of *C. gigas*. The
246 total PAH content in oysters (\sum PAHs) was equal to 7 ± 1 , 530 ± 75 , 198 ± 22 , 56 ± 50 and $8 \pm 3 \mu\text{g g}^{-1}$
247 dry weight for the control, the chemically dispersed oil (CD), the mechanically dispersed oil (MD), the
248 water soluble fraction (WSF) and the dispersant (D) treatments, respectively. Significant differences were
249 found between the control and the CD or the MD, but not with the WSF conditions ($F_{4,15} = 12.83$, $p =$
250 0.01). This lack of significant difference could be explained by the high variation in total PAH levels in
251 the WSF condition. Importantly, the total PAH content in oysters in the CD condition was almost 3 times
252 higher than in the MD condition.

253 After 15 days of depuration, the \sum PAHs was equal to 3 ± 0 , 15 ± 6 , 14 ± 6 , 2 ± 0 and $2 \pm 2 \mu\text{g g}^{-1}$ dry
254 weight in control, CD, MD, WSF and D treatments, respectively. Even if the PAH content in oysters had
255 decreased by 97 and 93% in CD and MD conditions, respectively, it remained significantly higher than
256 the control condition ($F_{4,15} = 10.27$, $p = 0.03$).

257 Independently of time and treatment, light PAHs (≤ 3 rings) and their alkylated homologues accounted
258 for at least 90% of the total PAHs in oyster tissues (Fig. 1). After 2 days of exposure to the CD condition,
259 alkylated naphthalenes (NaF), alkylated dibenzothiophenes (DBT) and alkylated fluorenes (Fl) accounted
260 for most of the PAH content in oyster soft tissues with 55 ± 6 , 19 ± 1 , $18 \pm 8\%$ for alkylated NaF,
261 alkylated DBT and alkylated Fl, respectively (Fig. 1). In the MD condition, alkylated DBT and alkylated
262 Fl accounted for most of the PAH content in the oysters' soft tissues, representing 38 ± 2 and $40 \pm 2\%$ of
263 the total PAHs, respectively. Alkylated DBT ($22 \pm 0\%$) and alkylated Fl ($53 \pm 0\%$) also accounted for a
264 large proportion of the PAH content in the oysters' soft tissues in the WSF condition. For the D condition,

265 alkylated DBT ($45 \pm 6\%$) and anthracene ($30 \pm 7\%$) accounted for most of the PAH content in oyster soft
266 tissues.

267 After 15 days of depuration, alkylated DBT were the predominant PAH compounds in oyster tissues of
268 the CD, MD, WSF and D conditions, representing $56 \pm 19\%$, $48 \pm 7\%$, $40 \pm 5\%$ and $50 \pm 16\%$ of the total
269 PAHs, respectively (Fig. 1).

270

271 **3.2. Enzymatic analysis**

272 Overall, no significant differences in enzymatic activities were observed between the control conditions
273 from both exposure and depuration periods, whatever the tissue (Figs. 2-7). In contrast, all enzyme
274 activities were affected by both treatment and period, independently of the considered tissue, except for
275 catecholase and laccase activities in the mantle, which were only affected by the treatment (Figs. 5 and 6).
276 No significant effect was observed for treatment for lysozyme activity in the gills and plasma (data not
277 shown).

278 After 2 days of exposure for the CD condition, and relative to the control condition, catalase (Fig. 3) and
279 GPx (Fig. 4) activities were completely inhibited and laccase activity (Fig. 6) was 1.4-fold higher in the
280 gills. SOD (Fig. 2) and GPx (Fig. 4) activities were 30% and 50% lower in the digestive gland,
281 respectively. Catalase activity (Fig. 3) was 75% lower in the mantle. Interestingly, results from the HLS
282 and the plasma differed. While no significant effect was observed in the HLS, SOD activity (Fig. 2) was
283 2-fold higher and catalase (Fig. 3) and laccase (Fig. 6) activities were completely inhibited in the plasma.
284 For the MD condition, SOD (Fig. 2) and laccase (Fig. 6) activities were 2.5-fold and 1.4-fold higher in the
285 gills, respectively. GPx activity (Fig. 4) was 1.5-fold higher in the mantle. While SOD (Fig. 2), GPx (Fig.
286 4), catecholase (Fig. 5) and laccase (Fig. 6) activities were 2-4-fold higher in the HLS, catalase activity
287 (Fig. 3) was completely inhibited in the plasma. For the WSF condition, SOD (Fig. 2) and laccase (Fig. 6)
288 activities were 2.5- and 1.7-fold higher in the gills, respectively. SOD (Fig. 2) and catecholase (Fig. 5)
289 activities were 4- and 2-fold higher in the HLS, respectively. It is important to notice that the dispersant
290 alone (D condition), which is considered as a harmless product, also modulated different enzyme
291 activities in *C. gigas*: laccase activity (Fig. 6) was 1.4-fold higher in the gills; SOD (Fig. 2) and lysozyme

292 (Fig. 7) activities (Fig. 2) were 70 and 55% lower in the digestive gland, respectively, and SOD (Fig. 2),
293 catecholase (Fig. 5) and laccase (Fig. 6) activities were 2.5- to 3-fold higher in the HLS.

294 After 15 days of depuration, some activities returned to control levels, depending on the tissue and the
295 treatment. In some tissues and for some activities, effects were observed only at this period, e.g. SOD
296 activity (Fig. 2) was 1.3-fold higher in the mantle for the MD condition; catalase activity (Fig. 3) was 2-
297 to 2.5-fold higher in the digestive gland for the CD, WSF and D conditions; SOD activity (Fig. 2) was
298 1.3-fold higher for the MD condition; lysozyme activity (Fig. 7) was 1.4- to 1.6-fold higher for the CD,
299 WSF and D conditions in the mantle; catecholase activity (Fig. 5) was inhibited by 20 to 30% in the
300 plasma for the CD, MD and WSF conditions, and 1.4-fold higher in the D condition.

301 In addition to these results, a significant treatment, but not period, effect was observed in the mantle for
302 catecholase (Fig. 5) and laccase (Fig. 6) activities. In this tissue, catecholase activity was inhibited by
303 20% for the CD and MD conditions and laccase activity was 1.2-fold higher for the CD and the D
304 conditions.

305

306 **3.3. Relationships between PAH contents in oyster soft tissues and enzyme activities**

307 At the end of the exposure period, significant correlations were observed between different enzyme
308 activities and PAH contents in oyster soft tissues and responses varied according to the tissue that was
309 analysed. The results of PCA and RDA are presented in Fig. 8 and Table 2, respectively. RDA indicated
310 PAH body burdens as the significant variable explaining 49%, 43%, 43%, 39%, 44% of total variation in
311 enzyme activities in the gills, digestive gland, mantle, plasma and HLS, respectively. Enzyme activities in
312 the gills, plasma and mantle were significantly correlated to parent PAHs. Enzyme activities in the plasma
313 were also significantly correlated to LMW PAHs. Four out of six enzyme activities analysed in the gills
314 and plasma were negatively correlated to body burdens of the various PAH categories: catalase, GPx,
315 lysozyme, and catecholase or laccase, in the gills and plasma, respectively (Fig. 8, Table 2). SOD, GPx,
316 catecholase and lysozyme in the mantle were positively correlated to body burdens of the various PAH
317 categories (Fig. 8, Table 2). Four out of five enzyme activities analysed in the HLS, i.e. SOD, GPx,
318 catecholase and laccase, were negatively correlated to body burdens of the various PAH categories, but
319 were positively correlated to parent PAHs (Fig. 8, Table 2). Enzyme activities in the digestive gland were

320 significantly correlated to HMW PAHs. Five out of six enzyme activities analysed in the digestive gland
321 were positively correlated to body burdens of the various PAH categories: SOD, catalase, catecholase,
322 laccase and lysozyme (Fig. 8, Table 2).

323

324 4. Discussion

325 4.1. PAH bioaccumulation and depuration in oyster tissues

326 The aim of this study was to assess 1) the bioaccumulation and 2) the effects of chemically dispersed
327 hydrocarbons on a species inhabiting coastal and estuarine zones, the Pacific oyster *C. gigas*. This marine
328 bivalve is a good indicator of the presence and bioavailability of oil in the water column and benthic
329 sediments. In this species, bioconcentration factors (BCF) of petroleum hydrocarbons range from 10 to
330 50 000 (Michel and Henry, 1997), so even low levels of exposure are likely to be detectable in oyster
331 tissues. PAHs, with high octanol-water partition coefficients ($\log K_{ow} > 3.5$), are readily taken up by
332 organisms (Meador, 2003). Thus, evaluating PAH bioaccumulation plays an important part in assessing
333 the risk that chemical dispersants are likely to pose to marine organisms inhabiting coasts and estuaries,
334 especially carcinogenic, mutagenic or teratogenic PAHs. Because no PAH measurements were carried out
335 in seawater, we were not able to calculate BCF, but PAH body burden analysis was conducted in oyster
336 soft tissues. Results showed that, independently of the treatment (i.e. CD, MD, WSF or D), heavy PAHs
337 were poorly accumulated in oyster tissues (0-3% of the total PAH content), while light PAHs and mainly
338 their alkylated homologues, which are generally more toxic than the parent compounds, were present in
339 large proportions in all the treatment conditions. Indeed, PAHs with low molecular weight (≤ 178.2
340 g mol^{-1}) such as NaF, DBT and Fl are more likely to be bioaccumulated due to high water solubility and
341 higher bioavailability for organisms (Neff, 2002).

342 A high heterogeneity in PAH body burden was observed for the WSF treatment. The WSF corresponds to
343 hydrocarbon molecules that are liable to naturally dissolve, meaning that the solution can be considered
344 as homogeneous. Therefore, the heterogeneity of results for the WSF treatment suggests that 1) organisms
345 depurate at different rates and/or that 2) organisms accumulate at different rates (Neff, 2002).

346 Interestingly, for the same amount of dispersed oil with (CD condition) or without (MD condition) a
347 chemical dispersant, and for the same exposure time, i.e. 2 days, oysters bioaccumulated approximately

348 2.7 times more PAHs in the presence of the chemical dispersant, showing that the dispersant increased the
349 bioavailability of PAHs and thus their uptake rate in *C. gigas*. The bioaccumulated PAH compounds were
350 completely different between both CD and MD conditions. In the CD condition, 55% of the total PAH
351 content was composed of high alkylated NaF. These compounds were poorly bioaccumulated in the MD
352 and WSF conditions (Fig. 1), whereas alkylated Fl and alkylated DBT represented between 70 and 80%
353 of the total PAH content (Fig. 1). Our results for the CD condition but not for the MD condition are in
354 agreement with a recent study carried out on the Iceland scallop *Chlamys islandica* where C3-NaF
355 accounted for a large proportion of the PAHs that were accumulated in the tissues after 15 days of
356 exposure to mechanically dispersed oil (Hannam et al., 2009). In the present study, the exposure
357 experiment was carried out for only 2 days, and, since uptake rate constants of PAHs in bivalves
358 generally increase with molecular weight or molecular complexity (McLeese and Burrige, 1987), it
359 cannot be excluded that a longer exposure period would result in a higher alkylated NaF uptake in oyster
360 tissues. Nonetheless, our results clearly demonstrated that the presence of the chemical dispersant
361 increased the bioaccumulation rate of alkylated NaF in oyster soft tissues. However, the processes that
362 could alter 1) the bioconcentration and/or 2) the type of components accumulated, when oil is chemically
363 dispersed, have been poorly described in the scientific literature. A possible contributing factor for
364 bioaccumulation of some components is that dispersing spilled oil converts the oil from a surface slick to
365 a plume of small oil droplets dispersed in the water column. These oil droplets might be more easily
366 ingested by filter-feeding organisms, such as oysters (Baussant et al., 2001). Additionally, particles
367 trapped on the oysters' gills are directed into the gut and later incorporated into endocytic vacuoles in the
368 digestive gland. Oil particles retained in intracellular vacuoles can also be assimilated to the tissue lipids
369 (Baussant et al., 2001). Thus, in the CD treatment, a significant amount of the larger PAHs bound to
370 particles could be retained and accumulated in intracellular vacuoles or in tissue lipids during the
371 exposure period.

372 After 15 days of depuration, more than 90% of the PAH burden had been depurated from oysters' soft
373 tissues. Even if PAH contents in CD and MD conditions were still significantly higher than the control
374 condition, this result clearly shows that oysters can eliminate high quantities of PAHs very rapidly.
375 Interestingly, no differences in the total PAH content between CD and MD were found for the depuration

376 period. This result is in agreement with a previous study carried out on mussels (Gilfillan et al., 1984).
377 Among PAHs, alkylated DBTs were the most persistent PAHs in all treatments (Fig. 1). This result is in
378 agreement with Berthou et al. (1987) who report that DBT persist in oyster tissues for at least one year.
379 As in the present study, C3-DBT was among the most persistent PAHs (Berthou et al., 1987), raising
380 questions about its potential toxic effects. Further studies are therefore needed in order to assess the long-
381 term toxicity of this compound.

382

383 **4.2. Effect on enzyme activities**

384 Disease emergence and organism survival are determined partly by the condition of the immune system.
385 Therefore, the measurement of defence mechanisms can provide important early warning signals of the
386 sub-lethal effects of exposure to contaminants and the susceptibility of animals to infectious diseases
387 (Hannam et al., 2009).

388 Bivalve molluscs possess two types of innate responses: 1) cellular, i.e. phagocytosis and encapsulation;
389 and 2) humoral, e.g. PO and lysozyme enzyme activities (Tryphonas et al., 2005). During phagocytosis,
390 reactive oxygen species (ROS), such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), play an
391 important role in protection against pathogens. However, ROS can also interact with hosts' biological
392 macromolecules which can result in enzyme inactivation, lipid peroxidation, DNA damage or cell death
393 (Cazenave et al., 2006). The extent to which oxyradical generation produces biological damage depends
394 on the effectiveness of antioxidant defences, such as SOD, which participates in the transformation of O_2^-
395 into H_2O_2 and H_2O , and of catalase and GPx, which participate in the transformation of H_2O_2 into O_2 and
396 H_2O (Neumann et al., 2001).

397 Environmental contaminants, such as PAHs, can lead to the formation of ROS and enhance oxidative
398 stress in aquatic organisms (Winston, 1991). Results from the present study showed a positive correlation
399 between PAH contents and SOD activities. Such a relationship has already been reported for different
400 bivalve species exposed to hydrocarbons (Sole et al., 1995; Orbea et al., 2002; Richardson et al., 2008)
401 and suggests that hydrocarbons induce oxidative stress by producing ROS such as O_2^- . However, in the
402 present study, catalase and GPx activities were generally negatively correlated with different PAH body
403 burdens. This may be due to the inhibition of enzyme synthesis by PAHs or to enzyme inactivation

404 caused by high tissue contaminant concentrations (Borg and Schaich, 1983). Moreover, since GPx and
405 catalase catalyze the transformation of H₂O₂ into H₂O, they may act on common substrates, and thus
406 competition may exist for the same group of substrates (Kappus, 1985). This may explain the positive
407 correlations of catalase activity and negative correlations of GPx activity with PAH content for the same
408 tissues in the present study (Table 2).

409 Among enzymes involved in humoral immune defences in bivalves, POs are the key enzymes of
410 melanization, participating in the entrapment of foreign material in a melanin capsule or in the direct
411 killing of microbes by the toxic quinone intermediates produced during the melanin production cascade
412 (Söderhäll and Cerenius, 1998). Tyrosinases, catecholases, and laccases belong to the family of POs.
413 While tyrosinase possesses monophenolase (hydroxylation of monophenols) and catecholase (oxidation
414 of diphenols) activities, catecholase only possesses catecholase activities and laccase possesses both
415 catecholase and laccase activities (oxidation of o-, p- and m-diphenols and other non phenolic substrates
416 such as diamines). In a recent study, two types of PO activity, catecholase and laccase, were detected in
417 *C. gigas* (Luna-Acosta et al., 2010b). In the present study, catecholase activities were generally inhibited
418 in the presence of hydrocarbons, except for HLS catecholase activity. These results are in agreement with
419 other studies on *C. gigas* where exposure to hydrocarbons induced an inhibitory effect in PO activities
420 (Bado-Nilles et al., 2008). Moreover, when using PPD as a substrate for PO assays in this study, laccase
421 activities were generally stimulated in the presence of hydrocarbons, except for laccase activity in plasma.
422 These results are in agreement with previous studies where the exposure of *C. gigas* to a light cycle oil
423 (LCO) soluble fraction induced an increase in the mRNA expression of a laccase (multicopper oxidase)
424 gene in haemocytes after 7 days of exposure (Bado-Nilles et al., 2010). Since laccases can catalyze
425 oxidation of aromatics, such as PAHs, by an indirect mechanism involving the participation of an
426 oxidative mediator (Dodor et al., 2004), an increase in laccase gene transcription in *C. gigas* may enable
427 protection against bioaccumulated PAHs. Laccase could therefore be a potential defence biomarker
428 candidate in ecotoxicological studies.

429 Lysozyme is one of the most important bacteriolytic agents against several species of Gram-positive and
430 Gram-negative bacteria, and has been recorded for various bivalve species (McHenery et al., 1986).
431 During phagocytosis, the release of lysosomal enzymes, such as lysozymes, participates in the

432 inactivation of invading pathogens. In the present study, inhibition of lysozyme activity was observed in
433 the digestive gland in the D condition. Previous studies have shown inhibition of lysozyme activity or
434 lysozyme gene expression in organisms exposed to hydrocarbons (Boutet et al., 2004; Gopalakrishnan et
435 al., 2009). However, in this study no lysozyme inhibition was observed in the presence of hydrocarbons,
436 except in the digestive gland for the D condition. Activation of mantle lysozyme activity was observed
437 during the depuration period. Although generally pollutant exposure has shown to inhibit lysozyme
438 activity in bivalves (for review, see Giron-Perez, 2010), some studies have shown an induction of
439 lysozyme activity (e.g., Oliver et al., 2003; Ordas et al., 2003; Hannam et al., 2009). However, to our
440 knowledge, no studies have shown the modulation of lysozyme activity during the depuration period. The
441 activation of this enzyme activity suggests a potential immunostimulation during the depuration period.

442 Uptake of xenobiotics from seawater is generally accomplished by filtration over the gill structure,
443 although diffusion through the integument may also contribute to tissue concentrations (Landrum and
444 Stubblefield, 1991). As the organism directs seawater over the gill surface to extract oxygen, hydrophobic
445 contaminants are very efficiently taken up because of the large surface area and lipid-rich membranes.
446 Moreover, bivalves possess an open circulatory system and are thus continually exposed to fluctuations of
447 environmental factors including contaminants (Cheng, 1981). As a general trend, enzyme activities in the
448 plasma and gills were generally inhibited, especially in the CD condition (Figs. 2-7), suggesting that
449 compartments that are the more exposed to the marine environment, such as the gills and plasma, are
450 likely to be more affected by the presence of hydrocarbons, in comparison to tissues less exposed to the
451 marine environment, such as the digestive gland, mantle and HLS (Cheng, 1981; Frouin et al., 2007).

452 Enzyme activities in the digestive gland were less modulated than in the other tissues but were strongly
453 correlated with PAH contents in this tissue, highlighting its role in xenobiotic detoxification (Chafai-El
454 Alaoui, 1994; Chu et al., 2003).

455 Enzyme activities in the mantle and haemocytes, which are known to play an important role in defence
456 mechanisms in oysters (Cheng, 1981), were generally activated in comparison to the control.

457 Among tissues that were analysed, haemolymph can be considered as a key tissue because this fluid
458 irrigates the whole body and, therefore, it can distribute contaminants and/or their metabolites throughout
459 the organism (Cheng, 1981). Interestingly, enzyme activities differed notably from the plasma and HLS

460 of the haemolymph. These differences could be due to alterations in the membrane integrity by PAHs
461 and/or their metabolites, as suggested by *in vitro* and *in vivo* investigations in the blue mussel *Mytilus*
462 *edulis* (Grundy et al., 1996). The extent of membrane alterations could be dependent on the physical (e.g.
463 linear versus angular or branched configuration of isomers) and/or chemical (e.g. low molecular weight
464 with high solubility versus high molecular weight with low solubility) properties of the compounds. In
465 addition, modulation of enzyme activities in the plasma can be attributed to 1) normal mechanisms, such
466 as secretion or 2) pathological features, such as cell lysis. Indeed, PAHs can cause cytolysis in
467 haemocytes (McCormick-Ray, 1987), due to 1) a depletion or stimulation of metabolites or coenzymes, 2)
468 an inhibition or stimulation of enzymes and other specific proteins, 3) an activation of a xenobiotic to a
469 more toxic molecular species, or 4) membrane disturbances (for review, see Moore 1985). Cytolysis may
470 lead to 1) an increase in haemocyte number indicating compensation for cell lysis and/or 2) the release of
471 cell contents in the plasma and consequently, a significant increase in plasma enzyme activities.
472 Alternatively, direct induction or repression by PAHs of humoral factors, such as lysozyme (Luna-
473 Gonzalez et al., 2004) and pro-phenoloxidase (Söderhäll and Cerenius, 1998), may contribute to the
474 modulation of enzyme activities found in the plasma.

475

476 **4.3. Effect of chemical dispersion (CD) and dispersant (D) conditions**

477 Increased activity of an enzyme involved in defence mechanisms can be interpreted as a response of the
478 organism to protect itself against a non-self molecule, e.g. xenobiotics. Persistent or excessive activation
479 can lead to overstimulation of the immune system, which can be detrimental to the organism. Inhibition
480 of the enzyme activity can be interpreted as saturation of the system because of the presence of a
481 xenobiotic, overpowering of antioxidant enzymes by oxygen radicals, or an immunodeficiency resulting
482 in direct or an indirect inhibition of mechanisms that modulate this enzyme activity (Huggett et al., 1992).
483 In both cases, changes in defensive enzymes may affect the survival of the organisms when challenged
484 with infectious pathogens (Thiagarajan et al., 2006).

485 Twenty nine enzymatic analyses were carried out in this study. As a general trend and relative to the
486 control, CD modulated a higher number of enzyme activities than D, following the exposure period.
487 Indeed, an equal or greater effect was observed in 13/18 (i.e. 13 out of 18) enzyme activities modulated

488 by CD and/or D: 5/5, 2/3, 3/3, 3/3 and 0/4 enzyme activities modulated by CD and/or D in the gills,
489 digestive gland, mantle, plasma and HLS, respectively (Figs. 2-7). When comparing CD and MD
490 conditions, following the exposure period, CD modulated a higher number of enzyme activities than MD,
491 relative to the control condition, with an equal or greater effect observed in 13/19 activities modulated by
492 CD and/or MD: 4/5, 2/2, 3/4, 4/5 and 0/4 enzyme activities modulated by CD and/or MD in the gills,
493 digestive gland, mantle, plasma and HLS, respectively (Figs. 2-6). Moreover, CD exerted an equal or
494 greater effect in a higher number of enzyme activities in the gills and plasma in comparison to other
495 tissues, i.e. 4/5 enzyme activities modulated by CD and/or MD (Figs. 2-6) , suggesting that the gills and
496 plasma are sensitive compartments in *C. gigas*. Moreover, some enzyme activities that were modulated by
497 the CD treatment following the exposure period were also modulated following the depuration period,
498 e.g. SOD activity in the gills, mantle and plasma (Fig. 2), or laccase activity in the plasma (Fig.6),
499 suggesting that CD may exert long-term effects. Importantly, D also modulated enzyme activities,
500 especially at the end of the depuration period, e.g. SOD activity in the plasma and HLS (Fig. 2), catalase
501 activity in the digestive gland (Fig. 3), GPx activity in the plasma (Fig. 4), catecholase activity in the
502 plasma and HLS (Fig. 5), laccase activity in the gills and digestive gland (Fig. 6) and lysozyme activity in
503 the mantle (Fig. 7), suggesting that the dispersant used in the present study could also induce long-term
504 effects, i.e. following 15 days of depuration. Our results are thus in agreement with previous studies that
505 have shown the effects of other third generation dispersants on biological functions in marine
506 invertebrates (Gilfillan et al., 1984; Shafir et al., 2007).

507 Differences between oysters from the control and the treatment conditions resided only in the presence of
508 oil and/or dispersants in the water column (i.e. experimental devices and acclimatization conditions were
509 identical in the control and the treatment conditions). Additionally, the D treatment (i.e. in the absence of
510 oil) induced effects in enzyme activities such as laccase in the gills, SOD, catalase and lysozyme in the
511 digestive gland, and SOD in the plasma, following the exposure or the depuration periods. Therefore,
512 significant differences in biological responses between the control and the different treatments could not
513 be considered as specific to contamination by hydrocarbons, but rather as indicators of unspecific stress in
514 oysters, induced by chemicals (oil and/or dispersants) that have entered the organism from the water
515 column. It is important to notice that transient effects were observed for different enzyme activities and

516 thus longer periods of exposure should be studied. Moreover, not all the enzyme activities returned to
517 control levels after the 15-day depuration period and thus longer depuration periods could give better
518 insight into long-term effects.

519

520 **5. Conclusions**

521

- 522 • Responses of enzyme activities involved in immune and detoxification mechanisms in juveniles
523 of the Pacific oyster *C. gigas* were highly variable depending on the treatment, the time and the
524 tissue that was studied, highlighting the importance of carrying out studies in different tissues and
525 with multiple enzymes involved in crucial biological responses.
- 526 • Enzyme activities were generally inhibited in the gills and plasma, while they were generally
527 activated in the mantle and haemocytes, suggesting that the gills and the plasma are sensitive
528 compartments in *C. gigas* and that the mantle and the haemocytes are likely to play an important
529 role in protection against PAHs.
- 530 • Among the various parameters and during the exposure period, enzyme activities in the digestive
531 gland, mantle and haemocytes were generally positively correlated with PAH body burdens.
532 Enzyme activities in the gills and plasma were generally negatively correlated with PAH body
533 burdens, suggesting potential suppressive effects of pollutants in immune and/or detoxification
534 mechanisms through the inhibition of enzyme activities involved in these biological responses.
- 535 • Finally, the presence of dispersants or of chemically dispersed oil modulates different biological
536 responses in *C. gigas*. These results raise questions as to the potential effects of chemically
537 dispersed oil in nearshore areas on immune and/or detoxification responses for this estuarine
538 species, such as in the case of the Deepwater Horizon oil spill, in which a high percentage of oil
539 was dispersed in the water column, with a large number of unknowns on the long-term impact of
540 the oil-dispersant association.

541

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553

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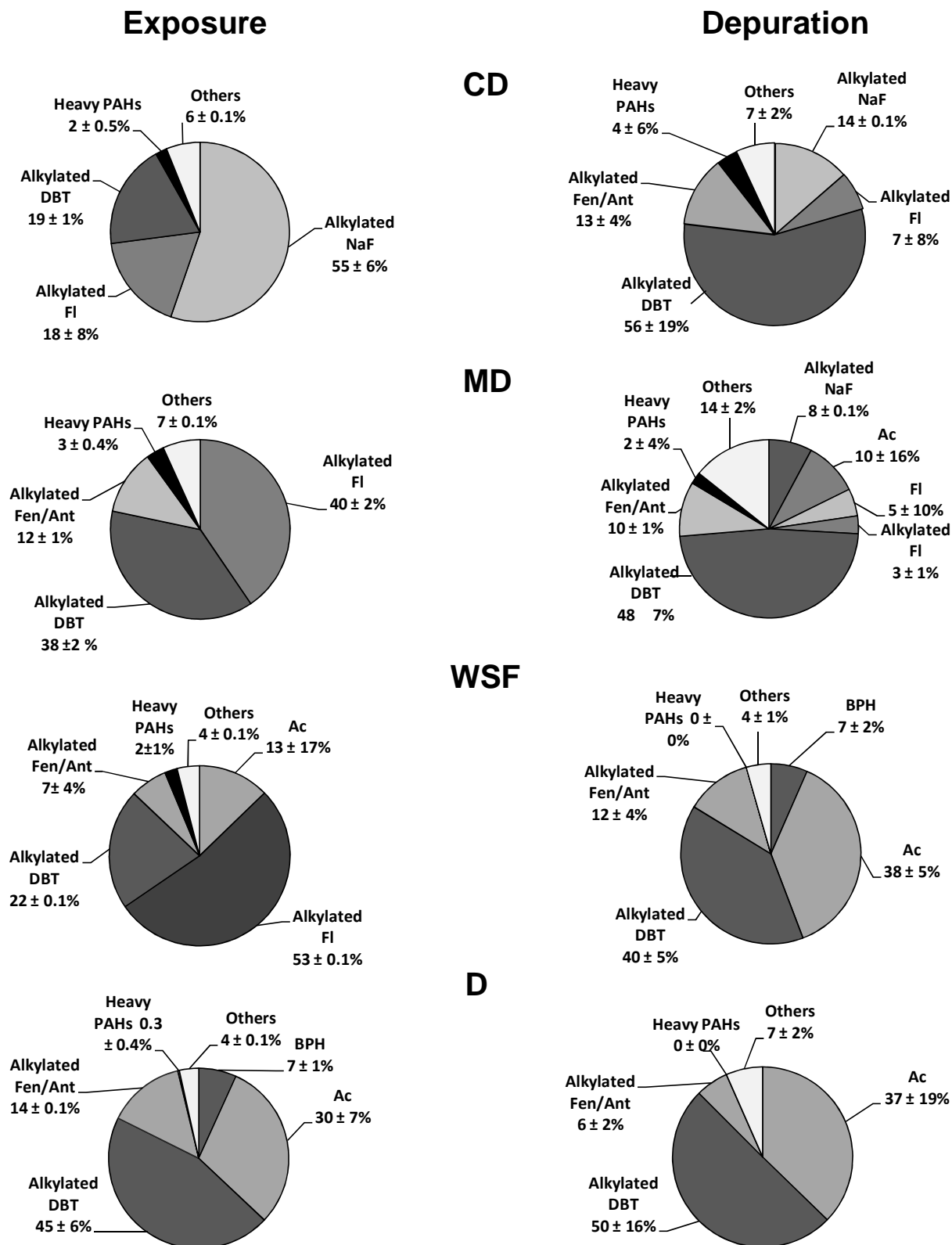
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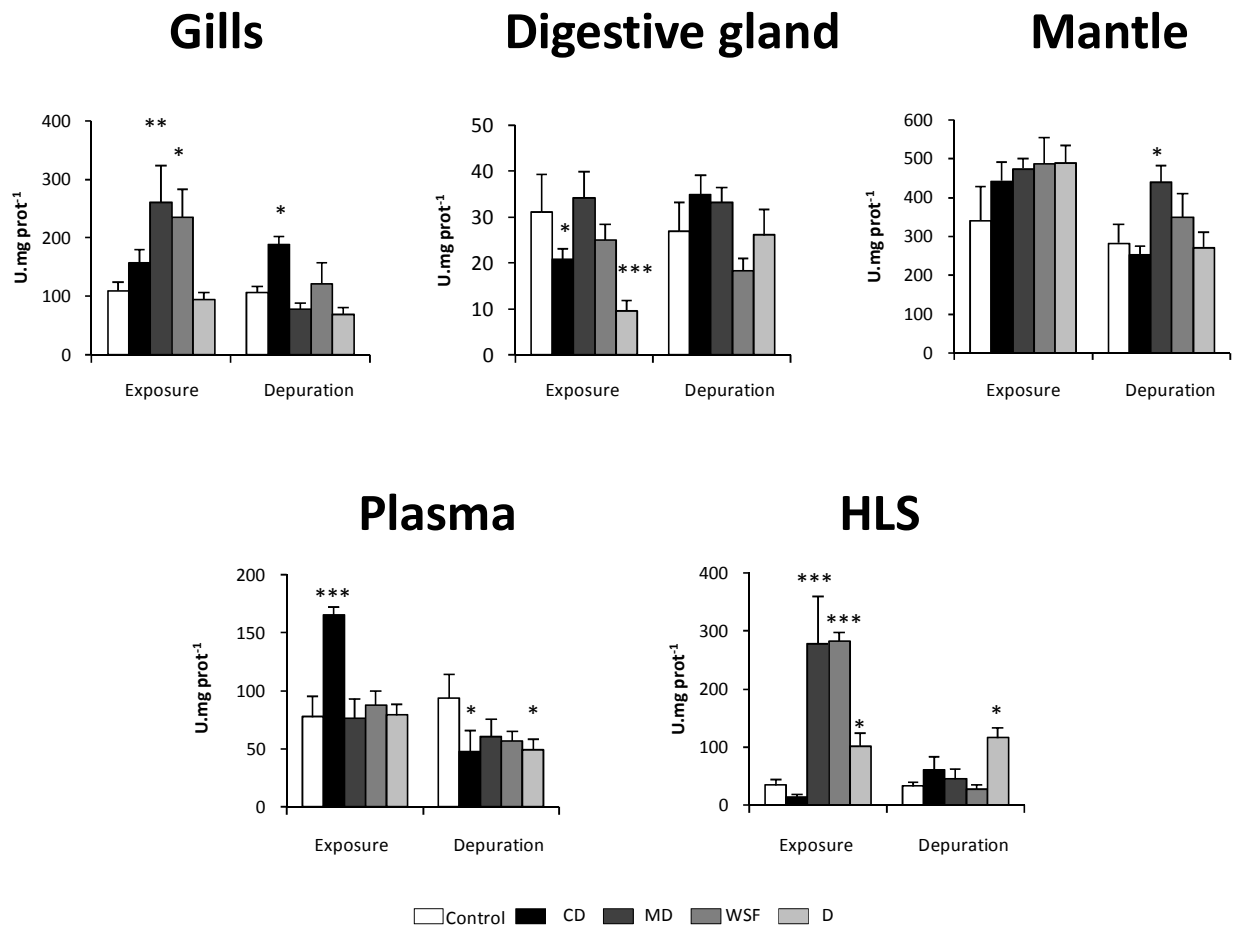
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716

717 **Figure 1. Proportion (% , mean \pm SD, n=3) of the main PAHs contained in oyster tissues after 2 days of**
 718 **exposure to chemical dispersion (CD), mechanical dispersion (MD), water soluble fraction (WSF) and**
 719 **dispersant (D) treatments, followed by 15 days of depuration. PAHs: polycyclic aromatic hydrocarbons; NaF:**
 720 **naphthalene; Ac: acenaphthylene; Fl: fluorene; Fen: phenanthrene; Ant: anthracene; DBT: dibenzothiophene.**



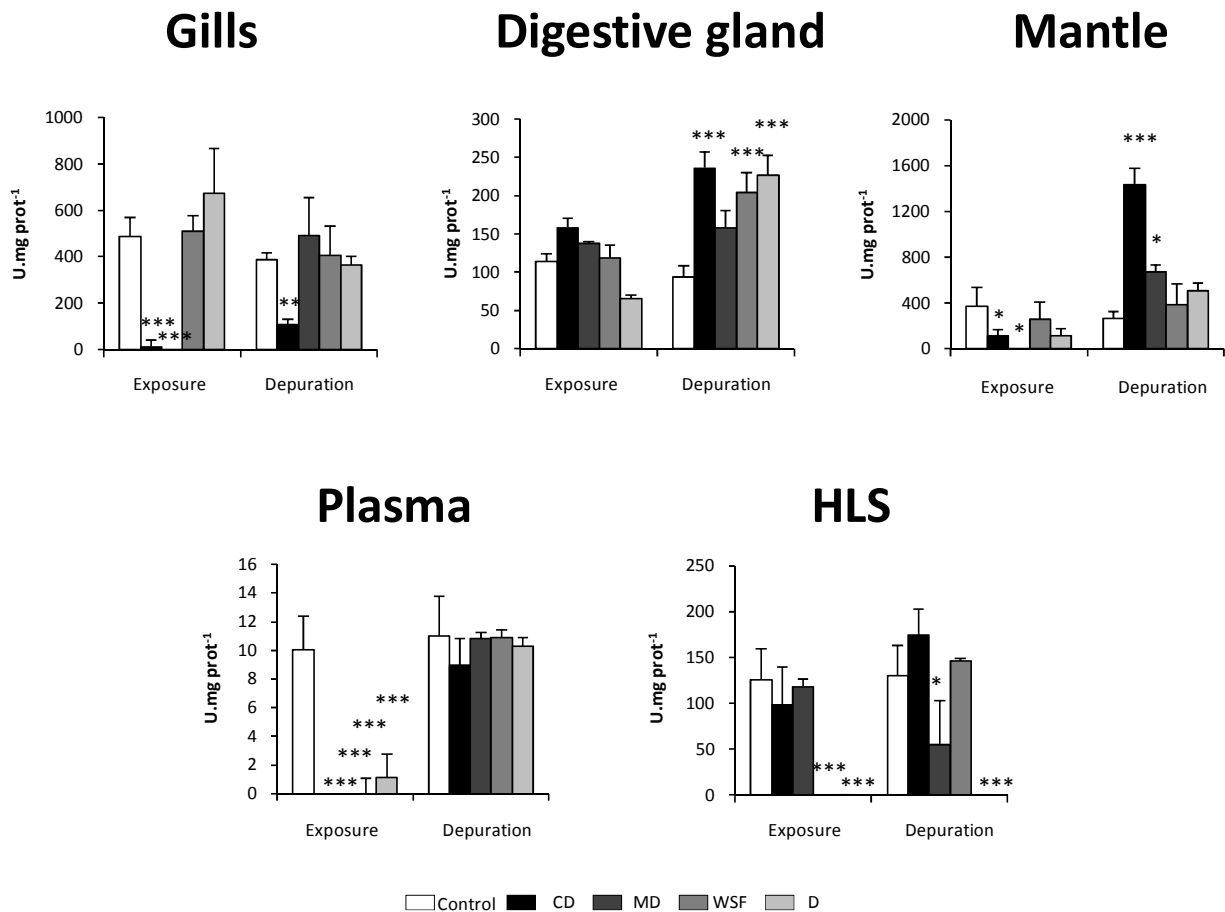
722 **Figure 2. Superoxide dismutase activity in the gills, digestive gland, mantle, plasma and haemocyte lysate**
 723 **supernatant (HLS) in *C. gigas* after 2 days of exposure to chemical dispersion (CD), mechanical dispersion**
 724 **(MD), water soluble fraction (WSF) and dispersant (D) treatments, followed by 15 days of depuration.** Data
 725 are expressed as mean \pm SD U.mg prot⁻¹, n=9 (i.e. 3 sample replicates from 3 experiment replicates). For clarity of
 726 results, only significant differences between control and treatment and for a given time (after 2 d of exposure or after
 727 15 d of depuration) are shown; * p<0.05, ** p<0.01, *** p<0.001.



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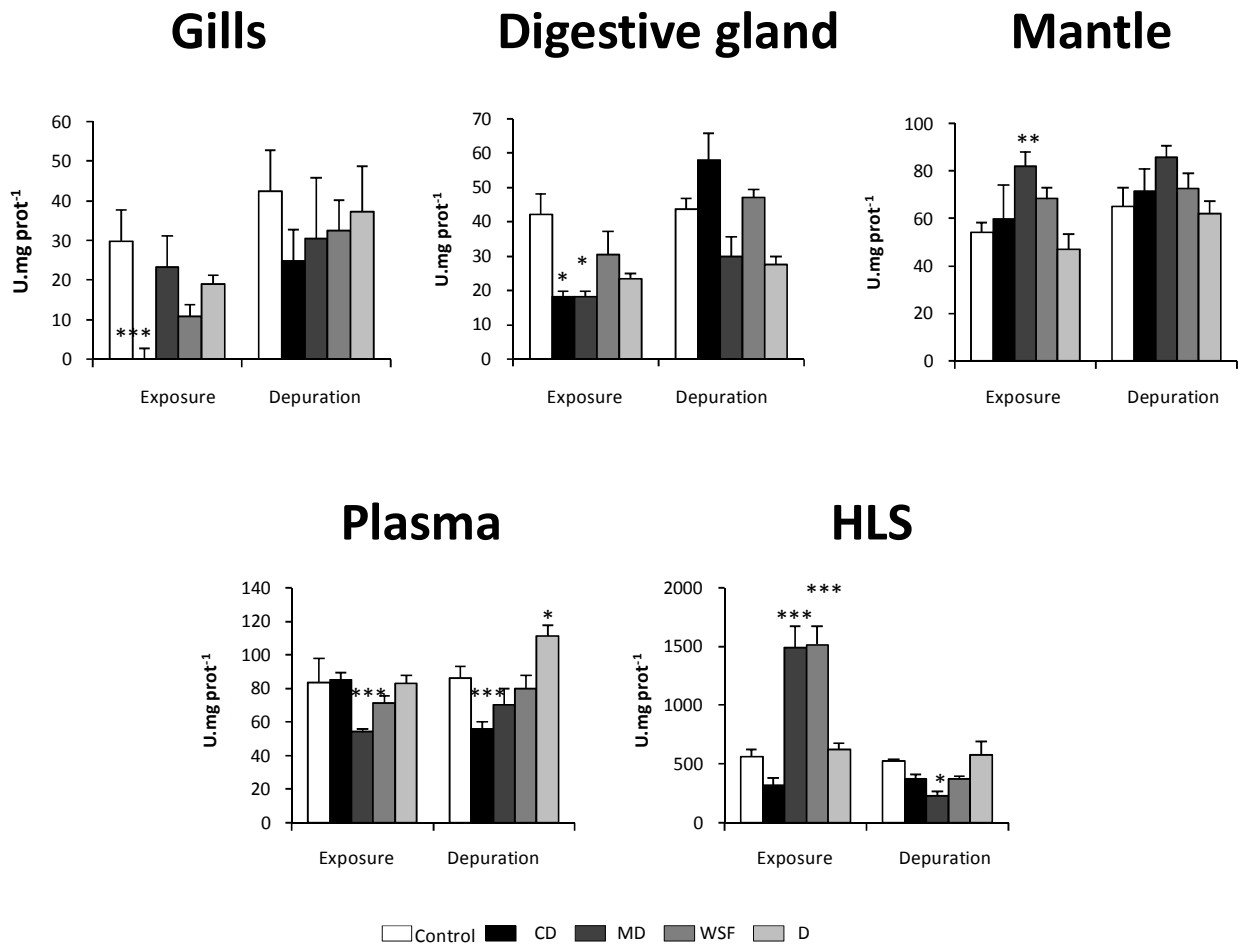
730 **Figure 3. Catalase activity in the gills, digestive gland, mantle, plasma and haemocyte lysate supernatant**
 731 **(HLS) in *C. gigas* after 2 days of exposure to chemical dispersion (CD), mechanical dispersion (MD), water**
 732 **soluble fraction (WSF) and dispersant (D) treatments, followed by 15 days of depuration.** Data are expressed
 733 as mean \pm SD U.mg prot⁻¹, n=9 (i.e. 3 sample replicates from 3 experiment replicates). For clarity of results, only
 734 significant differences between control and treatment and for a given time (after 2 d of exposure or after 15 d of
 735 depuration) are shown; * p<0.05, ** p<0.01, *** p<0.001.



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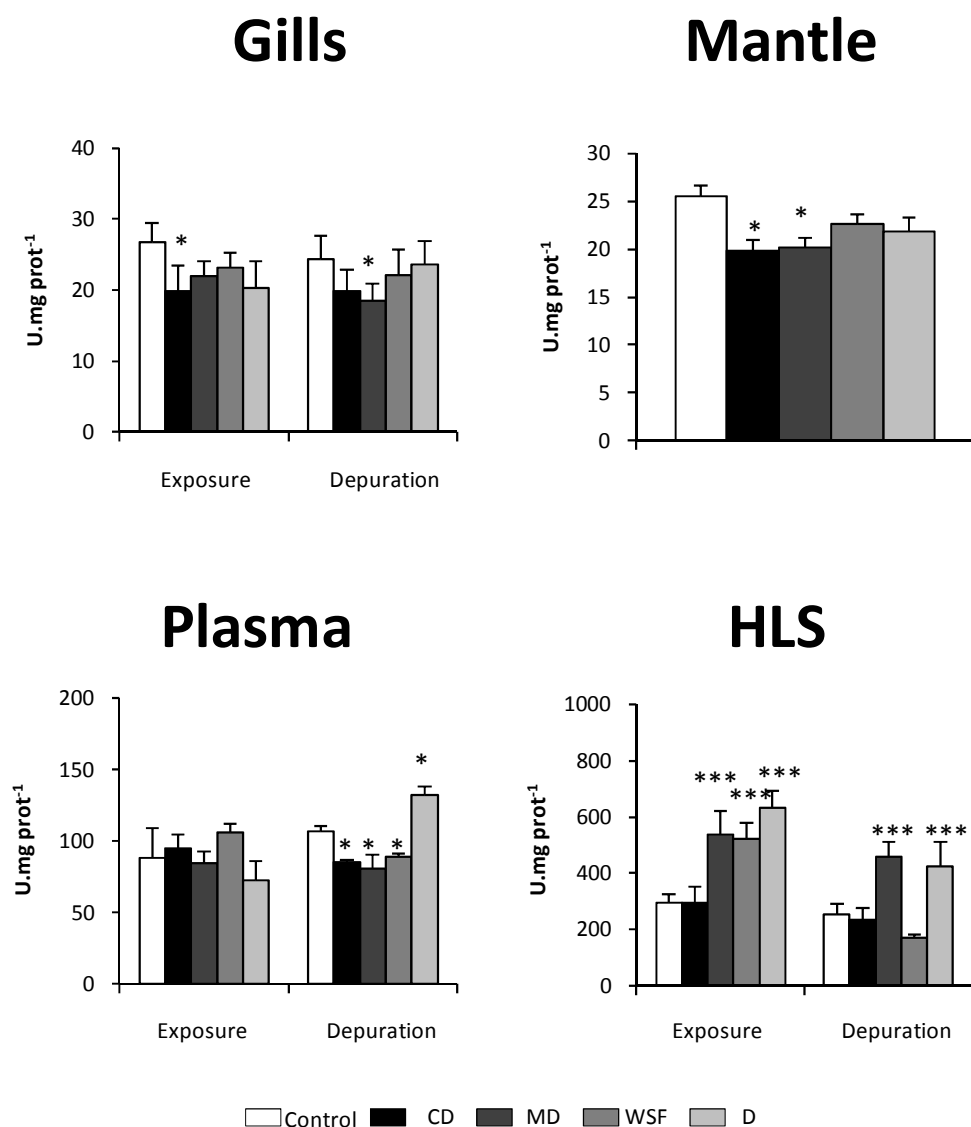
738 **Figure 4. Glutathione peroxidase activity in the gills, digestive gland, mantle, plasma and haemocyte lysate**
 739 **supernatant (HLS) in *C. gigas* after 2 days of exposure to chemical dispersion (CD), mechanical dispersion**
 740 **(MD), water soluble fraction (WSF) and dispersant (D) treatments, followed by 15 days of depuration.** Data
 741 are expressed as mean \pm SD U.mg prot⁻¹, n=9 (i.e. 3 sample replicates from 3 experiment replicates). For clarity of
 742 results, only significant differences between control and treatment and for a given time (after 2 d of exposure or after
 743 15 d of depuration) are shown; * p<0.05, ** <p0.01, *** p<0.001.



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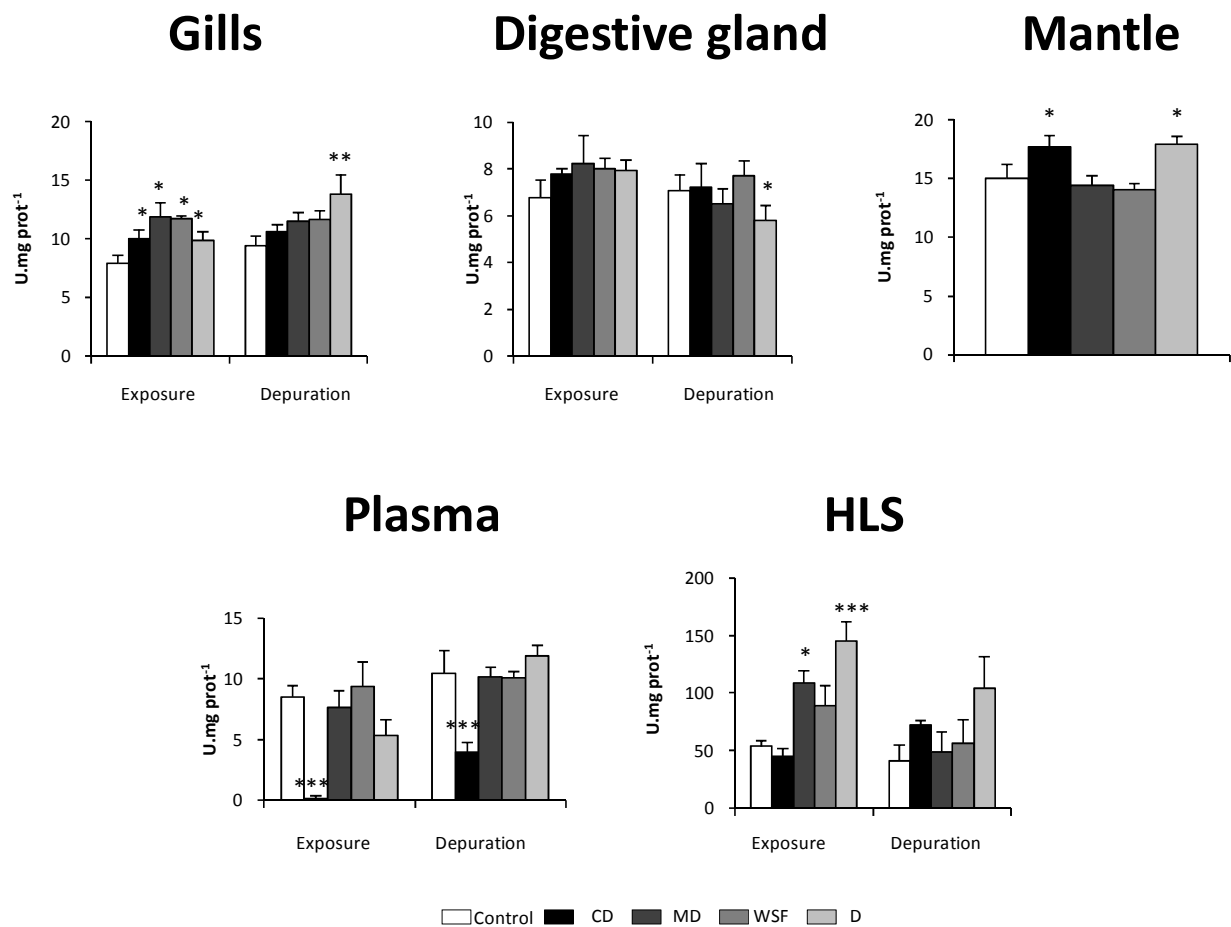
746 **Figure 5. Catecholase-type phenoloxidase activity in the gills, digestive gland, mantle, plasma and haemocyte**
 747 **lysate supernatant (HLS) in *C. gigas* after 2 days of exposure to chemical dispersion (CD), mechanical**
 748 **dispersion (MD), water soluble fraction (WSF) and dispersant (D) treatments, followed by 15 days of**
 749 **depuration.** Data are expressed as mean \pm SD U.mg prot⁻¹, n=9 (i.e. 3 sample replicates from 3 experiment
 750 replicates). For clarity of results, only significant differences between control and treatment and for a given time
 751 (after 2 d of exposure or after 15 d of depuration) are shown. For the mantle, the enzyme activity was not affected by
 752 both treatment and period, but only by treatment. Therefore, results represent the mean of overall data from
 753 exposure and depuration periods. * p<0.05, ** p<0.01, *** p<0.001.



754

755

756 **Figure 6. Laccase-type phenoloxidase activity in the gills, digestive gland, mantle, plasma and haemocyte**
 757 **lysate supernatant (HLS) in *C. gigas* after 2 days of exposure to chemical dispersion (CD), mechanical**
 758 **dispersion (MD), water soluble fraction (WSF) and dispersant (D) treatments, followed by 15 days of**
 759 **depuration.** Data are expressed as mean \pm SD U.mg prot⁻¹, n=9 (i.e. 3 sample replicates from 3 experiment
 760 replicates). For clarity of results, only significant differences between control and treatment and for a given time
 761 (after 2 d of exposure or after 15 d of depuration) are shown. For the mantle, the enzyme activity was not affected by
 762 both treatment and period, but only by treatment. Therefore, results represent the mean of overall data from
 763 exposure and depuration periods. * p<0.05, ** p<0.01, *** p<0.001.



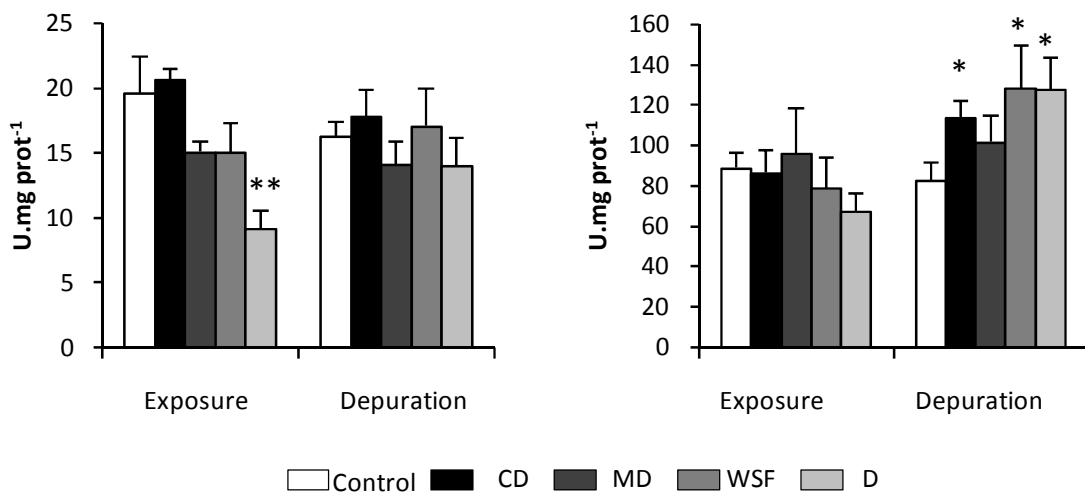
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766 **Figure 7. Lysozyme activity in the digestive gland and the mantle in *C. gigas* after 2 days of exposure to**
 767 **chemical dispersion (CD), mechanical dispersion (MD), water soluble fraction (WSF) and dispersant (D)**
 768 **treatments, followed by 15 days of depuration.** Data are expressed as mean \pm SD U.mg prot⁻¹, n=9 (i.e. 3 sample
 769 replicates from 3 experiment replicates). For clarity of results, only significant differences between control and
 770 treatment and for a given time (after 2 d of exposure or after 15 d of depuration) are shown; * p<0.05, ** p<0.01,
 771 *** p<0.001.

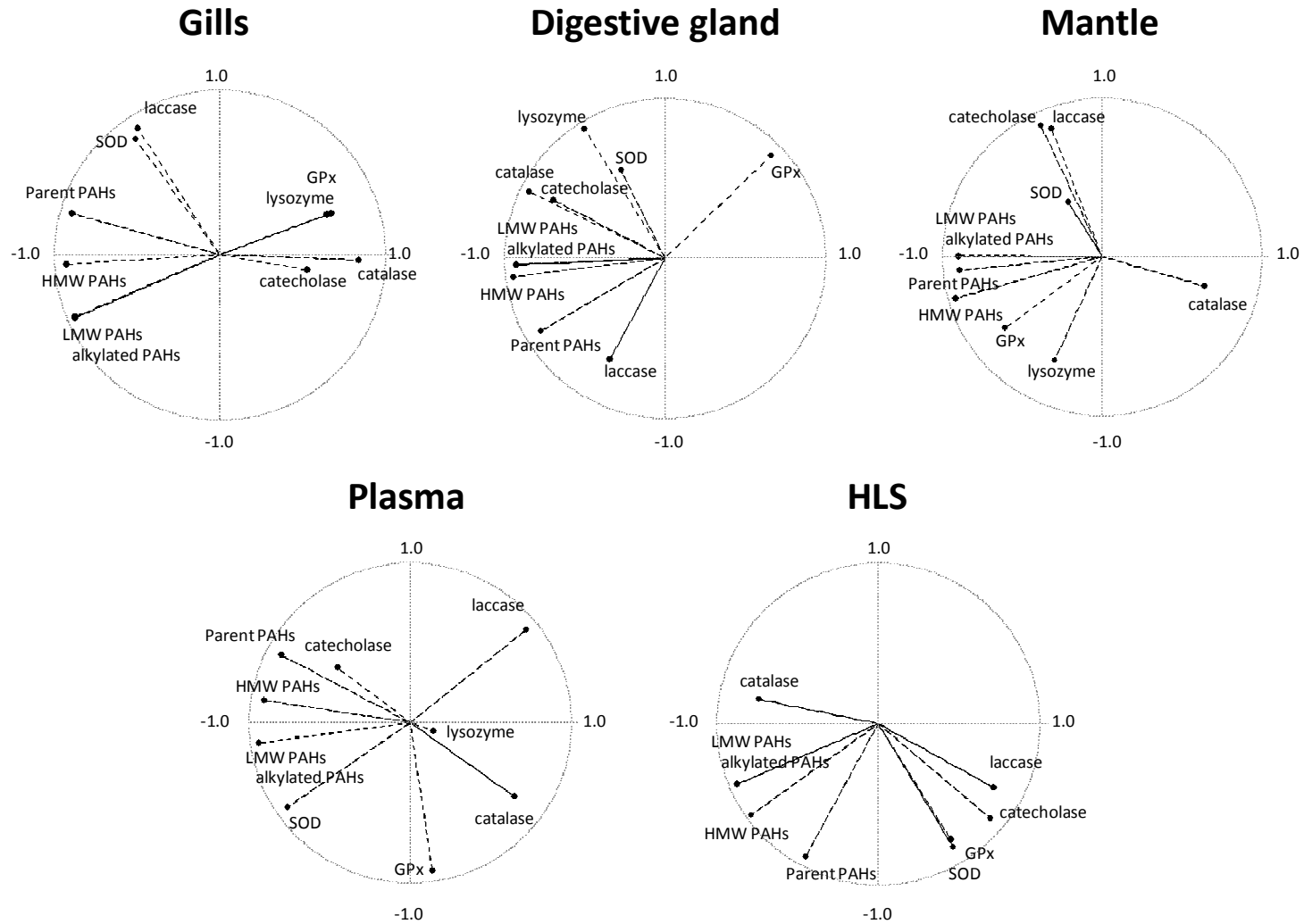
Digestive gland

Mantle



772

773 **Figure 8. Principal component analysis (PCA) of enzyme activities and PAH body burdens for the gills, digestive gland, mantle, plasma and HLS.** Eigen values of the
 774 first two axes are 5.54 and 1.57, for the gills, 5.30 and 2.30, for the digestive gland, 4.45 and 2.19, for the mantle, 4.99 and 2.03, for the plasma, and, 4.31 and 2.88, for the
 775 HLS, respectively.



777 **Table 1. Experimental conditions used in the study.** CD: chemical dispersion, MD: mechanical dispersion, WSF:
778 water soluble fraction and D: dispersant. BAL 110: Brut Arabian Light crude oil topped at 110°C.
779

	Quantity of the product added to the water column (mg.l ⁻¹) [#]	
	BAL 110	Dispersant
Control	0	0
CD	67	4
MD	67	0
WSF	67	0
D ((internal control of CD)	0	4

780 [#] In order to have a stable concentration of the products in the water column, the products were added 13 hours
781 before introducing animals in the tanks
782

783 **Table 2 Relationships between enzyme activities in tissues and haemolymphatic compartments and body**
 784 **burdens of the various PAH categories measured in the whole organism.**

785

	Gills	Digestive gland	Mantle	Plasma	HLS
Significance of enzyme activities-PAH body burden relationship (RDA; Monte Carlo test, <i>p</i> -value)	0.002	0.009	0.001	0.021	0.025
Amount of total variation in enzyme activities explained by PAH body burdens (%)	48.6	42.5	43.3	38.7	43.5
Correlation with RDA 1st axis					
HMW PAHs	0.778	0.822	0.382	0.673	0.183
LMW PAHs	0.631	0.756	0.620	0.776	0.369
Parent PAHs	0.871	0.618	0.873	0.623	-0.216
Alkylated PAHs	0.630	0.758	0.380	0.774	0.373
Trend with increasing body burden of the various PAH categories, i.e. HMW, LMW, parent and alkylated PAHs					
SOD	↗	↗	↗	↗	↘ (↗) ^a
Catalase	↘	↗	↘	↘	↗ (↘)
GPx	↘	↘	↗	↘	↘ (↗)
Catecholase	↘	↗	↗	↗	↘ (↗)
Laccase	↗	↗	↘	↘	↘ (↗)
Lysozyme	↘	↗	↗	↘	No data

786 ^a The arrows between parenthesis show the trend associated with body burden of parent PAHs