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An ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis*, for monitoring the impact of an offshore platform in the Adriatic sea.

Gorbi Stefania¹, Virno Lamberti Claudia², Notti Alessandra¹, Benedetti Maura¹, Fattorini Daniele¹, Molto Ginevra², Regoli Francesco^{1*}.

¹Istituto di Biologia e Genetica, Università Politecnica delle Marche, Ancona, Italy and

²Istituto Centrale per la Ricerca Scientifica Applicata al Mare (ICRAM), Via di Casalotti 300 Roma, Italy

*** Author to whom correspondence should be addressed:**

Dr. F. Regoli,
Istituto di Biologia e Genetica, Università Politecnica delle Marche,
Via Ranieri Monte d'Ago,
60100 Ancona, Italy
tel. +39-071-2204613; fax +39-071-2204609
e-mail: f. regoli@univpm.it

Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Experimental design

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

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

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

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

2.2. Chemical analyses

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

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

2.3. Biochemical analyses

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

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

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

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

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

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

2.4. Histological analyses

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

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

2.5. Analyses of genotoxic effects

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

$$\text{TD} = \sum [(n1 \times 5) + (n2 \times 20) + (n3 \times 40) + (n4 \times 95) + (n5 \times 100)]/100$$

where n1, n2, n3, n4, and n5 indicate the percentage of cells within each of five classes of damage. TD will thus range between 5, when the totality of cells are in Class 1 (corresponding to the 5% of DNA damage), and 100 when all the nuclei are in Class 5 (corresponding to the 100% of DNA damage).

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

2.6. "Stress on stress" analyses

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

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

2.7. Statistical analyses

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

3. Results

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

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

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

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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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)	<i>p</i> <0.005	<i>p</i> <0.000001	0.00005	<i>p</i> <0.000001	0.000005	<i>p</i> <0.000001
2 (Time)	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.000001
1 × 2 (Site × Time)	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> < 0.05	<i>p</i> <0.0001	<i>p</i> <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)	<i>p</i> <0.05	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.005	<i>p</i> <0.00005
2 (Time)	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.000001
1 × 2 (Site × Time)	<i>p</i> <0.0005	<i>p</i> <0.000001	<i>p</i> <0.000001	<i>p</i> <0.0005	<i>p</i> <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 peroxyl (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 ($\bullet$ OO $\cdot$) 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.

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