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32 **Abstract**

33 An ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis*, was developed
34 to evaluate the potential impact of an offshore gas platform in the central Adriatic Sea. Reference
35 organisms were collected on a seasonal basis from an unpolluted site and transplanted for 4 weeks
36 in both the sampling area and to the investigated platform. Chemical analyses of trace metals in
37 mussel tissues were integrated with a multi-biomarker approach for the early detection of biological
38 responses at several cellular targets. Induction of metallothioneins, peroxisomal proliferation and
39 activity of acetylcholinesterase were measured as markers for specific classes of chemicals. Special
40 attention was given to oxyradical metabolism and appearance of oxidative-mediated toxicity to
41 reveal a more general onset of cellular disturbance. In addition to individual antioxidants
42 (superoxide dismutase, catalase, glutathione S-transferases, glutathione reductase, Se-dependent and
43 Se-independent glutathione peroxidases, and levels of total glutathione), the total oxyradical
44 scavenging capacity (TOSC) allowed a quantification of the overall capability to neutralize specific
45 forms of intracellular reactive oxygen species (ROS; i.e. peroxy and hydroxyl radicals). Cellular
46 damages were evaluated as lysosomal destabilization (membrane stability, accumulation of
47 lipofuscin and neutral lipids), lipid peroxidation products (malondialdehyde) and DNA integrity
48 (strand breaks and micronuclei); the air survival test was finally applied to evaluate the overall
49 physiological condition of mussels. Concentration of trace metals (As, Ba, Cd, Cr, Cu, Fe, Hg, Mn,
50 Ni, Pb, Zn) revealed only limited variations in transplanted mussels during various experimental
51 periods and such changes appeared partly related to natural fluctuations. Among biological
52 responses, variations of antioxidants and lysosomal stability were confirmed as sensitive early
53 warning signals for biological disturbance of both natural and anthropogenic origin. The presented
54 protocol with caged mussels allowed marked biological effects caused by the investigated platform
55 to be excluded, and represented a useful approach that is easy to extend for monitoring the impact
56 of offshore activities in the Adriatic sea.

57

58 **Keywords:** biomonitoring, mussels, trace metals, biomarkers, ecotoxicology, offshore platforms,
59 Adriatic Sea.

60 1. Introduction

61 The exploitation of gas fields in the Adriatic sea began in the 1960's and more than 100
62 platforms have been installed since then. Several environmental issues are associated with the
63 offshore oil and gas industry, from the impact caused during installation, to various forms of
64 disturbance related to daily shipping movement, extraction activities, maintenance of structures and,
65 finally, decommissioning of old platforms. Produced water is the major discharged effluent and it is
66 comprised of formation water (naturally present in oil and gas reservoirs) and injection water,
67 forced into the well to improve recovery. At the surface, produced water is separated from
68 hydrocarbons and treated to reduce the oil content in water to a maximum of 40 ppm, before being
69 discharged into the sea or reinjected into the wells. Produced waters have a complex composition
70 depending on their origin, and contain different percentages of production chemicals, oil derived
71 hydrocarbons, non volatile dissolved organic material and elevated concentrations of trace metals
72 (Utvik, 1999).

73 Considering the importance of monitoring the effects of offshore activities in the Adriatic
74 Sea, the aim of this work was to develop an ecotoxicological protocol based on the use of caged
75 mussels, *Mytilus galloprovincialis*. Organisms were seasonally collected from an unpolluted site
76 and translocated for 4 weeks to the installation "Giovanna" selected as model platform for the
77 experiments. The structure is located 18 nautical miles offshore in central Adriatic (42° 46' 060N,
78 14° 27' 750E) on a gas reservoir at 117 m depth. Previous investigations on this platform revealed
79 trace metals in both produced waters and sediments, while polycyclic aromatic hydrocarbons
80 (mostly 2/3 benzenic rings) were detected in produced waters (range 50-90 µg/l) but without
81 significant influences on surrounding sediments (ICRAM, 2006); further analyses on native mussels
82 confirmed the presence of metals but very low levels of PAHs in these organisms.

83 Bioavailability of trace metals was characterized in the present study using transplanted
84 mussels and chemical data were integrated with a wide array of biological responses measured at
85 the molecular, cellular and organism levels; these biomarkers, representing the earliest warning
86 signals of environmental disturbance, can be very sensitive, specific toward particular classes of
87 contaminants and reflect different levels of cellular unbalance and toxicity (Cajaraville et al., 2000).

88 Induction of metallothionein-like proteins, acetylcholinesterase activity and peroxisomal
89 proliferation were measured as typical responses to metals and several forms of organic pollutants.
90 Metallothioneins are involved in intracellular homeostasis of trace metals and induced by elevated
91 intracellular concentrations of elements like zinc, copper, cadmium and mercury (Viarengo et al.,
92 1997); acetylcholinesterase, a crucial enzyme in the nervous system of vertebrates and
93 invertebrates, is commonly associated to organophosphate or carbamate pesticides, but other

94 chemicals have also been shown to modulate its activity (Rickwood and Galloway, 2004; Frasco et
95 al., 2005). Peroxisomes are involved in lipid metabolism and their proliferation caused by organic
96 xenobiotics is of particular interest in mussels, considering the limited biotransformation pathway
97 of cytochrome P450 in these invertebrates (Cajaraville et al., 2000; Bocchetti and Regoli, 2006).

98 An important role in environmental toxicity of both metals and organic contaminants is
99 assumed by the enhancement of intracellular reactive oxygen species (ROS), which modulate the
100 onset of several deleterious effects and cell damage; reactivity of ROS is normally counteracted by
101 antioxidants and changes in the levels or activities of these defences have been proposed as
102 biomarkers of contaminant-mediated prooxidant challenge (Regoli et al., 2002). Although
103 variations of antioxidants are difficult to predict and contradictory results can be obtained in
104 different experimental or field conditions, nonetheless a number of studies confirmed the utility of
105 oxidative stress biomarkers in several marine organisms, including Mediterranean mussels (Regoli
106 and Principato 1995; Regoli, 1998; Frenzilli et al., 2001; Nesto et al., 2004; Petrović et al., 2004;
107 Regoli et al., 2004). Analysed antioxidants in caged mussels were the activities of superoxide
108 dismutase, catalase, glutathione S-transferases, glutathione reductase, Se-dependent and Se-
109 independent glutathione peroxidases and the levels of total glutathione. Variations of the redox
110 status were further assessed with the total oxyradical scavenging capacity (TOSC) assay which,
111 measuring the overall capability to neutralise various oxyradicals, provides a quantitative index of
112 the susceptibility of biological tissues to oxidative stress (Regoli and Winston, 1999). Compared to
113 individual antioxidants, an impaired capability to neutralize ROS can anticipate the appearance of
114 oxidative damages at cellular level as widely demonstrated by the increased rate of lysosomal
115 alterations and genotoxic damages at reduced TOSC values (Frenzilli et al., 2001; Gorbi and
116 Regoli, 2003; Regoli et al., 2004).

117 Lysosomal impairment is largely recognised as a sensitive marker of chemical and oxidative
118 disturbance in mussels (Moore et al., 2006). These organelles are highly developed both in digestive
119 tissues and haemocytes, being responsible for fundamental processes, such as the intracellular food
120 digestion, degradation of cellular components during autophagic turnover, sequestration and
121 detoxification of xenobiotics, and immune responses (Moore, 1982; Cajaraville et al., 1995). In this
122 study lysosomal membrane stability was selected as a responsive biomarker toward oxidative
123 perturbations (Regoli et al., 2004); accumulation of lipofuscin and neutral lipids in tertiary
124 lysosomes and content of malondialdehyde were further investigated as indices of intensity of lipid
125 peroxidation processes and xenobiotic-mediated lipidosis (Moore, 1988; Regoli, 1992).

126 The appearance of genotoxic alterations in caged mussels was evaluated as loss of DNA
127 integrity by the Comet assay, which measures the early onset of strand breaks, and by the frequency

128 of micronuclei (MN) as less reversible effects resulting from chromosome breakage or aneuploidy
129 during cell division (Venier et al., 1997; Nigro et al., 2006).

130 A general index of physiological status was finally measured in caged mussels as their
131 capability to survive in air; several studies have already demonstrated that mussels exposed to
132 environmental disturbance exhibit a significantly reduced tolerance to air exposure and this simple
133 “stress on stress” test can be effectively included in a multi-biomarker approach (Viarengo et al.,
134 1995; Nesto et al., 2004; Pampanin et al., 2005).

135 The overall results of this work were expected to validate the use of caged mussels as an
136 additional contribution for monitoring offshore activities and in particular to provide an
137 ecotoxicological protocol based on cellular biomarkers for the early detection of biological
138 disturbance.

139

140 **2. Materials and methods**

141 *2.1. Experimental design*

142 Mussels *Mytilus galloprovincialis* (5.5 ± 0.5 cm shell length), were sampled from the
143 reference site of Portonovo (Ancona, Adriatic sea) at a depth of approximately 4 m. This area is
144 regularly monitored and assigned with the “blue flag” by the E.C.; moreover trace metals,
145 polycyclic aromatic hydrocarbons and a battery of cellular and biochemical markers have been
146 seasonally analyzed allowing to exclude any form of chemical pollution (Bocchetti and Regoli
147 2006).

148 After collection, a group of mussels was transported to the laboratory and dissected for
149 chemical and biological analyses; while another group was immediately caged in the same
150 sampling area of Portonovo and, within 12 hr, to the “Giovanna” offshore platform. Approximately
151 200 mussels were deployed in net bags (80 cm height x 25 cm diameter, mesh size 1.5 x 3 cm)
152 secured to a nylon rope; this system was reinforced by an external net cylinder (2 m height x 80 cm
153 diameter, mesh size 2 x 3 cm) to guarantee free circulation of seawater and protect mussels from
154 fish predation. Mussels were maintained at approximately 4 m depth and recovered after 4 weeks.
155 This translocation period allows the organisms to equilibrate with the new environmental conditions
156 (Regoli and Orlando 1994a, Regoli et al., 2004) and it is actually used in several national and
157 international (i.e. RAMOGE) Mussel Watch programmes. Experiments were carried out in April,
158 August, November 2004 and March 2005.

159 On each sampling occasion, whole tissues of 5 specimens were dissected and stored at -20°C
160 for chemical analyses of trace metals. For biochemical parameters, digestive glands were rapidly
161 removed from 30 specimens, pooled in 10 samples (each with tissues of 3 specimens), frozen in

162 liquid nitrogen and maintained at -80°C . Small pieces of digestive glands were rapidly excised from
163 10 mussels for histological analyses, placed on cork chucks, frozen in hexane precooled to -70°C in
164 liquid nitrogen, and maintained at -80°C . Haemolymph was withdrawn from adductor muscle of 10
165 specimens and immediately analysed for lysosomal membrane stability and DNA damages.

166 2.2. Chemical analyses

167 Trace metals (arsenic, barium, cadmium, chromium, copper, iron, lead, manganese, nickel,
168 zinc) were analysed by atomic absorption spectrophotometry (Regoli et al., 2004) with flame
169 (Varian, Spectraa 220FS) and flameless atomization (Varian Spectraa 300 Zeeman). Mercury
170 content was quantify by cold vapor atomic absorption spectrometry (Varian, VGA-76, Vapor
171 Generator Accessory). Quality assurance and quality control was assessed by processing blank
172 samples and reference standard material (Mussel Tissue Standard Reference Material SRM 2977,
173 National Institute of Standards and Technology). Concentrations obtained for standard reference
174 materials were always within the 95% confidence interval of certified values.

175 For each sampling point a representative sample of tissues was further analyzed for chemical
176 speciation of arsenic. After methanolic extraction and separation of various molecules by ion-
177 exchange HPLC, arsenic was measured by AAS (Fattorini and Regoli, 2004). Arsenic compounds
178 were identified by their retention times, using DORM-2 as standard reference material for
179 qualitative and quantitative control.

180 2.3. Biochemical analyses

181 All detailed conditions for sample preparation and analytical methods have been reported
182 elsewhere (Regoli et al., 2005; Bocchetti and Regoli, 2006).

183 *Metallothioneins (MT-like proteins)* were analyzed in digestive glands after acidic
184 ethanol/chloroform fractionation of the tissue homogenates, and spectrophotometrically quantified
185 using GSH as standard (Viarengo et al., 1997). The *activity of Acyl CoA oxidase (AOX)* was
186 measured as marker of peroxisomal proliferation in a coupled assay (Small, 1985). The H_2O_2
187 production was followed by the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalyzed by
188 an exogenous horseradish peroxidase and Palmitoyl-CoA was used as substrate.
189 *Acetylcholinesterase activity (AChE)* was assayed by the Ellman's reaction with acetylthiocholine
190 and 5,5-dithiobis-2-nitrobenzoic acid (DTNB).

191 Among *enzymatic antioxidants*, superoxide dismutase (SOD) was determined by monitoring
192 the decreased reduction of cytochrome c by O_2^- generated by xanthine oxidase/hypoxanthine
193 system. One unit of SOD is defined as the amount of enzyme inhibiting by 50% the reduction of
194 cytochrome c, and different volumes of sample were used to determine 50% inhibition of the
195 reaction rate. Catalase (CAT) was measured by the decrease in absorbance at 240 nm due to the

196 consumption of hydrogen peroxide. Glutathione reductase (GR) was determined following NADPH
197 oxidation during the reduction of oxidized glutathione, GSSG. Glutathione peroxidases activities
198 were measured in a coupled enzyme system where NADPH is consumed by glutathione reductase
199 to convert the formed GSSG to its reduced form (GSH); hydrogen peroxide or cumene
200 hydroperoxide were the substrates, respectively for the selenium-dependent and for the sum of Se-
201 dependent and Se-independent forms. Glutathione S-transferases (GST) were assayed using 1-
202 chloro-2,4-dinitrobenzene as substrate (CDNB). *Total glutathione* was enzymatically assayed by
203 following the GR-catalyzed reaction of GSH with DTNB and comparing this rate with a standard
204 GSH curve.

205 *The Total Oxyradical Scavenging Capacity (TOSC)* assay measures the overall capability of
206 cellular antioxidants to absorb different forms of artificially generated oxyradicals (peroxyl radicals,
207 ROO \cdot and hydroxyl radicals \cdot OH), thus inhibiting the oxidation of α -keto- γ -methiolbutyric acid
208 (KMBA) to ethylene gas (Winston et al., 1998; Regoli and Winston, 1999; Regoli et al., 2004).
209 Ethylene formation was analyzed by gas-chromatographic analyses and the specific TOSC values
210 (normalized to the content of protein) were calculated according to Regoli and Winston (1999).

211 Levels of *malondialdehyde (MDA)* were spectrophotometrically measured after
212 derivatization in 1-metyl-2-phenylindole (dissolved in acetonitrile/methanol 3:1) with HCl 32%,
213 and calibrated against a malondialdehyde standard curve. Protein concentrations were measured
214 according to the Lowry method, using bovine serum albumine (BSA) as standard.

215 2.4. Histological analyses

216 *The lysosomal membrane stability* was measured by the neutral red retention time (NRRT)
217 according to Lowe et al. (1995). At least 10 mussels were individually analysed for each sampling
218 point; haemocytes from the adductor muscle were incubated on a glass slide with a freshly prepared
219 neutral red (NR) working solution (2 μ l/ml saline from a stock solution of 20 mg neutral red dye
220 dissolved in 1 ml of dimethyl sulfoxide) and microscopically examined at 15 min intervals (for up
221 to 120) to determine the time at which 50% of cells had lost to the cytosol the dye previously taken
222 up by lysosomes. *Lipofuscin* content of tertiary lysosomes was determined on duplicate cryostat
223 sections (8 μ m thick) of digestive glands. Slides were fixed in Beker's fixative (+2.5% NaCl) and
224 stained by the Schmrol reaction (Moore, 1988) before mounting in glycerine gelatine. For analyses
225 of *neutral lipids*, duplicate cryostat sections (8 μ m thick) were fixed as above, and stained with the
226 Oil Red O (ORO) method (Moore, 1988). For both lipofuscin and neutral lipids, five measurements
227 were made on digestive tubules of each section (two sections for mussel, 10 mussels for sampling
228 point). Quantification of staining intensity was performed with Kodak 1D Image Software and then

229 normalized to the area of digestive tubules measured with Olympus DP software (Bocchetti and
230 Regoli, 2006).

231 2.5. Analyses of genotoxic effects

232 The DNA integrity was evaluated at molecular level as single strand breaks (SB) by the
233 Comet assay, and at chromosomal level by the micronucleus test. The *comet assay* was carried out
234 on haemocytes immediately diluted in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free buffer, centrifuged, resuspended in 0.6%
235 low-melting-point agarose, and included in 1% normal-melting-point agarose on glass slides
236 (Machella et al., 2006). Slides were placed into the lysing solution (2.5 M NaCl, 100 mM EDTA,
237 1% Triton X-100, and 10% DMSO, pH 10, 4°C in the dark for 90 min), and the DNA was unwound
238 in 75 mM NaOH, 10 mM EDTA (pH 13) before the electrophoresis (1 V/cm for 10 min). After
239 neutralization and fixation, slides were stained with SYBR Green 1 x (Molecular Probes), and 100
240 randomly selected cells per slide and two replicates per sample were observed under a fluorescence
241 microscope (200 x magnification; Olympus BX-51). Cells were classified as belonging to one of
242 five classes of damage, on the basis of length of DNA migration and the relative proportion of
243 head/tail fluorescence: Class 1, DNA fragmentation less than 5%; Class 2, DNA fragmentation
244 between 5% to 20%; Class 3, DNA fragmentation between 20 to 40%; Class 4, DNA fragmentation
245 between 40 to 95%; Class 5, DNA fragmentation between 95 to 100%. Comet results are given as
246 mean percentage distribution of cells in the various classes (\pm SD). A mean total damage (TD) was
247 summarized as a synthetic index of DNA integrity, allowing easier comparison between different
248 experimental conditions,

$$249 \quad \text{TD} = \sum [(n_1 \times 5) + (n_2 \times 20) + (n_3 \times 40) + (n_4 \times 95) + (n_5 \times 100)]/100$$

250 where n_1 , n_2 , n_3 , n_4 , and n_5 indicate the percentage of cells within each of five classes of damage.
251 TD will thus range between 5, when the totality of cells are in Class 1 (corresponding to the 5% of
252 DNA damage), and 100 when all the nuclei are in Class 5 (corresponding to the 100% of DNA
253 damage).

254 For the *micronucleus test* an aliquot of haemolymph was rapidly washed in saline buffer,
255 fixed in Carnoy's solution (3:1 ethanol, acetic acid), dispersed on glass slides and stained with the
256 fluorescent dye 4',6-diamidino-2-phenylindole DAPI (100 ng/ml). For each specimen, 2.000 cells
257 with preserved cytoplasm were scored to determine the frequency of micronuclei; micronuclei were
258 defined as round structures, smaller than 1/3 of the main nucleus diameter on the same optical plan
259 and clearly separated from it (Venier et al., 1997).

260 2.6. "Stress on stress" analyses

261 The capability to survive in air was used as a general physiological index in mussels from
262 different sampling periods and sites. Mussels (100 specimens) were maintained in a plastic chamber

263 with high humidity and at the constant temperature of $14 \pm 1^\circ\text{C}$. Mussels mortality was daily
264 checked, and animals were considered dead when their valves gaped and failed to close when
265 physically stimulated. Median survival time (LT_{50}) was calculated for each group.

266 2.7. Statistical analyses

267 Analysis of variance (2-way ANOVA) was applied to test differences between sites,
268 sampling periods and interactions “site x time” (level of significance at $p < 0.05$). Post-hoc
269 comparison (Newman-Keuls) was used to discriminate between means of values. Multivariate
270 statistical analyses (principal component analysis, PCA) of biomarkers and chemical data were
271 applied to discriminate between different sites and/or different sampling period.

272

273 3. Results

274 Concentration of trace metals in mussels before the translocation and in those caged to both
275 the reference site (Portonovo) and to the platform “Giovanna” are reported in Table 1. Regarding
276 mussels translocated to the sampling site of Portonovo, some differences were observed for all the
277 metals before and after the 4 weeks caging period; concentrations generally decreased during the
278 first sampling period and increased in the second, while more limited variations were obtained
279 during the third and the fourth periods when only a few elements slightly changed in mussels caged
280 in the reference site. Compared to mussels transplanted to the reference site, those from the
281 platform generally exhibited similar levels or some fluctuating variation for barium, copper,
282 mercury, nickel and lead. Higher bioaccumulation for caged mussels was detected close to the
283 platform for zinc and, to a more limited extent for cadmium and arsenic; for this latter element
284 chromatographic separation and chemical speciation revealed arsenobetaine as the most represented
285 molecule (>90%). On the other hand, levels of chromium and especially of iron and manganese
286 were higher in mussels from Portonovo than in those from the platform. Analysis of variance
287 revealed a significant effect of the sampling time (Table 2) with higher concentration of trace
288 metals generally measured in organisms at the third caging period in November-December 2004.

289 The comparison of mussels before and after the caging periods at both the reference site and
290 at the platform revealed the alteration of several biomarkers with significant effects of both the site
291 and seasonal period (Table 3). Levels of metallothioneins in caged mussel were not influenced by
292 the translocation site, while a significant effect of the period revealed generally higher values at the
293 III experiment (Fig.1a). A marked seasonal variability was observed also for the peroxisomal
294 proliferation (Fig.1b) with a lower acyl CoA oxidase activity in all the mussels sampled during the
295 II translocation period (August-September 2004); the effect of site demonstrated a significantly
296 reduced peroxisomal activity in organisms caged at the platform during the III and IV periods. The

297 activity of acetylcholinesterase exhibited significant temporal fluctuations with higher values in
298 mussels of the III period (November/December 2004); on the other hand, comparison between sites
299 did not revealed significant differences despite, after the translocation period, higher mean values
300 were always measured in mussels from the platform compared to those caged at the reference site
301 (Fig.1c).

302 The oxidative status of mussels was evaluated through the measurement of single
303 antioxidants, integrated with analyses of total oxyradical scavenging capacity which quantify the
304 overall efficiency to neutralize different forms of reactive oxygen species. All the antioxidants
305 showed significant seasonal fluctuations while more limited differences were observed between
306 mussels before and after the caging period at both the reference site and the platform (Table 3, Fig.1
307 d-n). Among these, glutathione reductase and glutathione peroxidases showed higher activities in
308 mussels from the platform compared to those caged at the reference site during the I and/or II
309 periods and a similar trend was exhibited also by superoxide dismutase although, due to the
310 elevated internal variability, differences between mean averages did not reach the statistical
311 significance.

312 When data on single antioxidants were integrated with the measurement of the total
313 oxyradical scavenging capacity, TOSC values were generally higher (toward both $\text{ROO}\cdot$ and/or
314 $\cdot\text{OH}$) in mussels transplanted to the platform during the I and II period, confirming a certain
315 prooxidant challenge, experienced by these organisms (Fig. 1m-n).

316 During the I translocation period, mussels from the platform showed a significant reduction
317 of the neutral red retention time while more similar levels of lysosomal destabilization were
318 measured at other sampling occasions between organisms caged at both the reference site and
319 platform (Fig. 2a). Analyses of lipid peroxidation revealed a peak of malondialdehyde in organisms
320 caged at the reference site during the III sampling period (November/December 2004) (Fig.3b).
321 Despite some differences in average values, accumulation of lipofuscin and neutral lipids in tertiary
322 lysosomes was not affected by the caging site, but markedly influenced by the seasonal period
323 (Table 3, Fig. 2c-d); in particular higher levels of lipofuscin were observed during the warmer
324 months (II sampling period), while the neutral lipid content significantly lowered in summer and
325 winter months (II and III periods) (Fig. 3d).

326 Genotoxic alterations were measured as occurrence of strand breaks (Comet assay) and the
327 percentage distribution of cells within 5 classes of damage was summarized by the Total Damage.
328 Mussels caged at the platform during the first period exhibited a higher degree of DNA alteration
329 despite, in the overall treatment of data, the effect of "site" did not reach the statistical significance
330 (Table 3, Fig 2e). More evident effects were obtained by the analysis of micronuclei which

331 exhibited a higher frequency in mussels caged at both the sites in the III period and close to
332 platform at the IV sampling time (Fig 3f).

333 The “Stress on stress response” revealed that the mussel ability to survive in air slightly
334 varied in different sampling periods with LT50 ranging between 8 days (during the winter) and 12
335 days (during the spring and summer period) (not shown), without any difference between organisms
336 before and after the translocation.

337 The Principal Component Analysis (PCA) of biomarkers and chemical data produced a two
338 dimensional pattern which explained 55% of the total variance; the plot score for all the sampling
339 periods, giving the position of the sites in the co-ordinates of PC1 and PC2, indicated a clear
340 separation between the different sampling periods (Fig. 3). The III sampling period (winter) was
341 discriminated for higher levels of trace metals and metallothioneins, higher activity of
342 acetylcholinesterase and glutathione reductase, increased levels of malondialdehyde, and lower time
343 of stress on stress response; on the other hand, the II sampling period (summer) was mostly
344 separated from the other periods due to the higher levels of lipofuscin and lower activities of
345 catalase and acyl CoA oxidase.

346 4. Discussion

347 The use of caged mussels has been demonstrated as an useful strategy for biomonitoring
348 marine pollution, either through the analysis of chemicals accumulation and biomarker responses
349 (Nasci et al., 2002; Romeo et al., 2003; Regoli et al., 2004; Nigro et al., 2006). Caged mussels
350 facilitate the investigation in areas where native organisms are absent, reduce the influence of
351 genetic / population differences, of seasonal variability or adaptive phenomena, all factors which
352 can attenuate the capability to discriminate between different levels of environmental disturbance.
353 The use of caged organisms provides a time-integrated assessment of environmental quality over
354 the 4-weeks translocation period, and reveals the early biological effects induced by accumulated
355 pollutants.

356 In this study, bioaccumulation of trace metals and a wide array of biomarkers were
357 seasonally investigated in mussels transplanted to both a reference site and a model offshore
358 platform, to demonstrate such ecotoxicological protocol as an additional contribution for monitoring
359 offshore activities in Adriatic sea. Mussels caged at the platform did not generally exhibit a
360 significant accumulation of trace metals with some exceptions for zinc and, to a more limited
361 extent, for cadmium, suggesting for these elements the influence of galvanic anodes installed for
362 cathodic protection of the platform structures. Also slightly higher arsenic concentrations were
363 measured in mussels caged at the platform compared to those maintained at the reference site.
364 However, chromatographic separation and chemical speciation revealed a natural origin of this
365 metal which was mostly present as arsenobetaine, a non toxic arsenic compound normally
366 accumulated by marine organisms through the diet and not released from anthropogenic activities
367 (Fattorini and Regoli 2004).

368 The relatively low concentrations of barium measured in mussels from the platform,
369 indicated that this metal is not accumulated by mussels and is probably not bioavailable, despite its
370 presence at high values in sediments around the structure as a result of its use as barite for
371 extraction activities (ICRAM, 2006). Environmental levels of Cu, Hg, Ni and Pb appeared similar
372 in the 2 sites, while more elevated concentrations of Cr, Mn and especially Fe were measured in
373 mussels from the reference area. These values were well within the natural range of control
374 organisms (Regoli, 1998) do not appear to represent an oxidative challenge for these organisms, but
375 would rather confirm a naturally greater bioavailability of these elements in coastal sites, partly due
376 to the higher sediment resuspension. In addition, Fe and Mn released with the discharge of
377 produced water (PW) from the platforms, can rapidly precipitate to the bottom sediments as
378 aggregates, thus being removed from the seawater column and filter-feeding bivalves (Azetsu-Scott
379 et al., 2007).

380 For all the investigated metals, a significant seasonality was observed in mussels from both
381 the reference and platform sites which exhibited the highest concentrations during the III sampling
382 period (November-December 2004). Such a trend confirms the natural influence of reproductive
383 phases, with higher levels of trace elements during the winter period after spawning (period III) and
384 before spring tissue growth and the development of gonadic tissues (period IV), which is known to
385 “biologically dilute” the body burdens of trace metals in mussels (Regoli and Orlando, 1994b;
386 Regoli, 1998).

387 The integration of chemical analyses with biomarkers in sentinel organisms is recommended
388 in the OSPAR guidelines for monitoring offshore exploitation activities (OSPAR, 2004). In a recent
389 workshop on Biological Effects of Contaminants in Marine Pelagic Ecosystems (BECPELAG),
390 several biochemical and cellular biomarkers have been applied in caged mussels and fish to assess
391 the impact of produced waters and pollutants released from oil and gas platforms in the North Sea
392 (Hylland et al., 2006). Intracellular accumulation of neutral lipids, peroxisomal proliferation,
393 lysosomal membrane stability, antioxidants defences, acetylcholinesterase, benzo(a)pyrene
394 hydroxylase (BPH) and histological changes were analysed in mussels caged at the Statfjord oil
395 extraction area (Bilbao et al., 2006; Burgeot et al., 2006; Regoli et al., 2006); in addition to these
396 responses, also biotransformation CYP450 enzymes, PAH metabolites in bile, liver pathology,
397 DNA damage and endocrine effects were measured in caged Atlantic cod, *Gadus morhua* (Aas et
398 al., 2006; Balk et al. 2006; Bilbao et al., 2006; Feist et al., 2006; Forlin and Hylland, 2006; Scott et
399 al., 2006).

400 The main objective of the present study was to test the suitability of a wide battery of
401 cellular biomarkers in caged Mediterranean mussels, as an additional tool for assessing the impact
402 of offshore activities also in the Adriatic sea. Levels of metallothioneins were generally comparable
403 in mussels caged in both the reference and platform sites and, according to data on trace metals
404 concentrations, higher levels of these proteins were observed during the III sampling period.
405 Although the induction of metallothioneins is commonly used as sensitive biomarker of trace metals
406 exposure, several studies revealed that biological factors like the reproductive cycle influence this
407 response in mussels (Cajaraville et al., 2000; Bocchetti and Regoli 2006). Seasonal variations
408 obtained in this study were similar to those reported by Ivankovic et al. (2005), confirming
409 pronounced fluctuations of these proteins in mussels from Adriatic coasts, with the highest values in
410 winter (at the end of the spawning season) and a progressive decrease from early spring to summer
411 period (during the gametogenesis).

412 Among biological effects potentially induced by organic chemicals, peroxisomal
413 proliferation is a potential biomarker particularly relevant for marine invertebrates (Cancio et al.,

414 1999). This study did not reveal any induction in mussels caged at the platform, allowing us to
415 exclude the significant exposure to chemicals acting as peroxisomal proliferators. In this respect,
416 our results are in agreement with previous studies indicating no changes in the levels of total PAHs
417 in tissues of native mussels with concentrations ranging between 20 and 75 ng/g dry weight for both
418 the reference and platform sites (ICRAM, 2005). The seasonal changes of Acyl CoA oxidase would
419 further support the natural influence of factors like reproductive cycle and/or food availability in
420 modulating this enzymatic activity. The lower peroxisomal activity measured in mussels from both
421 sites during the summer (II sampling period) has already been observed in Adriatic mussels
422 (Bocchetti and Regoli, 2006), while the reduced values measured in organisms from the platform
423 during the III and IV periods can reflect the reduced food availability at this site located 18 nautical
424 miles offshore.

425 Acetylcholinesterase has a fundamental role in the nervous system of both vertebrates and
426 invertebrates, and its inhibition is considered a typical effect of organophosphate and carbamate
427 pesticides (Rickwood and Galloway, 2004). Although activities of AChE were not statistically
428 different, organisms from the platform showed consistently higher mean values compared to those
429 caged at the reference area. Similar effects may be caused by other factors which are known to
430 modulate this enzymatic activity, including trace metals (cadmium, copper, mercury, zinc) and
431 variations of natural factors, i.e. seawater temperature and presence of biotoxins or cyanobacteria in
432 mussels tissues (Najimi et al., 1997; Dellali et al., 2001; Dailianis et al., 2003; Frasco et al., 2005).
433 Future investigations should get more insights in the seasonal variability of AChE in Adriatic
434 mussels, to support a different response to natural factors in coastal versus offshore sites.

435 The oxidative status of mussels was measured through the activity of several individual
436 antioxidants, integrated with the overall capability to neutralise specific oxyradicals (total
437 oxyradical scavenging capacity). Antioxidant responses generally showed limited variations in
438 mussels caged at the 2 different sites, demonstrating only a moderate effect of platform activities on
439 the oxidative status of transplanted mussels. Glutathione reductase and glutathione peroxidases
440 showed higher activities in mussels from the platform compared to those caged at the reference site
441 during the I and/or II periods; the overall significance of such responses was reflected by an
442 increased capability to neutralize peroxy or hydroxyl radicals, thus indicating a more integrated
443 unbalance of oxyradical metabolism (Regoli and Winston 1999; Gorbi and Regoli 2003). However
444 the limited extent of these variations can indicate only a slight prooxidant challenge in mussels
445 caged at the platform. More evident variations were observed for several antioxidant responses
446 (CAT, GR, GPx, TOSC •OOR, TOSC •OH) in different seasonal periods, confirming the natural
447 influence of biotic and abiotic factors (i.e. availability of nutrients, temperature or reproductive

448 status) in modulating the oxidative status of mussels from Adriatic sea (Petrović et al., 2004;
449 Bocchetti and Regoli, 2006).

450 Lysosomes represent major sites for intracellular sequestration and detoxification of
451 xenobiotics (Lowe et al., 1995; Petrović et al., 2004), and alterations of their membrane stability
452 have been widely demonstrated as sensitive biomarkers for chemical pollutants in both laboratory
453 and field studies (Petrovic et al., 2004; Regoli et al., 2004; Moore et al., 2006). According to
454 previous results on some antioxidant responses, the neutral red retention time was significantly
455 reduced in mussels caged at the platform during the I translocation period. On the other hand, site-
456 independent seasonal variations indicated a lower lysosomal stability during the summer (II
457 sampling period) when the more elevated temperature can act directly on the membrane
458 composition or through increased formation of oxyradicals (Moore, 1976; Regoli, 1992; Bocchetti
459 and Regoli, 2006). Analyses of malondialdehyde excluded the accumulation of such peroxidative
460 product in mussels caged at both the sites, with the exception of a peak in the reference area during
461 the III period (December 2005). An elevated variability of MDA levels has already been observed
462 in this mussel population during the winter, and it has been associated with the spawning event,
463 consequent degradation of digestive epithelia and increase of autophagic processes (Bocchetti and
464 Regoli, 2006). Lipofuscin is an intralysosomal nondegradable product, primarily composed of
465 cross-linked proteins and lipids accumulated under oxidative stress conditions. Although a marked
466 increase of lipofuscin can occur in polluted organisms (Moore, 1988), the higher levels observed in
467 this study during the II sampling period (August/September 2004) are in agreement with the
468 reduced lysosomal membrane stability in mussels from both the sites and the presence of a
469 biological disturbance due to the increased summer temperature (Regoli, 1992; Petrovic et al.,
470 2004). Similarly to lipofuscin, neutral lipids can be greatly accumulated in mussels collected from
471 contaminated sites, but this parameter also exhibits a marked seasonality with increased levels in
472 spring during gonadic development (Regoli, 1992; Cancio et al., 1999; Domouhtsidou and
473 Dimitriadis, 2001; Petrovic et al., 2004; Moore et al., 2006); our data confirmed these natural
474 fluctuations of neutral lipid content, higher during the I and IV sampling periods corresponding to
475 springs 2004 and 2005. Overall, the lysosomal biomarkers measured in this study, did not reveal a
476 marked biological impact in mussels caged at the “Giovanna” platform, but confirmed the
477 importance of natural seasonal fluctuations for a correct interpretation and use of these biological
478 responses.

479 Genotoxic biomarkers in sentinel species, are recommended in monitoring investigations
480 and both the Comet assay and the micronucleus test have been largely validated in Mediterranean
481 mussels (Frenzilli et al., 2001; Machella et al., 2006; Regoli et al., 2004; Nigro et al., 2006). In our

482 study, the Comet assay demonstrated a moderate increase of DNA fragmentation in mussels caged
483 to the platform during the first period, further supporting the slight oxidative challenge revealed by
484 antioxidant responses and lysosomal membrane stability. More evident effects were obtained by the
485 analysis of micronuclei, small and intracytoplasmatic masses of chromatin resulting from
486 chromosomal breakage, or aneuploidy during cell division (Venier et al., 1997). A higher frequency
487 of MN in mussels caged to both the sites during the winter suggests the influence of some natural
488 factors, potentially associated with spawning events and increase of autophagic processes. On the
489 other hand the increase of MN frequency in mussels caged at the platform in the IV sampling period
490 was the only altered parameter in these organisms and it will deserve further investigations.

491 The general lack of molecular and cellular effects in mussels caged at the platform was
492 confirmed at the organism level by the stress on stress response which always revealed a
493 comparable air survival capability in mussels before and after the caging period to both the sites.
494 Longer survival times at lower seawater temperature are normally explained in terms of reduced
495 metabolic activities and oxygen consumption (Eertman et al. 1993). However, our study revealed a
496 lower resistance in mussels during the winter, a feature already reported for mussels of the Northern
497 Adriatic, and probably influenced by the reproductive and spawning period (Nesto et al., 2004;
498 Pampanin et al., 2005).

499 The specific conclusion from assessment of the overall results of the present study is that we
500 can exclude any major pollution from the gas production platform with no marked biological
501 disturbance in caged mussels. However, given the current controversy over the acceptance of
502 biomarkers in European monitoring guidelines, some more general considerations can also be
503 summarized in clear messages for policy makers. All the biomarkers tested in caged mussels are
504 well known and methodologically simple analyses. They can reveal specific chemicals and different
505 levels of biological disturbance but they can also be used to assess the absence of anthropogenic
506 impact. The basic knowledge of biological responses used as biomarkers overcomes the
507 confounding of natural variability with environmental disturbance. In this study, variations of both
508 chemical and biological responses were influenced by fluctuations of biological and environmental
509 factors rather than the effects of platform activities. The effects of seasonality can be more
510 pronounced in certain periods and significantly influence responses of caged organisms. This effect
511 is easily accounted by comparison with organisms transplanted in the same 4 weeks to a reference
512 site. Several univariate and multivariate methods can be applied to obtained results allowing
513 researchers to discriminate impacted organisms and the huge amount of international scientifically
514 sound experiences should guarantee politicians on the efficacy of biomarkers as additional tools in
515 environmental monitoring. In particular, this study demonstrated the practical suitability of the

516 ecotoxicological protocol with caged mussels for implementing the monitoring and control of
517 offshore activities in the Adriatic sea.

518

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523

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Metal	Site	Sampling Period			
		I	II	III	IV
Arsenic	Before translocation	12.12 ± 1.74	11.99 ± 1.28 (a)	17.17 ± 0.84 (a)	16.89 ± 3.47 (a)
	Reference translocation	9.76 ± 0.83	14.48 ± 1.25 (b)	17.54 ± 3.25 (a)	7.48 ± 0.30 (b)
	Platform translocation	13.04 ± 3.72	18.44 ± 0.93 (c)	21.23 ± 1.10 (b)	9.87 ± 1.06 (c)
Barium	Before translocation	3.35 ± 1.23 (a)	1.03 ± 0.22 (a)	6.97 ± 2.57 (a)	1.76 ± 0.62 (a)
	Reference translocation	1.04 ± 0.19 (b)	1.65 ± 0.12 (b)	2.65 ± 0.39 (b)	2.28 ± 0.24 (a)
	Platform translocation	1.78 ± 0.51 (b)	0.55 ± 0.30 (c)	1.16 ± 0.13 (c)	0.82 ± 0.36 (b)
Cadmium	Before translocation	1.37 ± 0.21 (a)	0.82 ± 0.09 (a)	1.18 ± 0.27 (a)	0.74 ± 0.40
	Reference translocation	0.54 ± 0.07 (b)	0.89 ± 0.16 (a)	1.76 ± 0.52 (b)	0.45 ± 0.06
	Platform translocation	0.90 ± 0.22 (c)	1.21 ± 0.16 (b)	1.99 ± 0.35 (b)	0.57 ± 0.08
Chromium	Before translocation	0.71 ± 0.22 (a)	0.44 ± 0.22	1.08 ± 0.48	0.73 ± 0.45 (a)
	Reference translocation	0.46 ± 0.09 (b)	0.50 ± 0.13	1.22 ± 0.26	0.67 ± 0.09 (b)
	Platform translocation	0.31 ± 0.02 (c)	0.35 ± 0.06	0.70 ± 0.07	0.30 ± 0.06 (c)
Copper	Before translocation	5.57 ± 0.74 (a)	3.31 ± 0.57 (a)	7.13 ± 1.63	5.39 ± 1.06 (a)
	Reference translocation	4.23 ± 0.67 (b)	5.00 ± 0.57 (b)	8.02 ± 0.33	4.19 ± 0.36 (b)
	Platform translocation	3.97 ± 1.01 (b)	3.22 ± 0.62 (a)	6.61 ± 1.15	4.03 ± 2.92 (c)
Iron	Before translocation	353.95 ± 84.87 (a)	138.87 ± 25.95 (a)	507.39 ± 264.30 (a)	254.04 ± 168.50 (a)
	Reference translocation	126.25 ± 18.70 (b)	271.85 ± 65.73 (b)	423.89 ± 89.96 (a)	264.59 ± 43.54 (a)
	Platform translocation	64.27 ± 5.96 (b)	60.36 ± 11.98 (c)	142.67 ± 31.86 (b)	59.89 ± 7.34 (b)
Mercury	Before translocation	0.05 ± 0.01 (a)	0.03 ± 0.01 (a)	0.02 ± 0.01	0.02 ± 0.00 (a)
	Reference translocation	0.03 ± 0.00 (b)	0.04 ± 0.00 (ab)	0.02 ± 0.00	0.04 ± 0.00 (b)
	Platform translocation	0.04 ± 0.01 (b)	0.05 ± 0.01 (b)	0.02 ± 0.00	0.03 ± 0.00 (c)
Manganese	Before translocation	11.13 ± 4.10 (a)	8.16 ± 0.45 (a)	18.42 ± 6.99	10.29 ± 4.32 (a)
	Reference translocation	8.06 ± 1.31 (a)	12.11 ± 2.86 (b)	15.02 ± 2.07	8.87 ± 1.36 (a)
	Platform translocation	3.40 ± 0.62 (b)	5.80 ± 0.88 (c)	11.27 ± 1.89	2.09 ± 0.08 (b)
Nickel	Before translocation	2.09 ± 0.39 (a)	1.43 ± 0.04	3.45 ± 1.27	1.35 ± 0.20 (a)
	Reference translocation	6.04 ± 1.13 (b)	1.76 ± 0.46	3.90 ± 0.26	1.37 ± 0.04 (a)
	Platform translocation	1.01 ± 0.29 (c)	1.66 ± 0.31	3.49 ± 0.63	0.73 ± 0.06 (b)
Lead	Before translocation	1.16 ± 0.10 (a)	0.82 ± 0.08 (a)	1.51 ± 0.33	1.30 ± 1.13
	Reference translocation	0.75 ± 0.09 (b)	1.09 ± 0.12 (a)	1.46 ± 0.12	0.73 ± 0.05
	Platform translocation	0.85 ± 0.12 (b)	0.79 ± 0.21 (b)	1.44 ± 0.18	0.56 ± 0.06
Zinc	Before translocation	80.25 ± 32.21 (a)	154.12 ± 25.78 (a)	100.76 ± 36.99 (a)	63.11 ± 28.63
	Reference translocation	37.46 ± 26.24 (a)	154.12 ± 25.78 (a)	95.76 ± 15.38 (a)	54.08 ± 11.82
	Platform translocation	154.32 ± 54.69 (b)	162.66 ± 56.84 (a)	184.28 ± 78.14 (b)	62.02 ± 20.52

Table 1. Metals concentrations ($\mu\text{g/g}$ dry weight) in mussels collected during the 4 sampling periods (mean values \pm standard deviations, $n=5$). I April-May 2004; II August-September 2004; III November-December 2004; IV March-April 2005. Different letters indicate significant differences between sites during the same sampling period (Newman Keuls test; $p < 0.05$).

	As	Ba	Cd	Cr	Cu	Fe
Effect	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>
1 (Site)	$p < 0.005$	$p < 0.000001$	0.00005	$p < 0.000001$	0.000005	$p < 0.000001$
2 (Time)	$p < 0.000001$					
1 × 2 (Site × Time)	$p < 0.000001$	$p < 0.000001$	$p < 0.000001$	$p < 0.05$	$p < 0.0001$	$p < 0.000005$

	Hg	Mn	Ni	Pb	Zn
Effect	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>
1 (Site)	$p < 0.05$	$p < 0.000001$	$p < 0.000001$	$p < 0.005$	$p < 0.00005$
2 (Time)	$p < 0.000001$				
1 × 2 (Site × Time)	$p < 0.0005$	$p < 0.000001$	$p < 0.000001$	$p < 0.0005$	$p < 0.005$

Table 2. Results of two-way analysis of variance for the metals measured in mussels, *M. galloprovincialis*, before and after the 4 translocation periods at both the reference site (Portonovo) and the Giovanna platform.

	MT	AOX	AChE	SOD	CAT	GST
Effect	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>
1 (Site)	n.s.	<i>p</i> <0.05	<i>p</i> <0.001	n.s.	n.s.	n.s.
2 (Time)	<i>p</i> <0.005	<i>p</i> <0.000001	<i>p</i> <0.000001	n.s.	<i>p</i> <0.000001	n.s.
1 × 2 (Site × Time)	n.s.	<i>p</i> <0.005	n.s.	n.s.	n.s.	<i>p</i> <0.000001

	GR	GPx H ₂ O ₂	GPx CHPx	GSH	TOSC ROO•	TOSC •OH
Effect	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>
1 (Site)	<i>p</i> <0.05	<i>p</i> <0.05	<i>p</i> <0.05	n.s.	<i>p</i> <0.05	<i>p</i> <0.05
2 (Time)	<i>p</i> <0.000001	<i>p</i> <0.001	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.0005	<i>p</i> <0.000005
1 × 2 (Site × Time)	<i>p</i> <0.001	<i>p</i> <0.05	<i>p</i> <0.0005	<i>p</i> <0.01	n.s.	<i>p</i> <0.05

	NRRT	MDA	Lipofuscin	Neutral lipids	DNA SB	MN
Effect	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>	<i>p</i>
1 (Site)	<i>p</i> <0.05	<i>p</i> <0.01	n.s.	n.s.	n.s.	<i>p</i> <0.005
2 (Time)	<i>p</i> <0.000001	<i>p</i> <0.00005	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.001
1 × 2 (Site × Time)	<i>p</i> <0.000001	<i>p</i> <0.0001	<i>p</i> <0.01	<i>p</i> <0.005	n.s.	<i>p</i> <0.0005

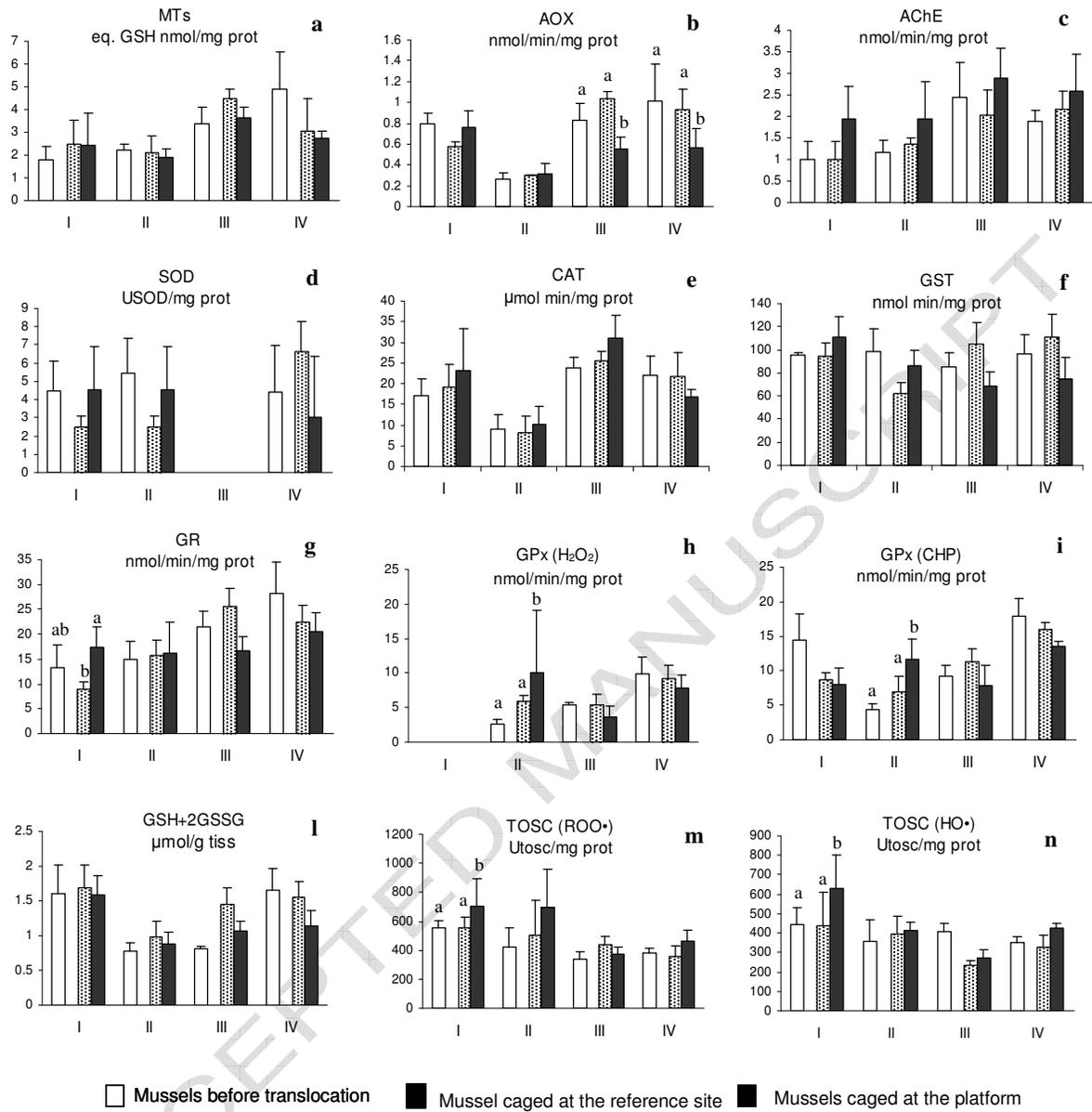
Table 3. Results of two-way analysis of variance for the biological responses in mussels, *M. galloprovincialis*, before and after the 4 translocation periods at both the reference site (Portonovo) and the Giovanna platform. MT – metallothioneins; AOX - acyl CoA oxidase; AChE - acetylcholinesterase; SOD - superoxide dismutase; CAT - catalase; GST - glutathione S-transferases; GR - glutathione reductase; GPx H₂O₂ and GPx CHPx - selenium-dependent and sum of Se- dependent and Se-independent glutathione peroxidases; GSH - level of total glutathione; TOSC - total oxyradical scavenging capacity toward peroxy (ROO•) and hydroxyl (•OH) radical; NRRT – Neutral Red Retention Time; MDA – content of malondialdehyde; DNA SB – single DNA strand breaks; MN - micronuclei.

LEGENDS OF FIGURES

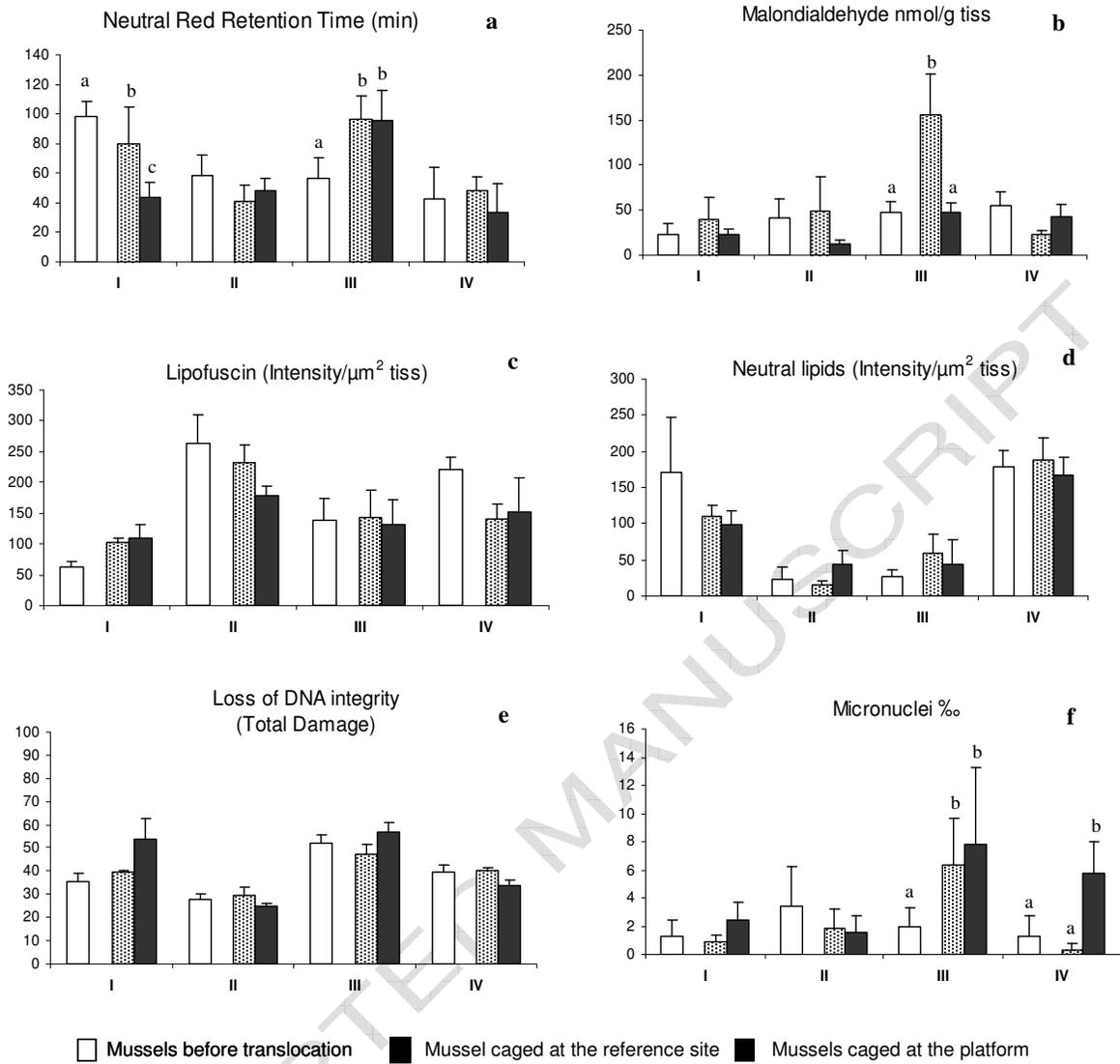
Figure 1: Variations of biomarkers in mussels before and after the 4 caging periods at both the reference site (Portonovo) and the platform Giovanna (mean values \pm standard deviations, $n=10$). MTs: level of metallothioneins; AOX: activity of the peroxisomal enzyme acyl CoA oxidase; AChE: acetylcholinesterase activity; SOD: superoxide dismutase activity; CAT: catalase activity; GST: glutathione S-transferases activity; GR: glutathione reductase activity; GPx: glutathione peroxidases activity; GSH+2GSSG: level of total glutathione; TOSC: total oxyradical scavenging capacity toward peroxyl (\bullet OOR) and hydroxyl (\bullet OH) radical. Different letters indicate significant differences between means of values within the same sampling period (Newman Keuls test; $p<0.05$).

Figure 2: Variations of cellular biomarkers in mussels before and after the 4 caging periods at both the reference site (Portonovo) and the platform Giovanna (mean values \pm standard deviations, $n=10$). Different letters indicate significant differences between means of values within the same sampling period (Newman Keuls test; $p<0.05$).

Figure 3: Separation of sites and sampling periods obtained from PCA analyses on chemical parameters and biomarkers data. to: mussels from reference site before the caging period; tp: mussels from reference site caged for 4 weeks in the same location; tg: mussels from reference site caged for 4 weeks to “Giovanna” platform; I April/May 2004, II August/September 2004, III December/January 2004, IV March/April 2005.

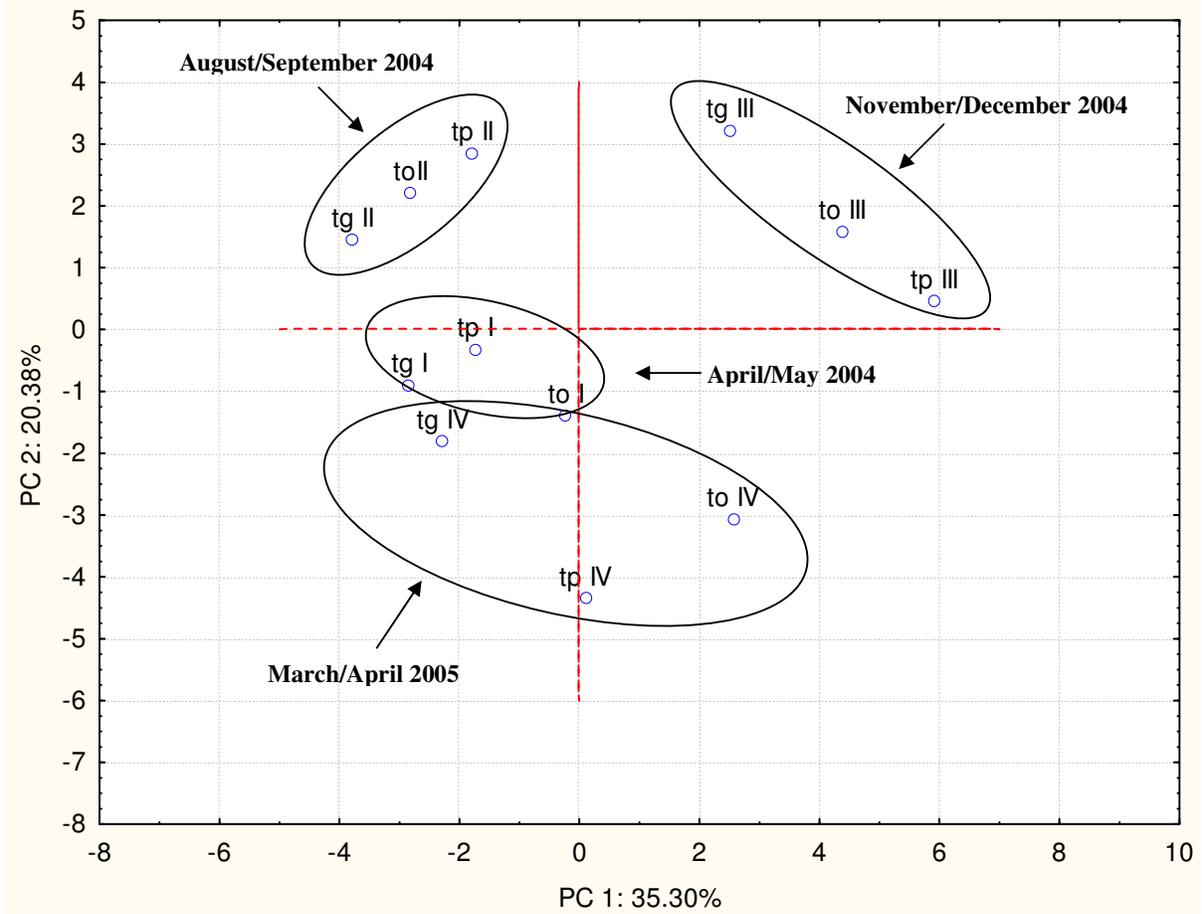


Gorbi et al., Figure 1



I April/May 2004; II August/September 2004; III November/December 2004; IV March/April 2005

Gorbi et al., Figure 2



Gorbi et al., Figure 3