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Effect of exposure to benzo[a]pyrene on SODs, CYP1A1/1A2- and CYP2E1 immunopositive proteins in the blood clam *Scapharca inaequivalvis*

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1 **Effect of exposure to benzo[a]pyrene on SODs, CYP1A1/1A2- and CYP2E1**
2 **immunopositive proteins in the blood clam *Scapharca inaequalis***

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28 **Abstract**

29 The effects of water-borne exposure to benzo[a]pyrene (36 hrs; celite-bound 0.44 mg L⁻¹ B[a]P) on
30 cytochrome P450 (CYP) and superoxide dismutases (SODs) were examined in digestive gland of
31 the blood clam, *Scapharca inaequivalvis*. B[a]P accumulation and elimination were rapid, with
32 maximum whole-body concentrations of 1.78 ng g⁻¹ wet wt. after 12 hours of treatment, followed
33 by a progressive decline to 0.89 ng g⁻¹ at 36 hours. The presence of B[a]P resulted in an increase in
34 total CYP of digestive gland microsomes from 54 ± 14 to 108 ± 21 pmol mg⁻¹ protein (mean ± SD;
35 p<0.05, 24 hrs). Increases were also seen in microsomal CYP1A1/1A2-immunopositive protein
36 (50.5 kDa app. mol. wt; p<0.05), but not CYP2E1-immunopositive protein (49 kDa app. mol. wt.),
37 indicating a specific response of the former isoform. Exposure to B[a]P produced a steady increase
38 in Mn-SOD digestive gland activity (p<0.01; p<0.05) but no significant change in Cu/Zn-SOD
39 activity. The respective proteins, measured by western blotting, weren't significant induced after
40 B[a]P exposure.

41 Cu/Zn-SOD and Mn-SOD activities were correlated with total CYP levels (r = 0.96 and 0.63,
42 respectively), indicating a role for CYP in reactive oxygen species (ROS) production during
43 exposure. Both 'NADPH-independent' and NADPH-dependent metabolism of B[a]P by digestive
44 gland microsomes was seen, producing mainly 1,6-, 3,6- and 6,12-diones, with some phenols and
45 7,8-dihydrodiol; putative protein adducts were also formed. Redox cycling of the diones may also
46 have contributed to ROS production, leading to the increased SOD activities.

47

48 **Keywords:**

49 Benzo[a]pyrene metabolism, cytochrome P450, CYP1A1/1A2- and CYP2E1-immunopositive
50 proteins, SOD isoforms, *Scapharca inaequivalvis*, digestive gland..

51 **1. Introduction**

52 Polycyclic aromatic hydrocarbons (PAHs), particularly benzo[a]pyrene (B[a]P), are common
53 pollutants in the environment (Caricchia et al., 1993, Magi et al., 2002), including the Adriatic
54 coast, and of great concern for their toxicity, mutagenicity and carcinogenicity (Gomez-Mendikute
55 et al., 2002) and consequently have been widely studied in aquatic ecotoxicology.

56 Bivalve molluscs bioaccumulate lipophilic organic pollutants such as B[a]P (Durand et al., 2002)
57 and in these aquatic organisms *in vivo* and *in vitro* B[a]P biotransformation to primary metabolites
58 (phenols, dihydrodiols, and mainly quinones) and conjugates has been widely demonstrated
59 (Livingstone, 1998). This first step in the metabolism of PAHs was catalysed by cytochrome P450
60 (CYP) monooxygenase (or mixed-function oxygenase) system (EC 1.14.14.1).

61 CYPs exist in multiple forms classified into families, subfamilies and individual members, that
62 catalyse many reactions involved in both endogenous metabolism and oxidative metabolism of
63 xenobiotics (Mansuy, 1998; Gonzalez, 2005). Families CYP1 to CYP4 are involved in the
64 metabolism of xenobiotics (Nelson et al., 2004; Gonzalez, 2005), with the CYP1A subfamily being
65 of major importance in all of vertebrate classes in the metabolism and detoxication/activation of
66 PAHs and organochlorines (Teramitsu et al., 2000). Both these latter types of compounds in
67 vertebrates bind to the aryl hydrocarbon receptor (AHR) leading to increased CYP1A
68 expression/activity and increased toxicity by several mechanisms, including enhanced reactive
69 oxygen species (ROS) production (Chae et al., 1993; Liu et al., 2001). Moreover vertebrate
70 CYP2E1 is involved in the metabolism and activation of a large number of low molecular weight
71 substances, such as aliphatic, aromatic and halogenated hydrocarbons (Guengerich et al., 1991), and
72 it is one of the most active CYPs in ROS production (Gonzalez, 2005).

73 The CYP monooxygenase system and associated catalytic activities, such as BaP hydroxylase, are
74 widely distributed throughout aquatic invertebrate taxa overall in the digestive gland, the main
75 organ of xenobiotic metabolism (Solé and Livingstone, 2005)

76 The CYP1A-like protein in mytilids has been implicated in the metabolism of B[a]P (Wootton et
77 al., 1996 Canova et al., 1998). No complete CYP1A sequence has yet been detected in molluscs
78 (Herron, 2004), indicating that the protein(s) responsible for B[a]P metabolism and/or recognised
79 by fish anti-CYP1A (see above) likely belong to a different, possibly unique, CYP group.

80 Enhanced production of ROS, is thought to be an important mechanism of pollutant-mediated
81 toxicity in aquatic organisms (Livingstone, 2001). An imbalance of ROS and other pro-oxidant
82 production over antioxidant defences results in increased oxidative damage to unsaturated lipid,
83 DNA and other key molecules, resulting in a condition of oxidative stress (Halliwell and
84 Gutteridge, 1999). The extent of such damage is dependent on the effectiveness of the antioxidant
85 defences (Michiels and Remacle, 1988), including the key antioxidant enzyme superoxide
86 dismutase (SOD; EC. 1.15.1.1) (Halliwell and Gutteridge, 1999). CYP-catalysed metabolism of
87 B[a]P, resulting in ROS generation, (Sjölin and Livingstone, 1997), has been proposed as a
88 pollutant-mediated mechanism of toxicity in molluscs (Livingstone, 2001). SOD is widely
89 distributed in molluscs (Doyotte et al., 1997; Regoli et al., 1997), and changes in its activity and
90 form have been proposed as a biomarker of pollutant-mediated oxidative stress (Livingstone, 2001;
91 Orbea et al., 2002).

92 The main aim of this research was to study the response of *S. inaequalvis* to short-term exposure
93 to water-borne B[a]P, in terms of i) total CYP content, ii) responses of putative CYP isoenzymes;
94 and iii) response of SOD activity and expression. Additionally, the metabolite profile of digestive
95 gland microsomal metabolism of B[a]P was determined in control animals to examine whether
96 redox cycling quinones were major products.

97 The blood clam, *Scapharca inaequalvis* is a species of bivalve mollusc of Indo-Pacific origin,
98 known to be present in the Adriatic Sea region since at least 1972. It is an infaunal organism, living
99 burrowed in sandy-muddy sediments, with some unusual physiological characteristics, including in
100 particular haemoglobin-containing blood cells and a high tolerance to hypoxia/anoxia (Cattani and
101 Vitali 1995). It is known to be exposed to PAHs, with levels in sediments in the region varying

102 from 24 to 501 ng per g dry weight in coastal areas (Chioggia and Ancona) (Magi et al., 2002) and
103 up to 557 ng per g dry weight in harbours (Trieste) and river mouths (Caricchia et al., 1993).

104 The novelty was represented by the study and discussion of these global relationships among
105 different detoxification and antioxidant systems in *Scapharca inaequivalvis*, lack in similar works,
106 in fact to date most research on CYP responses in bivalves has been carried out on *Mytilus* sp., with
107 relatively little known for other species (Peters and Livingstone, 2001).

108 The paper focuses on several aspects of B[a]P metabolism in an important species of mollusc that
109 plays a crucial role in Adriatic ecological systems. Since the clam (*Scapharca inaequivalvis*) was
110 introduced some 30 yr ago it has been rapidly outcompeting native bivalves and in now the
111 dominant species (Ghisotti and Rinaldi, 1976). The presence of red blood cells (RBCs), obviously
112 determine a great advantage in resisting the main side effects of eutrophication (frequently in
113 Northern Adriatic sea), so during cycles of normoxia and severe lack of oxygen, *Scapharca*
114 activates fermentative pathways for supplementing ATP production at much lower pO₂ than RBC
115 lacking bivalves (de Zwaan et al., 1991).

116 This work provides a complete evaluation that considers the accumulated xenobiotic, P450 content
117 and expression, and the antioxidant defence involved in radical production. No comparable studies
118 have been conducted for *Scapharca inaequivalvis* therefore the result are discussed in relation to
119 information available for other bivalve species.

120

121 **2. Materials and methods**

122 2.1. Chemicals

123 All the reagents used for the determination of B[a]P levels, total CYP content and SOD activity
124 were obtained from Sigma Aldrich (USA), Roche Diagnostics (USA) and Merck &Co (USA).
125 Protein determination was performed with a Bio-Rad DC Protein Assay kit (USA). For
126 electrophoresis and western blotting: minigels, specific buffers and the chromogenic
127 immunodetection kit were purchased from Invitrogen (UK); polyvinylidene difluoride (PVDF)

128 membranes were from Bio-Rad (USA); the polyclonal rabbit anti-rat Mn-SOD, rabbit anti-human
129 Cu/ZnSOD and rabbit anti-rat CYP 2E1 antibodies were obtained from Stressgen (CAN), and the
130 polyclonal goat anti-rabbit CYP 1A1/1A2 was obtained by Oxford Biomedical Researches (USA);
131 and CuZnSOD standard was from Sigma Aldrich (USA). HPLC (High Performance Liquid
132 Chromatography) chemicals were from Merck or Packard UK and were HPLC or Scintran grade.
133 Authentic B[a]P metabolite standards were obtained from the NCI Chemical Carcinogen
134 Respository (Kansas City, USA).

135 2.2. Animals

136 Clams were collected in an uncontaminated area of Northern Adriatic Sea, by local fishermen using
137 a hydraulic dredge dragged over the sandy sea bottom. After collection, animals were suspended in
138 open baskets in wide aquaria with filtered sea water at a temperature of 17-18°C and at a salinity of
139 34‰, the sea water was pumped in from an inlet approximately 300 m offshore. Every second day,
140 50% of the water was changed. Bivalves were allowed to acclimatize to laboratory conditions for
141 one week before their utilization in exposure experiments. Clams for microsomal B[a]P metabolism
142 studies were collected separately and held in filtered sea water for a few days, without feeding, to
143 clear gut contents, prior to dissection; digestive glands were removed as described below for the
144 exposure experiment.

145 2.3. B[a]P exposure and animal dissection

146 Specimens of 4.5 ± 0.4 cm shell length were put in tanks containing filtered seawater (4 animals/L)
147 at a temperature of 17-18°C. Because of its low solubility in water, B[a]P was first adsorbed to a
148 solid phase constituted of celite[®] 545 (Merck & Co). B[a]P was added to celite as an acetone
149 solution and the organic solvent subsequently evaporated by rotary evaporation. After drying, celite
150 was added in a single administration to the sea water, to give a final B[a]P concentration of 0.44
151 mg/L, and maintained in suspension with the aid of a magnetic stirrer. Treated animals (not fed)
152 were compared with controls maintained in the same experimental condition except for the absence
153 of B[a]P bound to the added celite.

154 Animals were sampled at 0, 12, 24 and 36 hours of exposure. Two sets of animals were taken at
155 each sampling time for biochemical and chemical analyses. Digestive glands from the first set of
156 animals were immediately dissected out, damp-dried, frozen in liquid nitrogen and stored at -70°C .,
157 In order to have enough material for the biochemical analyses, a total of 18 clam were sampled for
158 each experimental condition. Digestive glands from each condition were pooled (three
159 pools/condition).

160 Analogous analyses of the B[a]P body-burden concentration were performed on whole tissues from
161 a second set of 18 animals for each experimental condition (three pools/condition).

162 2.4. Determination of tissue B[a]P levels

163 All solvents were distilled before use. Solid phase extraction (SPE) was performed with Isolute™
164 (SI 500 mg/3.0 ml) cartridges obtained from International Sorbent Technology Ltd. (Hengoed, UK).

165 A B[a]P standard (Min. 97%, HPLC grade, Sigma) was used.

166 Analyses were carried out using a Kontron System Pump 325 ternary liquid chromatograph
167 equipped with a 20 μl loop, combined with a Thermoquest FL3000 fluorescence detector. A
168 Supelcosil LC-PAH 5 μm column (250 x 4.6 mm I.D.) (Supelco, Bellefonte, PA, USA) was used.

169 After defrosting, 20-30g of sample (corresponding to one pool constituted to 6 animal) were
170 submitted to lipid extraction (Christie, 1982). The lipid fraction, after solvent evaporation, was
171 submitted to the partition, cleanup and chromatographic analyses after Moret et al., (1999).

172 For BaP fluorescence determination, excitation (Ex) and emission (Em) were: Ex=268 nm, Em=398
173 nm. The linearity and the quantification of the instrumental response were checked by injecting the
174 B[a]P standard prepared in five different concentrations ranging from 20 $\mu\text{g/L}$ up to 800 $\mu\text{g/L}$.

175 The recovery of B[a]P was of 80% and the CV (variation of coefficient) was of 7-8%.

176 2.5. Biochemical analyses

177 Digestive gland subcellular fractions were prepared essentially as described in Livingstone (1988).

178 All procedures were carried out at 4°C . Frozen pooled digestive gland was homogenized with an
179 Ultraturrax (or Potter-Elvehjem) homogenizer in 1:4 (w/v) homogenization buffer (20 mM Tris-

180 HCl pH 7.6, 0.5M sucrose, 0.15M KCl, 1mM EDTA). The homogenate and resulting supernatants
181 were progressively centrifuged at 500 g x 30 min, 12,000g x 45 min and 100,000g x 90 min to
182 obtain the microsomal and cytosolic fractions. The mitochondrial and cytosolic fractions were
183 combined together in the same ratio as in the original homogenate and used for the SOD
184 determinations (activity measures and western blotting). The microsomal pellet was washed by
185 resuspension in homogenization buffer and further centrifuged at 100,000g x 30 min. Finally the
186 washed microsomal pellet was suspended in 10mM Tris-HCl pH 7.6, 20% w/v glycerol, 1mM
187 EDTA to give a protein concentration of ~ 5-10mg protein/ml. Microsomal samples were used for
188 determination of total CYP content, CYP isoforms and B[a]P metabolism. If not used immediately,
189 the microsomal samples were aliquotted into Cryovials and stored under liquid nitrogen (-196°C),
190 until required.

191 2.6. Cytochrome P450 content

192 Total CYP content was measured after Livingstone (1988) in a temperature-controlled UV/VIS
193 Uvikon 941 spectrophotometer (Kontron Instruments). Spectra were measured in the range of 400
194 to 500 nm at 25±1°C, and total CYP calculated using an extinction coefficient of 91 mM⁻¹ cm⁻¹
195 ($A_{450-490}$).

196 2.7. Superoxide dismutase activity assay

197 The activity of SOD was measured in a UV/Vis Beckman DU 530 spectrophotometer, temperature-
198 controlled to 25±1°C. SOD activity was determined with the xanthine oxidase-cytochrome c
199 method according to Crapo et al. (1978). The Cu/Zn-containing form of SOD was assayed by the
200 inhibitory effect of 1mM KCN on SOD activity (Fridovich, 1982).

201 2.8. Protein assay

202 Total soluble protein concentration was measured according to Lowry et al. (1951) by a Bio-Rad
203 DC Protein Assay kit, using a microassay procedure and bovine serum albumin as the standard. The
204 protein content was determined using 96-well microtiter plates. The extinction was read at 750 nm

205 in a Labsystem Multiskan EX spectrophotometer. Values were reported as mg protein per ml and
206 were used to normalize enzyme activity and expression.

207 2.9. Electrophoresis and western blotting

208 SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) of different subcellular
209 fractions, (section 2.5) were performed in an Invitrogen Xcell SureLock Mini-Cell using minigels
210 (Bis-Tris gels) consisting of 12% running gel with MES [2-(N-morpholino)ethane sulfonic acid]
211 running buffer at pH 7.3, under reducing conditions. Samples were added with LDS (lithium
212 dodecyl sulphate) buffer and reducing agent (0.5 M dithiothreitol) and heated for 10 min at 70°C.
213 Apparent molecular weights of immunopositive bands were determined from simultaneously run
214 pre-stained molecular weight markers. Aliquots of partially purified Mn-SOD (obtained by gel
215 chromatography using a Sephadex G-75 column, 1x90cm) from the bivalve mollusc *S.*
216 *inaequivalvis*, and Cu/Zn-SOD from bovine erythrocytes, were loaded in the gels as standards to
217 identify the two SOD isoforms. Samples were loaded to the gel in volumes corresponding to a
218 protein content range of from 30 to 40 µg.

219 After electrophoresis, proteins were transferred for 1h to polyvinylidene difluoride (PVDF)
220 membranes in an Invitrogen Xcell SureLock Blot Modul using transfer buffer pH 7.2 (Bicine, bis-
221 Tris, EDTA, chlorobutanol). After blotting, the PVDF membranes were treated with specific
222 chromogenic western blot immunodetection kits. Blots were blocked for 45 min with concentrated
223 casein solution in buffered saline solution and then incubated with the diluted primary specific
224 antibody for 1h. To identify the SOD isoforms, rabbit polyclonal anti-Cu/Zn-SOD, diluted
225 1:10,000, and rabbit polyclonal anti-Mn-SOD, diluted 1:7,000 were used. CYP detection was
226 carried out using goat polyclonal anti-CYP 1A1/1A2, diluted 1:100 and by rabbit polyclonal anti-
227 CYP 2E1, diluted 1:2,500. Membranes were washed with a specific antibody wash solution and
228 incubated with the secondary antibody solution consisting of alkaline phosphatase-conjugated anti-
229 rabbit or anti-goat IgG for 45 min. Blots were then visualised using chromogenic substrate
230 containing 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitro blue tetrazolium (NBT).

231 Immunopositive bands were semi-quantified by Quantity One Software (Bio-Rad) and results per
232 unit protein were expressed in arbitrary units.

233 2.10. Microsomal benzo[a]pyrene metabolism

234 Microsomes were prepared from pooled frozen digestive glands as described in section 2.5. Assays
235 and B[a]P metabolite and putative protein adduct analysis were performed on approximately 2 mg
236 of microsomal protein as described in Lemaire et al., (1993). After extraction of metabolites,
237 putative protein adducts of B[a]P were quantified by repeated washing of precipitated microsomal
238 material in methanol to remove loosely bound ^3H -B[a]P, dissolution in 1 M NaOH (40 °C
239 overnight), neutralization and counting of radioactivity (Lemaire et al., (1993).

240

241 2.11. Statistical analyses

242 Results were statistically analysed using one-way analysis of variance (ANOVA) followed by the
243 Student's Neuman Keuls test for multiple comparisons using statistical package STAT software;
244 only data obtained from microsomal benzo[a]pyrene metabolism analyses were compared by
245 Student's t-test for independent samples with the same software. Values of $p < 0.05$ was considered
246 to be significant. Data are reported as means \pm SD.

247

248 3. Results

249 3.1. B[a]P concentration

250 The results of B[a]P analyses performed on four specimen pooled whole tissues, sampled at 0
251 (control) 12, 24 and 36 hours of exposure are reported in Figure 1. Tissue B[a]P concentration
252 levels were maximal after 12 hours of exposure (1.78 ± 0.06 ng/g ww), followed by a decrease at 24
253 and 36 hours of treatment, with levels of 1.1 ± 0.5 and 0.9 ± 0.3 ng/g ww respectively. Statistical
254 analyses showed that values after 12 h of exposure were significantly higher ($p < 0.01$) than the 24
255 and 36 hour ones. In contrast, differences between B[a]P content at 24 and 36 hours were not

256 significant ($p > 0.1$). In control animals B[a]P was absent (0.2 ng/g ww was the instrument
257 detection limit).

258 3.2. Cytochrome P450 content

259 Total CYP concentration was measured in microsomes from pooled digestive glands. During the
260 treatment with B[a]P, a rapid increase of total CYP content was recorded, with levels ranging from
261 64 ± 11 pmol/mg protein to 108 ± 21 pmol/mg protein at 12 and 24 hours of exposure, respectively
262 (Fig. 2), the latter value being significantly higher than the ones recorded both in control animals
263 (54 ± 15 pmol/mg protein) and at 12 hours exposure ($p < 0.05$). Total CYP content decreased at 36
264 hours (87 ± 11 pmol/mg protein) compared to 24 hours exposure but the levels were not significantly
265 higher than the control samples.

266 3.3. SOD activity

267 The specific activities of Cu/Zn-SOD and Mn-SOD were measured in the combined mitochondrial
268 and cytosolic fractions of the digestive gland (Fig. 3). Trends of increasing activity of both SOD
269 isoforms were observed throughout the experiment. Mn-SOD activity showed a significant steady
270 increase ($p < 0.05$; $p < 0.01$) at 36 hours of exposure respect to the other experimental conditions,
271 (895 ± 27 U/g ww in control animals and to 1290 ± 25 U/g ww after 36 h of exposure).

272 No significant differences were found in Cu/Zn-SOD activity during the B[a]P exposure compared
273 to control animals with the other exposure conditions.

274 3.4. Immunoblotting detection

275 Western blots of digestive gland extract revealed single major bands for both Mn-SOD (Fig. 4) and
276 Cu/Zn-SOD (Fig. 5), at the expected apparent molecular weights, indicating a high specificity of
277 recognition of the cross-species antibodies. The Mn-SOD band had an apparent molecular weight of
278 26 kDa, corresponding to the monomeric form of the enzyme (Mn-SOD exists as a homotetramer
279 with an individual subunit molecular weight of about 23 kDa - Barra et al., 1984) (Fig. 4A). Results
280 of densitometric analyses showed that the expression of Mn-SOD appeared not to change between
281 12 and 24 hours of exposure; however, slightly less Mn-SOD protein was seen at 36 hours of

282 exposure compared to control animals ($p < 0.01$; Fig. 4B). The Cu/Zn-SOD band had an apparent
283 molecular weight of 16 kDa (human Cu/Zn-SOD is homodimeric with a molecular weight of 31.2
284 kDa - Crapo et al., 1992) (Fig. 5A). The expression of the Cu/Zn-SOD band showed no variation
285 ($p > 0.1$) during the time-course of the experiment (Fig. 5B).

286 Less specificity of recognition was seen with the cross-species antibodies for CYP1A1/1A2- and
287 CYP2E1-immunopositive proteins in digestive gland microsomes as multiple bands were observed
288 (Figs. 6 and 7). However, these included major bands in the apparent molecular weight range
289 expected for CYPs, i.e. 40.6-50.5 kDa. Two major bands were found using anti-CYP1A1/1A2,
290 which had apparent molecular weights of 50.5 and 40.6 kDa (Fig. 6A). After densitometric analysis,
291 the 50.5 kDa band of CYP1A1/1A2-immunopositive protein was significantly more expressed after
292 36 h of B[a]P exposure with respect to the control and the other exposure sampling times ($p < 0.05$ -
293 0.01; Fig. 6B), whereas the 40.6 kDa band showed the same expression in all conditions (data not
294 shown). The anti-CYP2E1 recognized one immunopositive band present in all conditions with an
295 apparent molecular weight of 49 kDa (Fig. 7A). After the densitometric analyses of this band, no
296 differences were found in the levels of CYP2E1-immunopositive protein between the control and
297 the treated conditions (Fig. 7B).

298 3.5. Microsomal benzo[a]pyrene metabolism

299 The results of the *in vitro* metabolism of B[a]P by digestive gland microsomes of field animals are
300 presented in Table 1. Metabolism both in the absence of NADPH (so-called 'NADPH-independent
301 metabolism') and presence of NADPH (NADPH-dependent metabolism) showed a similar range of
302 primary metabolites, including 7,8-dihydrodiol (diol); 1,6-, 3,6- and 6,12-quinones (diones); and at
303 least two phenols that likely included the 3- and 9-phenols. The major metabolites formed in both
304 cases were diones, comprising 89 to 90 % of the total. Rates of production of total primary
305 metabolites were inhibited 41 % by the addition of NADPH due to a marked reduction in dione
306 formation. In contrast, no differences were seen in diol formation, but a slight increase in 'phenol a'
307 production occurred in the presence of NADPH ($p < 0.1$). Tetrols were only detected in the absence

308 of NADPH. In addition to primary metabolite formation, a substantial putative covalent binding of
309 B[a]P-derived metabolites to protein to form adducts was seen, equivalent to 48 to 55 % of the rates
310 seen for primary metabolites. Putative protein adduct formation was inhibited 49 % in the presence
311 compared to the absence of NADPH.

312

313 **4. Discussion**

314 Responses of the CYP-dependent monooxygenase system and antioxidant enzymes have been seen
315 in bivalve and other molluscs after organic pollutant exposure (Peters and Livingstone, 2001).

316 4.1. Bioaccumulation of B[a]P

317 Hydrophobic compounds such as B[a]P are readily taken up into the tissues of bivalve molluscs, the
318 results seen in this study for *S. inaequivalvis* are comparable with those reported for other bivalves,
319 both in laboratory and field exposures (Nasci et al., 2000; Porte et al., 2001), e.g. rapid
320 accumulation of B[a]P was found in *Mytilus edulis* after six hours of water-borne B[a]P exposure
321 (Durand et al., 2002). Differences also occur, very little bioaccumulation of B[a]P by the mussel
322 *Elliptio complanata* being seen after five days of exposure (Gewurtz et al., 2002).

323 The decrease in whole body B[a]P levels in *S. inaequivalvis* between 12 and 36 hours is likely
324 mainly due to depuration and/or biotransformation. Half-lives of loss of hydrophobic compounds
325 such as B[a]P depend on many factors, including species and exposure period (Livingstone, 1991).
326 Thus, 26 % of B[a]P was lost from *S. inaequivalvis* between 12 and 24 hours after exposure
327 compared to 0 % for *M. edulis* for the same time period (Durand et al., 2002). Similarly, the half-
328 life of loss of B[a]P from *S. inaequivalvis* was 24 hours (tissue B[a]P levels at 36 hours were 50 %
329 of those at 12 hours) compared to six to ten days for the clam *Rangia cuneata* following a one-day
330 exposure period (Neff et al., 1976). *In vivo* biotransformation of B[a]P occurs in molluscs, but
331 considerable species variation is reported (Magnusson et al., 2000; Varanasi et al., 1985).

332 A relationship has been determined for the dependence of *in vivo* biotransformation rates of
333 hydrocarbons on tissue parent compound concentration (Livingstone, 1998). From this it can be

334 calculated that a whole tissue B[a]P concentration of $6.75 \text{ pmol g}^{-1} \text{ ww}$ (i.e. $1.7 \text{ ng g}^{-1} \text{ ww}$.) would
335 produce an *in vivo* biotransformation rate of $0.0027 \text{ pmol min}^{-1} \text{ g}^{-1} \text{ ww}$, which compares well with
336 the rate of $0.00243 \text{ pmol min}^{-1} \text{ g}^{-1} \text{ ww}$ required to reduce the B[a]P levels in *S. inaequalvis* from
337 6.75 to $5.0 \text{ pmol g}^{-1} \text{ ww}$ (i.e. $1.26 \text{ ng g}^{-1} \text{ ww}$) over 12 hours. Thus the possibility exists that
338 biotransformation may make a significant contribution to the reduction in B[a]P levels observed in
339 *S. inaequalvis* between 24 and 36 hours following exposure.

340 4.2. Total CYP content and SOD activities

341 Total CYP content of digestive gland microsomes was maximal after 24 hours of exposure, and
342 double the levels of control animals, indicative of an inductive effect by B[a]P. Similar levels of
343 increase in total CYP content with exposure to various PAHs have been seen for *Mytilus* sp. and the
344 periwinkle *Littorina littorea* (Livingstone, 1991). The peak of total CYP (24 hours exposure) was
345 not coincident with maximal B[a]P tissue levels (12 hours exposure), possibly due to the time
346 required for full induction, or to the decrease in B[a]P levels caused by biotransformation.

347 With regard to CYP level/xenobiotic concentration, a correlation between variation in CYP content
348 and tissue level of hydrocarbons was found in *M. edulis* and *L. littorea* (Livingstone, 1988).

349 During Phase I biotransformation the involvement of CYP and flavoprotein reductases leads to the
350 production of ROS (Livingstone, 2001). Hepatic and other microsomes of many aquatic organisms
351 have been shown to catalyse the monovalent reduction of xenobiotics, with the resultant generation
352 of $\text{O}_2^{\cdot-}$ and other ROS (Livingstone, 2001). In particular, digestive gland microsomes of *M. edulis*
353 catalyse the redox cycling of PAH-quinones, including the 1,6-, 3,6- and 6,12-B[a]P-quinones, to
354 produce $\text{O}_2^{\cdot-}$ and H_2O_2 (Sjölin and Livingstone, 1997).

355 Protection against ROS is afforded by several antioxidant enzymes (included SODs studied in *S.*
356 *inaequalvis*) detected in many bivalve species (Orbea et al., 2002; Manduzio et al., 2004).

357 Mn-SOD activity showed a significant steady increase in digestive gland of *S. inaequalvis* from
358 control to 36 hours exposure; no significant differences were seen in Cu/Zn-SOD activity between
359 control and other experimental conditions. Wenning and Di Giulio (1988) found a two-fold increase

360 in total SOD activity in the freshwater bivalve *Geukensia demissa* exposed to 129 to 354 ppm
361 paraquat, although in this case the increase was transient, being maximal between 6 and 24 hours of
362 exposure. The increase in SOD antioxidant enzyme activities in *S. inaequalvis* with B[a]P
363 exposure indicates biotransformation mechanisms that enhance ROS production. Shick and Dykens
364 (1985) found in several tissues of bivalve *Tridacna crocea* the positive relationship between tissue-
365 specific SOD activity and index of superoxide production (R^2 : 0.759). Considering SOD activity as
366 an indirect measure of $O_2^{\cdot-}$ production (Yu and Rengel, 1999), we found correlations between total
367 CYP content and SOD isoform activities (Mn-SOD/CYP: $r = 0.63$; Cu/Zn-SOD/CYP: $r = 0.96$),
368 possibly indicating a role for CYP in putative $O_2^{\cdot-}$ generation. Several studies have showed increase
369 in SOD activity in digestive glands of mussels (*Mytilus* sp.) exposed to PAHs and PCBs (Porte et
370 al., 1991), or collected from polluted sites (Porte et al., 1991). Cheung et al. (2001) observed an
371 increase in SOD activity in digestive gland of the mussel *Perna viridis* that was positively
372 correlated with B[a]P tissue concentrations, indicative again of $O_2^{\cdot-}$ production related to xenobiotic
373 in the tissue.

374 4.3. Immunoblotting detection

375 The expression of Mn-SOD protein in *S. inaequalvis* did not parallel its enzyme activity during
376 B[a]P exposure. The reasons for this divergent behaviour are not clear. Mn-SOD and its responses
377 to pollutants have been little investigated in aquatic organisms, even though it plays a central role in
378 cellular responses to oxidative stress and mitochondrial protection (Reed, 2001). Recent studies
379 indicate that Mn-SOD is expressed in many cell types and tissues at relatively high levels and is
380 highly regulated by various intracellular and environmental cues (Zelko et al., 2002), including
381 cytokines and damaging cellular agents such as hyperoxia and chemical prooxidants (Halliwell and
382 Gutteridge, 1999). The lack of correlation between Mn-SOD activity and expression, in the present
383 study might be due to protein modification and/or associations to other proteins such as chaperonin
384 (Manduzio et al., 2003). Probably SODs degeneration by ROS may have resulted in less active
385 SOD isoforms that were still recognised by the antibodies used in the western blotting (Chan et al.,

386 1999). Also, several factors involved in the post-translation control of SOD enzyme biosynthesis
387 have to be considered (Chan et al., 1999).

388 Again, differently from Mn-SOD, Cu/Zn-SOD activity was coherent with expression, which
389 showed no change in Cu/Zn-SOD protein levels with B[a]P exposure. Manduzio et al. (2003) found
390 changes in Cu/Zn-SOD isoform pattern in *M. edulis* exposed to copper, but no changes in total SOD
391 activity. Several authors, despite considering the enzyme to be constitutively expressed, stated that
392 its mRNA levels might be up- and down-regulated by several physiological conditions such as the
393 presence of organic xenobiotics, metals and NO (Zelko et al., 2002). Cu/Zn-SOD have a widespread
394 distribution in many type of cells, furthermore, it seems that cytoplasmatic Cu/Zn-SOD expression
395 is stable and its activity is often considered to be an internal control for Cu/Zn-SOD gene
396 expression (Zelko et al., 2002). With respect to Cu/Zn-SOD, variable results have been seen with
397 other aquatic animals exposed to PAHs (McFarland et al., 1999; Meyer et al., 2003).

398 Western blot analyses of CYP isoforms in digestive gland microsomes of *S. inaequalvis* were
399 performed using polyclonal antibodies for rabbit CYP1A1/1A2 and rat CYP2E1. Results showed a
400 reasonable cross-reactivity between the mammalian anti-CYP antibodies and the CYP1A1/1A2-
401 and CYP2E1-immunopositive proteins in *S. inaequalvis*. The putative CYP bands ranged in
402 apparent molecular weight from 40.6 to 50.5 kDa in agreement with Porte et al., (1995). Two major
403 CYP bands were detected by anti-CYP1A/1A2 at 50.5 and 40.6 kDa, the expression of the former
404 being increased at the end of the B[a]P exposure, whereas no differences were found in the latter.

405 The occurrence of a CYP1A like-protein in digestive gland of bivalve molluscs that can apparently
406 be induced by PAHs and PCBs has been seen in a number of laboratory and field studies with
407 *Mytilus* sp. (Livingstone et al., 1997; Peters et al., 1998). The conditions of exposure resulting in
408 increased levels of digestive gland microsomal CYP1A-immunopositive protein vary, but the 36
409 hours B[a]P exposure observed for *S. inaequalvis* compares well with the 4 days seen for *M.*
410 *galloprovincialis* exposed to PCBs (Livingstone et al., 1997). Several literature data indicate a
411 strong dependence of CYPs induction on tissue xenobiotic concentration and exposure time

412 (Canova et al., 1998). In fish and other vertebrates, CYP1A induction is regulated through a ligand-
413 activated transcription factor known as the Aryl Hydrocarbon Receptor (AHR) (Hahn, 2002).
414 Relatively little is known of this mechanism in molluscs, but recently AHR homologs have been
415 found in the bivalves *Mya arenaria*, *Dreissena polymorpha* and *M. edulis* (Wiesner et al., 2001). A
416 high affinity of the inducing xenobiotic to AHR results in high CYP1A induction (Billiard et al.,
417 2002). However, the observed responses, and the existence of AHR homologs, argue that an AHR-
418 dependent mechanism may exist in molluscs, and the response may have biomarker application
419 (Peters and Livingstone, 2001).

420 A single major anti-CYP2E1 band of apparent molecular weight 49 kDa, unaffected by 36 hours
421 exposure to B[a]P, was seen in digestive gland microsomes of *S. inaequivalvis*. In *Mytilus* sp.,
422 higher levels of CYP2E-immunopositive protein were seen in polluted compared to clean
423 populations in the Venice Lagoon (Peters et al., 1998) and the UK (Shaw et al., 2000), but in the
424 latter case only at one time of the year (May), indicating a seasonal regulation of gene expression.
425 Additionally, unlike CYP1A-immunopositive protein, CYP2E-immunopositive protein was not
426 elevated in *M. galloprovincialis* after 3 weeks transplantation to an industrially polluted site (Peters
427 et al., 1998), indicating a slow response to PAHs, PCB and other pollutants. However, the absence
428 of a response in *S. inaequivalvis* indicates that elevation of the CYP2E-immunopositive protein
429 does not contribute to toxicity with B[a]P exposure.

430 4.4. Microsomal benzo[a]pyrene metabolism

431 Bivalve and other molluscan digestive gland microsomes show the unusual properties of a) CYP-
432 catalysed monooxygenation in the absence of added NADPH (termed 'NADPH-independent'
433 metabolism), and b) metabolism of B[a]P to predominantly diones (Livingstone, 1991). NADPH-
434 independent metabolism has been seen in over twenty molluscan species for a variety of
435 monooxygenation reactions (Livingstone, 1991; Solé and Livingstone, 2005). Metabolism of B[a]P
436 to 70 % or more diones, with the remaining metabolites comprising tetrols, diols and phenols, has
437 been seen for *Mytilus* sp., *Crassostrea virginica* and *Cryptochiton stelleri* (Livingstone, 1991;

438 Lemaire et al., 1993; Michel et al., 1993). This is in contrast to microsomal metabolism of
439 vertebrates, echinoderms, crustaceans and polychaetes which produce mainly diols and phenols
440 (Livingstone, 1991). Results similar to the ones observed in other molluscs were obtained for *S.*
441 *inaequivalvis*, which showed NADPH-independent metabolism of B[a]P to 90 % diones. Putative
442 protein adducts were also formed in the absence of added NADPH, as has been observed for
443 *Mytilus* sp. (Lemaire et al., 1993). The inhibition of dione and putative protein adduct, but not diol
444 or phenol, formation by added NADPH is consistent with results for *Mytilus* sp. (Livingstone,
445 1991). These results for *Mytilus* sp. led to the hypothesis that the CYP-catalysed metabolism of
446 B[a]P may proceed by two pathways, namely one-electron oxidation leading to mainly dione and
447 protein adducts, and two-electron monooxygenation leading to phenols (Livingstone, 1992).
448 Despite conjugation by Phase II metabolism, substantial quantities of free B[a]P-diones are formed
449 *in vivo* in *M. galloprovincialis* (Michel et al., 1995), indicating that B[a]P-dione-mediated ROS
450 production, via redox cycling (Garcia Martinez and Livingstone, 1995), may be a significant
451 mechanism of toxicity in whole animals.

452

453 5. Conclusion

454 Rapid uptake of B[a]P was followed by a decrease in levels in the tissues, which could have been
455 due to depuration, but also to significant biotransformation. The presence of B[a]P resulted in
456 increases in both total CYP (24 hours) and CYP1A-immunopositive protein (36 hours). The latter
457 response was isoenzyme-specific as no change was seen in CYP2E-immunopositive protein.
458 However, other unknown CYPs must also have been induced because maximum CYP1A-
459 immunopositive protein levels did not correspond with total CYP levels. Metabolism of B[a]P to
460 redox-cycling diones, combined with elevation of total CYP levels, could have been mechanisms
461 leading to enhanced ROS production and the concomitant increase in Mn-SOD activity. Overall, the
462 results for *S. inaequivalvis* are consistent with those for other molluscs, and indicate that it may be a
463 useful organism for ecotoxicological studies.

464

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471

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TABLES

Table 1 Metabolism of tritiated benzo[a]pyrene (^3H -BaP) by digestive gland microsomes (pmol mg^{-1} protein; mean \pm SD) of *Scapharca inaequalvis*.

Metabolite	Without added NADPH (pmol mg^{-1} protein)	With added NADPH (pmol mg^{-1} protein)
Tetrol	5.1 \pm 4.5	0 \pm 0
Unknown diol	7.5 \pm 8.1	1.5 \pm 1.5
7,8-diol	7.2 \pm 6.9	3.3 \pm 2.7
1,6-dione	81.3 \pm 3.3	42.0 \pm 3.3 ***
3,6-dione	81.0 \pm 9.6	52.2 \pm 6.3 **
6,12-dione	90.6 \pm 8.1	53.7 \pm 9.0 ***
Phenol a	5.4 \pm 1.5	10.2 \pm 3.0 *
Phenol b	5.4 \pm 7.8	1.8 \pm 3.3
Total diols	14.7 \pm 5.1	4.8 \pm 4.2
Total diones	252.9 \pm 6.0	147.9 \pm 10.8 ***
Total phenols	10.8 \pm 9.0	12.0 \pm 5.1
Total metabolites	283.5 \pm 17.7	164.7 \pm 15.6 ***
Putative protein adducts	154.8 \pm 3.3	79.2 \pm 5.7 ***

Metabolites formed over 30 minutes of incubation; retention times (min) under the specified chromatographic conditions were tetrol (5.0), unknown diol (9.6), 7,8-diol (14.6), 1,6-dione (21.8), 3,6-dione (23.6), 6,12-dione (26.4), phenol a (29.2) and phenol b (32.0); phenol peaks include 3-hydroxy- and 9-hydroxy-B[a]P. Values are mean \pm SD (triplicates); comparison of metabolites formed with or without NADPH showed significant differences where * p <0.1, ** p <0.05 and *** p <0.01.

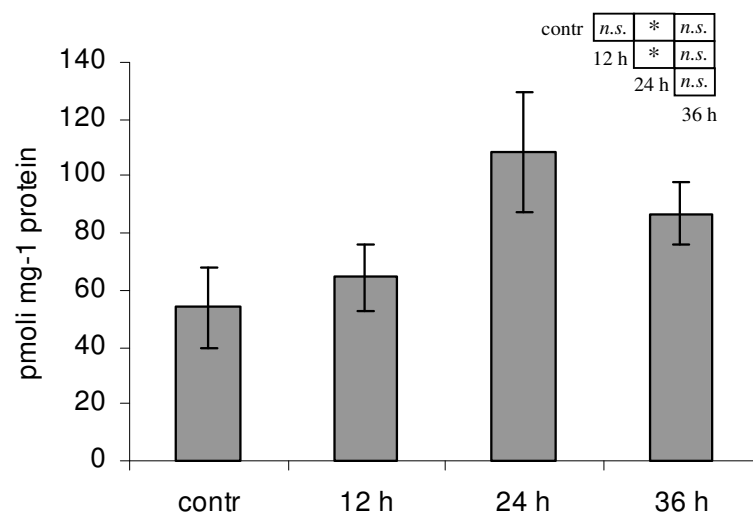
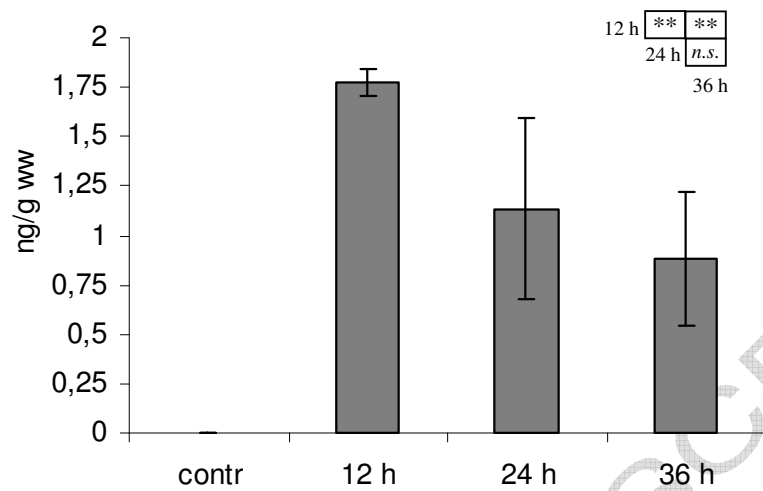


Figure 2

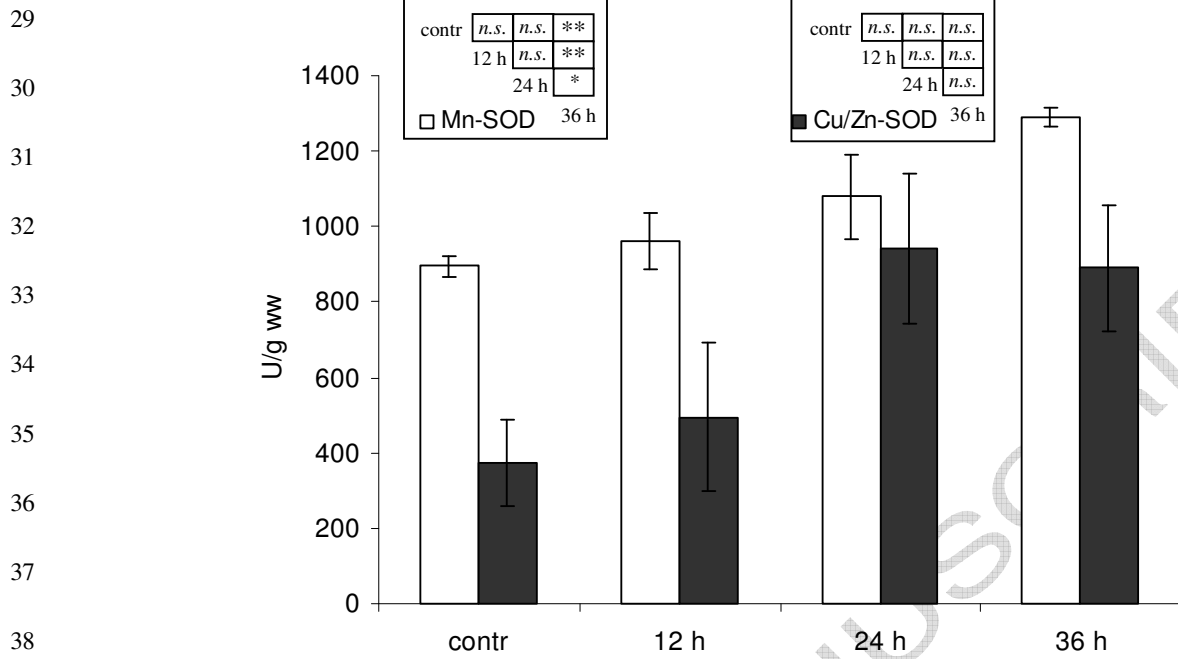


Figure 3

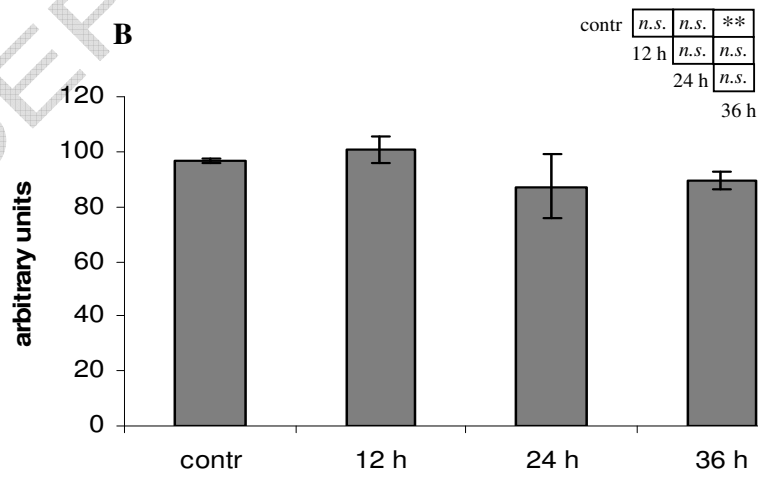
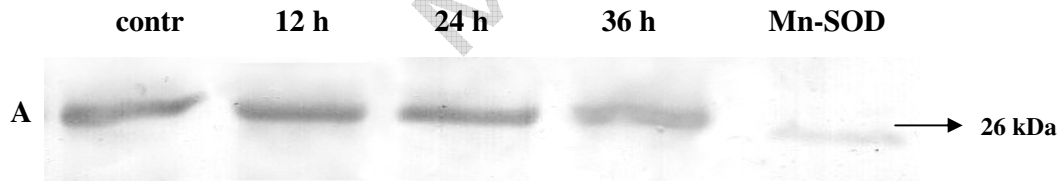


Figure 4 A, B

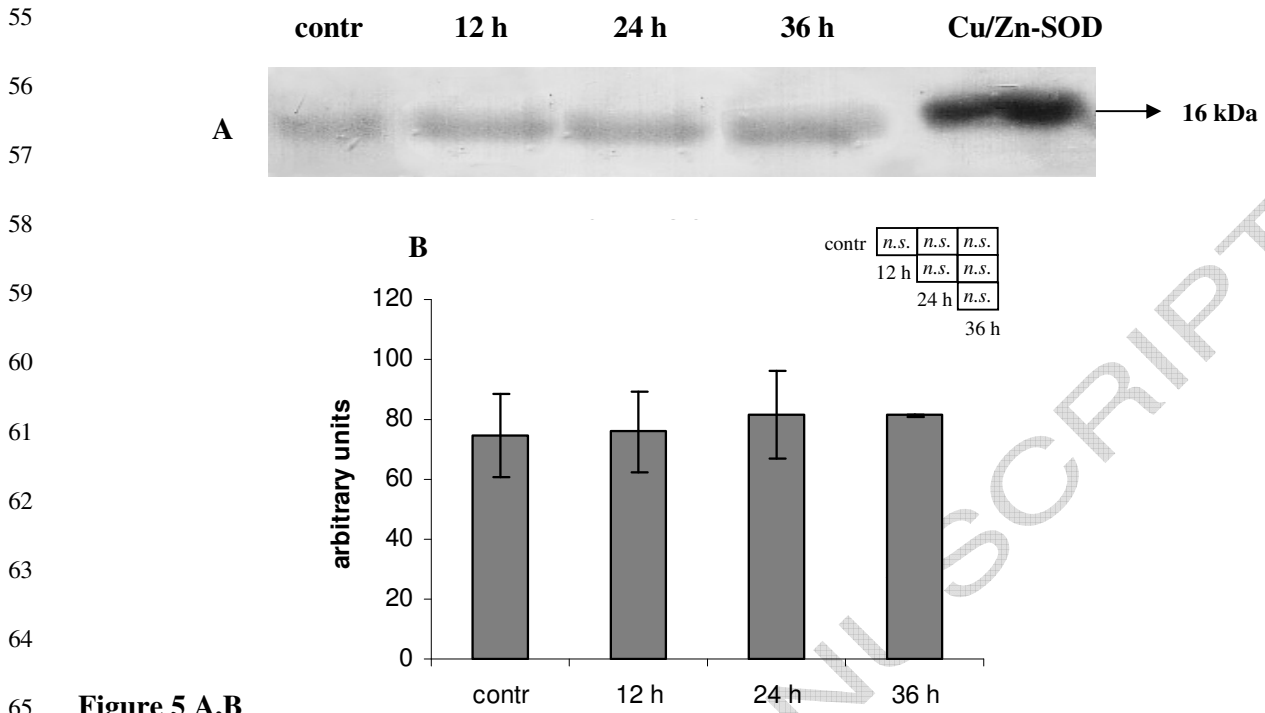


Figure 5 A,B

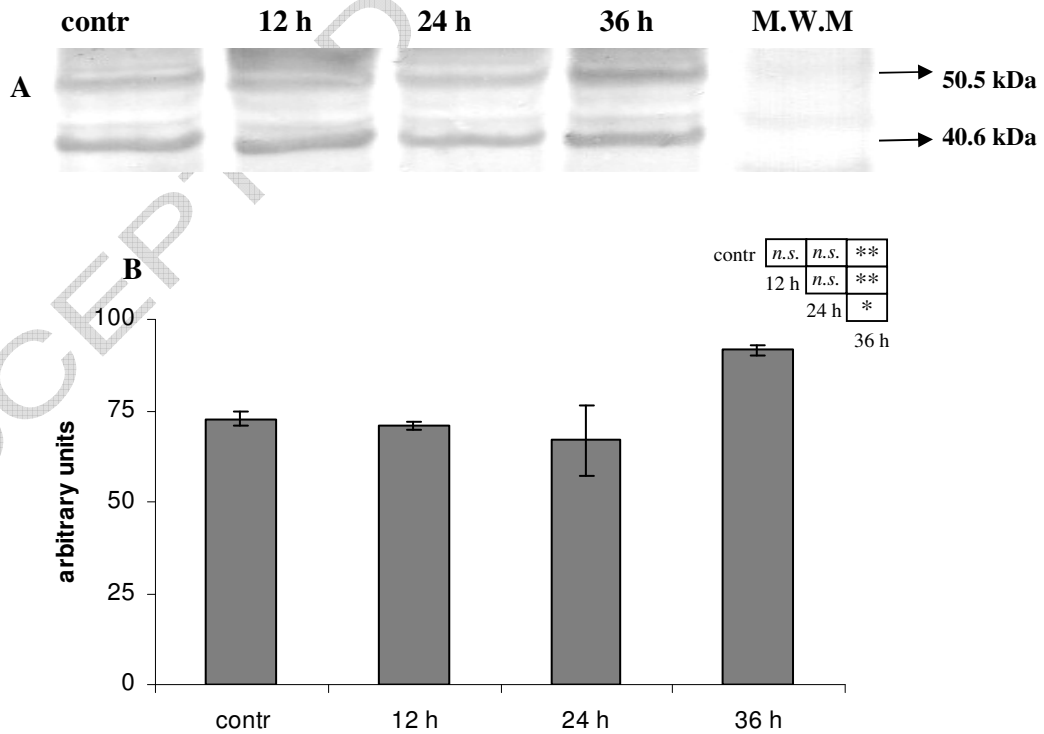


Figure 6 A,B

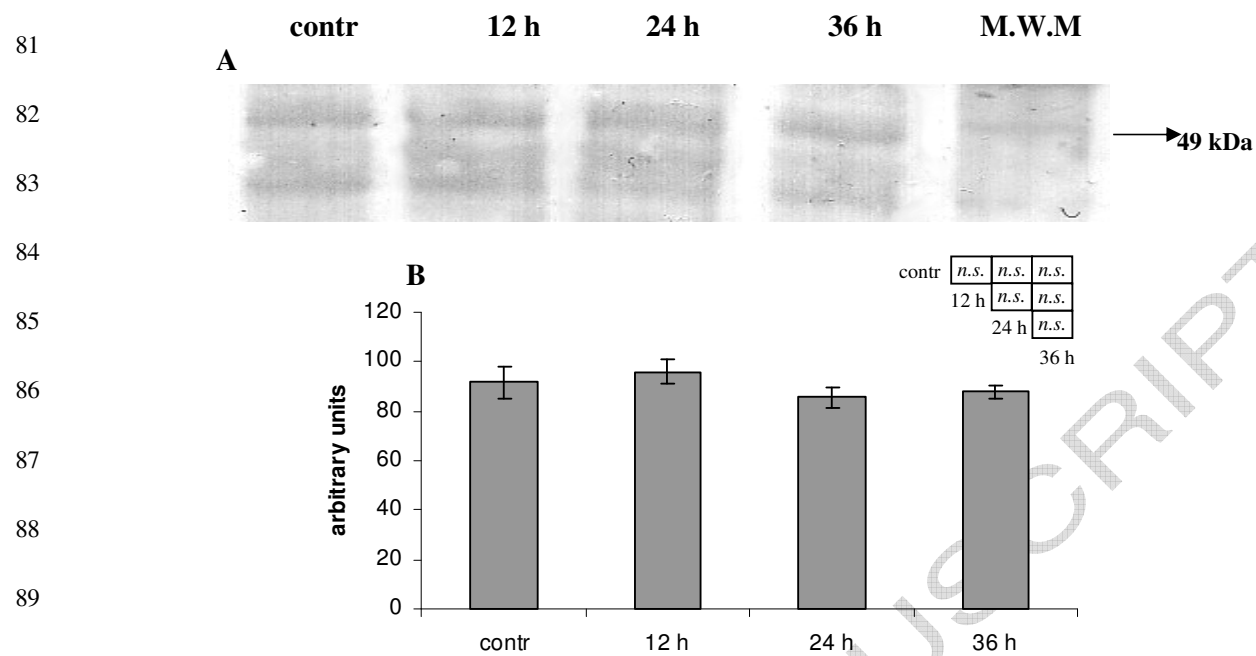


Figure 7 A,B

CAPTIONS

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108

109 **Fig. 1.** *Scapharca inaequivalvis*. B[a]P content in whole soft tissues in control animals (contr) and
110 after 12, 24 and 36 hours of B[a]P exposure (ng/g ww; mean \pm SD, n = 4). Asterisks: significant
111 difference: **p<0.01. Inset: significance of comparisons between experimental groups.

112

113 **Fig. 2.** Total CYP content in digestive gland microsomes of *Scapharca inaequivalvis* in control
114 animals (contr) and after 12, 24 and 36 hours of B[a]P exposure (pmol mg⁻¹ protein). Each
115 histogram represents the mean \pm SD for three pools measured in triplicates. Asterisks: significant
116 difference: *p<0.05 Inset: significance of comparisons between experimental groups.

117

118 **Fig. 3.** Mn-SOD and Cu/Zn-SOD activities in subcellular fraction (cytosolic/mitochondrial) of
119 digestive gland of *Scapharca inaequivalvis* in control animals (contr) and after 12, 24 and 36 hours
120 of B[a]P exposure (U mg⁻¹ protein). Each histogram represents the mean \pm SD for three pools
121 measured in triplicates. Asterisks: significant difference: ** p<0.01; *p<0.05. Inset: significance of
122 comparisons between experimental groups.

123

124 **Fig. 4. A.** Western blots of Mn-SOD in subcellular fraction (cytosolic/mitochondrial) of digestive
125 gland of *Scapharca inaequivalvis* in control animals (contr) and after 12, 24 and 36 hours of B[a]P
126 exposure **B.** Densitometric analyses of blot expressed in arbitrary units. Each histogram represents
127 the mean \pm SD for three pools measured in triplicates. Asterisks: significant difference: **p<0.01
128 Inset: significance of comparisons between experimental groups.

129

130 **Fig. 5. A.** Western blots of Cu/Zn-SOD in subcellular fraction (cytosolic/mitochondrial) of
131 digestive gland of *Scapharca inaequivalvis* in control animals (contr) and after 12, 24 and 36 hours
132 of B[a]P exposure **B.** Densitometric analyses of blot expressed in arbitrary units. Each histogram

133 represents the mean \pm SD for three pools measured in triplicates. Inset: significance of comparisons
134 between experimental groups.

135

136 **Fig. 6. A.** Western blots in digestive gland microsomes of *Scapharca inaequivalvis* incubated with
137 anti-CYP1A1/A2, in control animals (contr) and after 12, 24 and 36 hours of B[a]P exposure **B.**
138 Densitometric analyses of higher band (app. mol. wt. 50.5 kDa) expressed in arbitrary units. Each
139 histogram represents the mean \pm SD for three pools measured in triplicates. Asterisks: significant
140 difference: ** $p < 0.01$; * $p < 0.05$. Inset: significance of comparisons between experimental groups.

141

142 **Fig. 7. A.** Western blots in digestive gland microsomes of *Scapharca inaequivalvis* incubated with
143 anti-CYP2E1 in control animals (contr) and after 12, 24 and 36 hours of B[a]P exposure **B.**
144 Densitometric analyses of blot expressed in arbitrary units. Each histogram represents the mean \pm
145 SD for three pools measured in triplicates. Inset: significance of comparisons between experimental
146 groups.

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