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Contaminant accumulation and biomarker responses in caged mussels, *Mytilus galloprovincialis*, to evaluate bioavailability and toxicological effects of remobilized chemicals during dredging and disposal operations in harbour areas

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ABSTRACT

Remobilization of chemicals from contaminated sediments is a major risk associated with dredging and disposal operations in harbour areas. In this work caged mussels, *Mytilus galloprovincialis*, were chosen as bioindicator organisms to reveal the impact and recovery of organisms from these activities in the harbour of Piombino (Tuscany, Italy) where approximately 100,000 m^3 of sediments were removed and disposed in a local confined disposal facility (CDF). Organisms were deployed before, during and after the end of operations, selecting sites differently impacted by these activities. Temporal changes in environmental bioavailability and biological effects of pollutants were assessed by integrating analyses of trace metals and polycyclic aromatic hydrocarbons (PAHs) accumulated in tissues of caged mussels with a wide array of biomarkers reflecting exposure to specific classes of pollutants and different levels of cellular unbalance or toxicity. Such biological responses included levels of metallothioneins, activity of acyl CoA oxidase (AOX) as a marker of peroxisome proliferation, oxidative stress biomarkers (content of glutathione, enzymatic activities of catalase, glutathione *S*-transferases, glutathione reductase, glutathione peroxidases), total oxyradical scavenging capacity (TOSC) toward peroxyl and hydroxyl radicals, lysosomal membrane stability and genotoxic effects measured as DNA strand breaks and frequency of micronuclei. Obtained results indicated that a general disturbance was already present in the whole harbour area and especially in the inner site before the beginning of operations, when caged mussels exhibited a significant accumulation of PAHs and Pb, lower TOSC values and higher levels of both lysosomal and genotoxic damages. Bioavailability of trace metals and PAHs markedly increased during dredging activities with values up to 40 μg/g for Pb and up to 2200 ng/g for PAHs in tissues of caged mussels, a significant inhibition of antioxidant efficiency and increase of oxidative damages. While bioavailability of trace metals returned to the pre-dredging values after the end of operations, the accumulation of PAHs, oxidative effects and genotoxic damages remained elevated in mussels caged in the inner area and in front of CDF. Overall this study confirmed the utility of caged mussels to assess the remobilization of chemicals from dredged sediments and the onset of potentially harmful biological effects.

1. Introduction

Harbour areas represent critical environments in the Mediterranean with strategic economic importance but also pose major concerns because of the presence of toxic chemicals and their harmful effects on the marine ecosystems. Several industrial and urban sources (i.e. shipping, loading and bunkering operations, shipyards, accidental spills, wastewater emissions) are responsible for typically elevated concentrations of pollutants which accumulate in sediments due to the limited hydrodynamic energy on the inside portions of harbours. In this respect, dredging operations, which are regularly needed to maintain accessibility and navigational depths, require a proper assessment and management of contaminated sediments.

In Italy dredging activities are regulated by Legislative Decree n. 152/2006 and specific guidelines support management decisions on the disposal of harbour-dredged sediments (ICRAM-APAT, 2007). According to results of chemical–physical characterization of dredged materials integrated with acute and chronic toxicity bioassays, disposal options may include beneficial use (i.e. beach

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nourishments, construction of fill, landscaping, landfill cover, etc.), specific treatments or clean-up procedures, storage in confined disposal facilities (CDF). The direct open water discharge can be considered as the last option only for noncontaminated sediments when there is no technical or economical possibility for their re-utilization (ICRAM-APAT, 2007).

The chemical and toxicological evaluations of sediments can indicate the hazard and the most appropriate disposal strategy to some degree, but they do not reflect the risks during the dredging operations. Remobilization of pollutants from sediments can greatly affect their mobility, bioavailability and effects on organisms, which should be adequately monitored, especially when the disposal option is the confinement of dredged materials within on-site CDFs. In fact, the more frequent concern about these artificial structures is their efficacy in isolating materials from recontamination of surrounding environments.

The measurement of bioaccumulation and biological responses in sentinel organisms is thus of great utility and a similar approach has already been reported in various harbours as a valuable tool to assess the impact and toxicological effects of chemicals remobilized from sediments (Stephensen et al., 2000; Regoli et al., 2002b, 2004; Stronkhorst et al., 2003; Frenzilli et al., 2004; Almroth et al., 2005; Sturve et al., 2005; Barsiene et al., 2006).

In this work, an active biomonitoring strategy with transplanted mussels, *Mytilus galloprovincialis*, has been applied during various phases of dredging in the harbour of Piombino (Tuscany, Italy) where approximately 100,000 m^3 of sediments were removed between January and May 2005 and disposed in a local CDF. Organisms were deployed before, during and after the end of dredging and disposal operations, selecting sites differently influenced by these activities.

Chemical analyses of trace metals and polycyclic aromatic hydrocarbons (PAHs) in caged mussels were expected to reflect changes of their bioavailability during different phases and in different areas of the harbour. These results were integrated with a multi-biomarker approach, measuring a wide array of biological responses at the molecular and subcellular levels. Such biomarkers are the earliest warning signals of chemical disturbance, being often very sensitive or specific toward particular classes of pollutants, and reflecting different levels of cellular unbalance and toxicity (Cajaraville et al., 2000). The induction of metallothionein-like proteins and peroxisome proliferation were chosen as specific responses toward exposure to trace metals and organic compounds, respectively. Metallothioneins are low molecular weight, cytosolic proteins rich in –SH groups, normally involved in homeostasis of trace metals and over-expressed by elevated intracellular concentrations of Hg, Cu, Cd and Zn (Viarengo et al., 1997). Peroxisomes are heterogeneous organelles containing various oxidases and antioxidant enzymes with a metabolic function associated to lipid metabolism and inactivation of ROS (Cajaraville et al., 2003). Peroxisome proliferation in mussels exposed to PAHs and polychlorinated biphenyls (PCBs) has been shown by the increased number and volume of peroxisomes, and by the induction of enzymes involved in fatty acid oxidation, such as acyl CoA oxidase (Cajaraville et al., 2003; Orbea and Cajaraville, 2006).

A typical pathway of chemical toxicity is mediated through the increased intracellular generation of reactive oxygen species (ROS) and modulation of antioxidant defences (Regoli et al., 2002a). Although variations of antioxidants can be difficult to predict and often exhibit contradictory results in field conditions, nonetheless such oxidative biomarkers have also been shown to be sensitive in revealing a prooxidant chemical challenge in Mediterranean mussels (Regoli and Principato, 1995; Regoli, 1998, 2000; Frenzilli et al., 2001; Roméo et al., 2003; Petrović et al., 2004; Regoli et al., 2004; Gorbi et al., 2008; Viarengo et al., 2007). In this study, prooxidant effects of chemicals potentially released from dredging activities were assessed by measuring enzymatic activities of catalase, glutathione *S*-transferases (GST), glutathione reductase, glutathione peroxidases and levels of glutathione. These data were integrated with the analyses of total oxyradical scavenging capacity (TOSC) to quantify the overall cellular resistance toward peroxyl radicals, $ROO^{\bullet}$, and hydroxyl radicals, $HO^{\bullet}$ (Regoli and Winston, 1999; Regoli, 2000). Compared to individual antioxidants, TOSC is less sensitive but has a greater prognostic value since an impaired capability to neutralize ROS has been associated with the onset of various forms of oxidative damage like lysosomal alterations and genotoxic damages (Regoli, 2000; Frenzilli et al., 2001; Gorbi and Regoli, 2003; Regoli et al., 2004).

Toxic effects on lysosomal systems are generally included in all the ecotoxicological studies with mussels; these organelles are widely developed in digestive tissues and haemocytes, and have a fundamental role in cell physiology, food digestion, intracellular turnover, immune function, sequestration and excretion of harmful compounds (Moore, 1988). On the other hand, lysosomes are also the target of several contaminants which can act directly on the membranes or, indirectly, through generation of ROS or signalling pathways (Regoli, 1992, 2000; Canesi et al., 2004; Regoli et al., 2004; Viarengo et al., 2007). In the present work, lysosomal membrane stability in harbour-caged mussels was selected as a sensitive biomarker already validated in several field studies and well characterized in terms of natural variability and seasonal fluctuations for Mediterranean organisms (Regoli, 1992, 2000; Lowe et al., 1995; Bocchetti and Regoli, 2006; Viarengo et al., 2007; Gorbi et al., 2008).

The presence of a genotoxic risk during dredging and disposal operations was evaluated by the loss of DNA integrity in caged mussels. While DNA strand breaks reflects a sensitive and potentially repairable effect caused by chemicals and/or enhanced prooxidant challenge, the frequency of micronuclei indicates an irreversible genetic damage from chromatin breakage or anueploidy during cell division (Bolognesi et al., 2004).

The overall results of this study were expected to demonstrate the utility of an ecotoxicological approach with caged mussels as an additional tool to better assess the impact and risks during dredging and disposal operations in harbour areas, allowing to measure temporary variations in bioavailability of chemicals released from dredged and disposed sediments, and the early onset of harmful effects for the organisms.

2. Materials and methods

2.1. Sites and experimental design

The harbour of Piombino, located along the Tyrrhenian coast, is characterized by the presence of one of the largest European metallurgy and steel complex, carbon coke production, thermoelectric energy plant and intensive maritime traffic related to industrial activities, commercial trade and tourism. In the period January–May 2005 approximately 100,000 m^3 of sediment was dredged from the industrial area of the harbour and discharged in an artificial confined disposal facility, CDF (Fig. 1).

Mussels, *M. galloprovincialis* (6 ± 0.5 cm shell length), were obtained from a commercial farm and translocation experiments were performed in September 2004, February, April and July 2005 to monitor variations in the bioavailability and biological effects of pollutants before, during and after the end of dredging and disposal operations. In these periods mussels were caged for 4 weeks in the inner part of the harbour affected by the activities, in front of the CDF and in a more external location separated by a quay

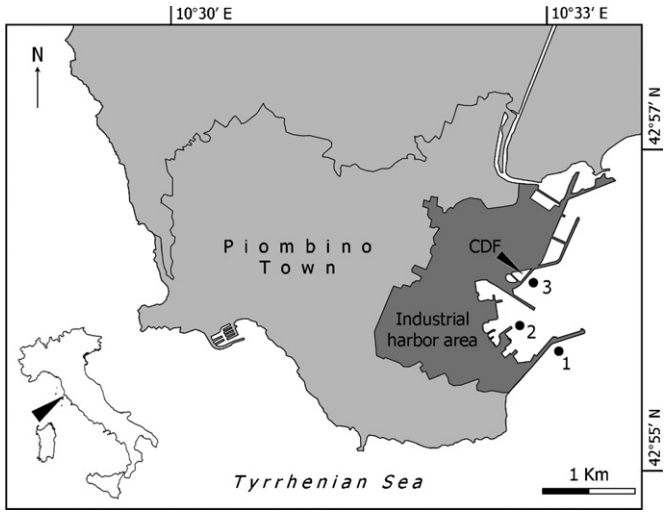

Fig. 1. Sites of translocation experiments within the harbour of Piombino. (1) The "outer" location, on the external side of a quay; (2) the "inner" area interested by dredging activities; (3) the site located in front of the confined disposal facility "CDF", where dredged sediments were disposed.

(Fig. 1). Chemical concentrations in tissues of caged mussels were compared with those measured before the various translocation periods (time 0, T0) to assess the incremental hazard within harbour area associated with dredging. Since biomarkers responses are characterized by a marked seasonal variability, to discriminate the onset of anthropogenic disturbance from natural fluctuations results obtained in harbour-caged mussels were compared to values measured in organisms caged in a pristine area not influenced by harbour activities (Bocchetti and Regoli, 2006).

After collection, mussels were transported in cold, humid containers to the laboratory and dissected within 5–6 h for chemical measurements; for each site, whole tissues were removed from 30 specimens, pooled in 10 samples and stored at −20 °C. Samples dissected for biomarkers analyses were obtained after maintaining the organisms overnight in aquaria with running filtered seawater at the constant temperature of 18 ± 1 °C. For biochemical parameters, digestive glands were rapidly removed from 30 specimens, pooled in 10 samples (each with tissues of three specimens), frozen 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

Mussel tissues were analysed for the content of cadmium, lead, mercury, zinc and polycyclic aromatic hydrocarbons, since these chemicals were those found in more elevated concentrations in sediments to be dredged (ICRAM, 2006).

For trace metals analyses, tissues were dried at 50 °C to constant weight and minced to obtain homogenous samples (Regoli et al., 2004); about 0.3 g were digested with 5 ml nitric acid and 1 ml hydrogen peroxide in a microwave digestion system (Milestone 1200, Shelton, CT, USA). Quality assurance and quality control were done by processing blank samples and certified reference material (CRM 278, mussel tissue, Community Bureau of Reference, Brussels, Belgium). Trace metals were measured by atomic absorption (AAS) or inductively coupled plasma (ICP) spectrometry. Cadmium (Cd) and lead (Pb) were analyzed using graphite furnace atomization and Zeeman effect (Varian SpectraAA 200Z, Varian, Mulgrave, VIC, Australia); when necessary, palladium solution (1 g/l, 10% nitric acid, 5% citric acid) was added as chemical matrix modifier and the standard addition technique applied for resolution of matrix effects. Zinc (Zn) was measured by ICP atomic emission (Varian Liberty AX Sequential ICP-AES), while mercury (Hg) was assayed using cold vapour atomic absorption spectrometry with a Bacharach Coleman model 50B Mercury Analyser System. The concentrations were expressed as μg/g dry weight (dw); the values obtained for the standard reference material were always within the 95% confidence interval of certified values.

For PAHs, about 1 g wet tissues were extracted in 5 ml 0.5 M potassium hydroxide in methanol using a microwave system (55 °C for 15 min) (Mars CEM, CEM Corporation, Matthews NC) according to Regoli et al. (2005). After centrifugation at 1000 × *g* for 5 min, the methanolic solutions were concentrated in a SpeedVac (RC1009; Jouan, Nantes, France) and purified with solid phase extraction (Octadecyl C18, 500 mg × 6 ml, Bakerbond; Mallinckrodt Baker, Phillipsburg, NJ, USA). A final volume of 1 ml was recovered with acetonitrile, and HPLC analyses were carried out with water–acetonitrile gradient and fluorimetric detection. PAHs were identified by the retention time of appropriate pure standard solutions (EPA 610 Polynuclear Aromatic Hydrocarbons Mix), and classified as low molecular weight (LMW: naphthalene, acenaphthene, fluorene, phenanthrene, anthracene) and high molecular weight (HMW: fluoranthene, pyrene, benzo[*a*]antrhacene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenzo[*a*,*h*]anthracene, benzo[*g*,*h*,*i*]perylene). Quality assurance and quality control were done by processing blank and reference samples (mussel tissues SRM 2977, NIST), and concentrations obtained for the SRM were always within the 95% confidence interval of certified value. The water content in tissues was determined and concentrations of PAHs expressed as ng/g dw.

2.3. Biomarker analyses

Metallothioneins (MT) were analyzed in the digestive glands homogenized (1:3, w/v) in 20 mM Tris–HCl buffer, pH 8.6, 0.5 M sucrose, 0.006 mM PMSF (phenylmethylsulphonylfluoride) and 0.01% β-mercaptoethanol. After acidic ethanol/chloroform fractionation of the tissue homogenate, MTs were spectrophotometrically quantified using GSH as standard (Viarengo et al., 1997).

Total glutathione was analysed in samples homogenized (1:5, w/v ratio) in 5% sulfosalicylic acid with 4 mM EDTA, maintained for 45 min on ice and centrifuged at 37,000 × *g* for 15 min. The resulting supernatants were enzymatically assayed (Regoli et al., 2004).

The activity of the peroxisomal enzyme Acyl-CoA oxidase (AOX) was measured in samples homogenized in 1 mM sodium bicarbonate buffer (pH 7.6) containing 1 mM EDTA, 0.1% ethanol, 0.01% Triton X-100 and centrifuged at 500 × *g* for 15 min at 4 °C. The H_2O_2 production was measured in a coupled assay (Bocchetti and Regoli, 2006) by following the oxidation of dichlorofluorescein-diacetate (DCF-DA) catalyzed by an exogenous horseradish peroxidase (HRP). The reaction medium was 0.5 M potassium phosphate buffer (pH 7.4), 2.2 mM DCF-DA, 40 μM sodium azide, 0.01% Triton X-100, 1.2 U/ml HRP in a final volume of 1 ml. After a pre-incubation at 25 °C for 5 min in the dark with an appropriate volume of sample, reactions were started adding the substrates Palmitoyl-CoA, at final concentrations of 30 μM; readings were carried out against a blank without the substrates at 502 nm.

For antioxidant enzymes, samples were homogenized (1:5, w/v ratio) in 100 mM K-phosphate buffer (pH 7.5), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mg/ml bacitracin, 0.008 TIU/ml aprotinin, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin, NaCl 2.5%, and centrifuged at 110,000 × *g* for 1 h at 4 °C. Measurements were made with a Varian (model Cary 3) spectrophotometer at a constant temperature of 18 °C (Regoli et al., 2004). Cata-

lase (CAT) was measured by the decrease in absorbance at 240 nm (extinction coefficient, $\varepsilon = 0.04\ mM^{-1}\ cm^{-1}$) due to the consumption of hydrogen peroxide, H_2O_2 (12 mM H_2O_2 in 100 mM K-phosphate buffer pH 7.0). Glutathione *S*-transferases were determined at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate (CDNB). The assay was carried out in 100 mM K-phosphate buffer pH 6.5, 1.5 mM CDNB, 1 mM GSH ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$). Glutathione reductase (GR) was determined from NADPH oxidation during the reduction of oxidized glutathione, GSSG ($\lambda = 340$ nm, $\varepsilon = -6.22\ mM^{-1}\ cm^{-1}$). The final assay conditions were 100 mM K-phosphate buffer pH 7.0, 1 mM GSSG, and 60 μM NADPH. The sum of Se-dependent and Se-independent glutathione peroxidases were assayed in a coupled enzyme system where NADPH is consumed by glutathione reductase to convert the formed GSSG to its reduced form (GSH). The decrease of absorbance was monitored at 340 nm ($\varepsilon = -6.22\ mM^{-1}\ cm^{-1}$) in 100 mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 2 mM GSH, 1 unit glutathione reductase, 0.24 mM NADPH, and 0.8 mM cumene hydroperoxide as substrate. The rate of the blank reaction was subtracted from the total rate.

The total oxyradical scavenging capacity assay measures the overall capability of cellular antioxidants to absorb different forms of artificially generated oxyradicals, thus inhibiting the oxidation of 0.2 mM α-keto-γ-methiolbutyric acid (KMBA) to ethylene gas (Winston et al., 1998; Regoli and Winston, 1999). Peroxyl radicals ($ROO^{\bullet}$) were generated by the thermal homolysis of 20 mM 2-2′-azo-bis-(2-methylpropionamidine)-dihydrochloride (ABAP) in 100 mM K-phosphate buffer, pH 7.4. Hydroxyl radicals ($^{\bullet}OH$) were produced by the Fenton reaction of iron–EDTA (1.8 μM Fe^{3+}, 3.6 μM EDTA) plus ascorbate (180 μM) in 100 mM K-phosphate buffer. Under these conditions the different oxyradicals produced quantitatively similar yields of ethylene in control reactions, thus allowing to compare the relative efficiency of cellular antioxidants toward a quantitatively similar radical flux (Regoli and Winston, 1999). Ethylene formation in control and sample reactions was analyzed at 10–12 min time intervals by gas-chromatographic analyses according to Regoli and Winston (1999). The TOSC values are quantified from the equation $TOSC = 100 - (\int SA / \int CA \times 100)$, where $\int SA$ and $\int CA$ are the integrated areas calculated under the kinetic curves for samples (SA) and control (CA) reactions. For all the samples, a specific TOSC (normalized to content of protein) was calculated by dividing the experimental TOSC values by the relative protein concentration contained in the assay. Protein concentrations were measured with the Lowry method using bovine serum albumin (BSA) as standard.

Among biomarkers of cellular alterations, the lysosomal membrane stability was measured as Neutral Red Retention Time (NRRT) as reported in Regoli et al. (2004). Haemolymph collected from the adductor muscle of 10 specimens was incubated on a glass slide with a freshly prepared neutral red working solution (2 μl/ml saline from a stock solution of 20 mg neutral red dye dissolved in 1 ml of dimethyl sulfoxide); the haemocytes were microscopically examined at 20 min intervals (for up to 120) to determine the time at which 50% of cells had lost into the cytosol the dye previously taken up by lysosomes.

The DNA integrity was evaluated at molecular level as single strand breaks (SB) by the Comet assay, and at chromosomal level by the frequency of micronuclei. The comet assay was immediately carried out on haemocytes collected from the adductor muscle of organisms according to Gorbi et al. (2008). Cells were diluted in Ca^{2+}/Mg^{2+}-free buffer at 4 °C in the dark (20 mM Hepes, 500 mM NaCl, 12.5 mM KCl, 10 mM EDTA, pH 7.3), centrifuged at 1000 rpm for 1 min at 4 °C, resuspended in 0.6% low-melting-point agarose, and added with a sandwich stratification to glass slides coated with 1% normal-melting-point agarose. After gel solidification, slides were placed into the lysing solution (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, and 10% DMSO, pH 10) at 4 °C in the dark for 90 min. DNA was unwound in 75 mM NaOH, 10 mM EDTA (pH 13), and the electrophoretic migration was carried out in the same buffer at 1 V/cm for 10 min. Slides were then neutralized for 10 min in 0.4 M Tris, pH 7.5, fixed in cold methanol for 3 min at −20 °C, and dried. After staining with SYBR Green 1 × (Molecular Probes), 100 randomly selected cells per slide and two replicates per sample were observed under a fluorescence microscope (200× magnification; Olympus BX-51). Cells were classified as belonging to one of the 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 then 5%; Class 2, DNA fragmentation between 5 and 20%; Class 3, DNA fragmentation between 20 and 40%; Class 4, DNA fragmentation between 40 and 95%; Class 5, DNA fragmentation between 95 and 100%. Comet results are calculated as mean percentage distribution of cells in the various classes. A mean total damage (TD) was summarized as a synthetic index of DNA integrity, allowing easier comparison between different experimental conditions:

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

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 frequency of micronuclei, an aliquot of haemolymph collected from the adductor muscle of organisms was rapidly washed in saline buffer (20 mM Hepes, 500 mM NaCl, 12.5 mM KCl, 10 mM EDTA, pH 7.3) and centrifuged at 1000 rpm for 1 min at 4 °C. Cells were immersed in a fixative solution (3:1 methanol, acetic acid) and washed twice. Suspended cells were dispersed on glass slides, air dried and stained with the fluorescent dye 4′,6-diamidino-2-phenylindole DAPI (100 ng/ml). For each specimen, 2000 cells with preserved cytoplasm were scored to determine the frequency of micronuclei, defined as round structures, smaller than 1/3 of the main nucleus diameter on the same optical plan and clearly separated from it (Nigro et al., 2006).

2.4. Statistical analyses

Analysis of variance was applied to test differences of chemical and biochemical parameters between sites within sampling periods (level of significance at $p < 0.05$). The homogeneity of variance was analyzed by Cochran C and 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. Statistical analyses of data were performed using the software Statistica 6.0 (Stat Soft, Tulsa, USA).

3. Results

Concentrations of trace metals and polycyclic aromatic hydrocarbons in caged mussels revealed significant differences between harbour areas ($p < 0.0001$) and translocation periods ($p < 0.0001$). Before the beginning of operations, Pb and PAHs were significantly accumulated in mussels caged in the inner site (Fig. 2) and, to a lower extent, tissue levels of PAHs increased also in mussels translocated in the outer area and in front of CDF. No variations were observed for concentrations of Cd, Zn, and Hg between reference and harbour-caged mussels.

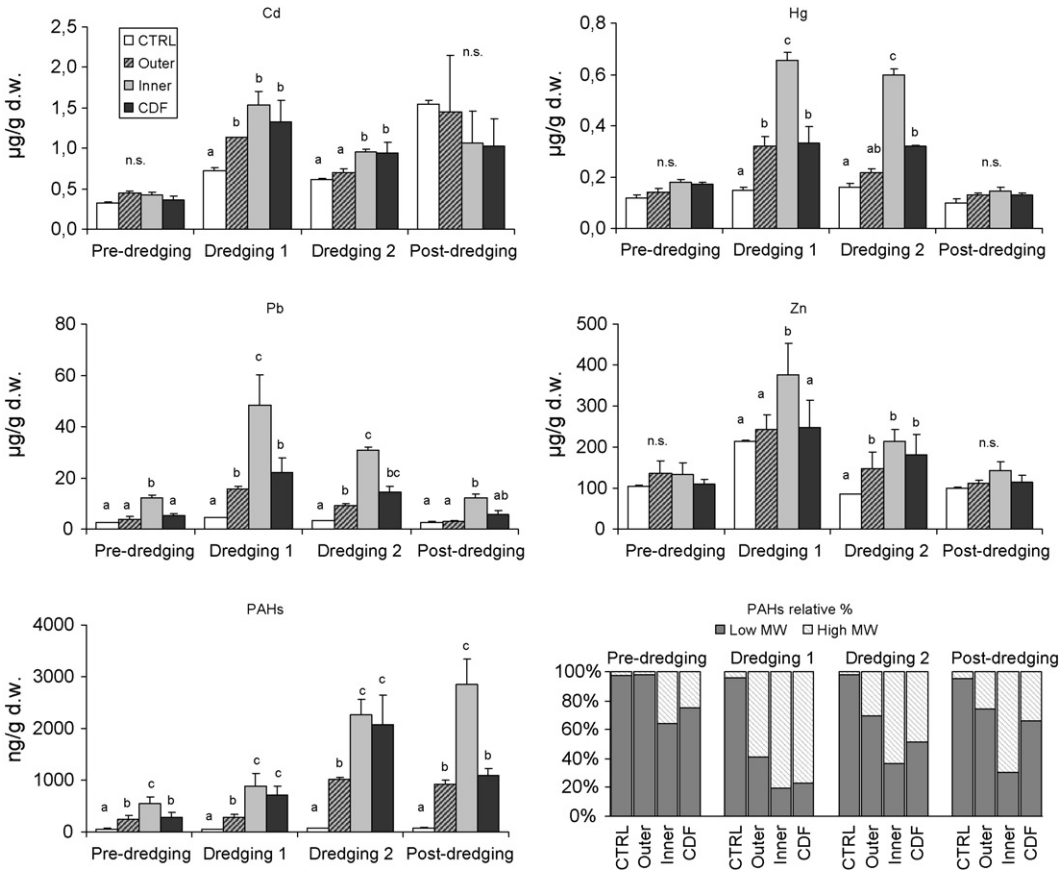

Fig. 2. Concentrations of trace metals (Cd, Hg, Pb, and Zn), polycyclic aromatic hydrocarbons (PAHs) and percentage contribution of low molecular weight and high molecular weight hydrocarbons in reference and harbour-caged mussels. Chemical concentrations are given as mean values ± standard deviations ($n = 5$). Different letters indicate significant differences between groups of means (post hoc comparison using Newman-Keuls test); n.s., not significant.

During the dredging periods, bioavailability of trace metals was clearly enhanced in all the harbour areas, with a particularly marked increase in the inner station, the more closely influenced by sediments removal. Two months after the end of operations, trace metals accumulation in harbour areas returned similar to the pre-dredging period, with higher levels of lead only in organisms caged in the inner station. Although concentrations of cadmium were higher than in other periods, such values reflected a more elevated basal content in control organisms rather than a different bioavailability in harbour sites.

Dredging operations significantly influenced also the bioavailability of PAHs which were greatly accumulated by mussels caged in the inner harbour area, in front of the CDF and, to a lower extent, in the more external site (Fig. 2). After the end of activities, bioavailability of these chemicals remained elevated in all the sites and especially in the dredged area. Comparing various phases of activities, concentrations of PAHs in caged mussels exhibited significant variations in the ratio between low molecular weight and high molecular weight compounds (Fig. 2). Before the beginning of operations LMW PAHs were always predominant in reference and harbour-caged mussels, while the contribution of HMW hydrocarbons increased during dredging, representing up to 80% of total PAHs in mussels caged in the inner area and in front of CDF. After the end of operations, the levels of LMW compounds returned to be the higher close to the CDF and in the outer location, while HMW hydrocarbons continued to account for almost 70% of the total PAHs in organisms transplanted in the inner station.

Variations of biomarkers responses according to site and period of translocation are summarized in Figs. 3–5. Levels of metallothioneins did not change in mussels caged in harbour areas compared to reference organisms and the generally low values observed in all the specimens after the last translocation period, did not appear to be related to dredging or disposal activities (Fig. 3). The analyses of glutathione revealed a significant increase of –SH groups in harbour-caged mussels during the dredging, while no differences were seen in reference organisms before and after the end of operations (Fig. 3). The activity of acyl CoA oxidase, as a marker of peroxisome proliferation, was similar in harbour-caged and reference mussels before the beginning of dredging (Fig. 3); a slight, significant increase of this enzyme was observed during the operations in the inner area and close to the CDF, remaining higher than in other sites in organisms caged in the dredged area after the end of activities.

Among the oxidative stress biomarkers, catalase was similar between harbour-caged and reference mussels during the pre-dredging period, being significantly induced after the first phase of operations (Fig. 4). A biphasic response was observed in organisms from the inner site which showed lowered values of this enzymatic activity during the second period of dredging operations. After the end of activities, catalase was lower in all the harbour than in reference mussels. Biphasic responses were also obtained for glutathione *S*-transferases, similar in reference and harbour-caged mussels before the dredging, induced during the first phase of activities in the inner area and close to the CDF and lowered during the second part and after the end of operations (Fig. 4). Glutathione reductase exhibited a more general decrease in all harbour-caged mussels during the dredging (Fig. 4), an effect still evident after the end of operations only close to the CDF. The activities of glu-

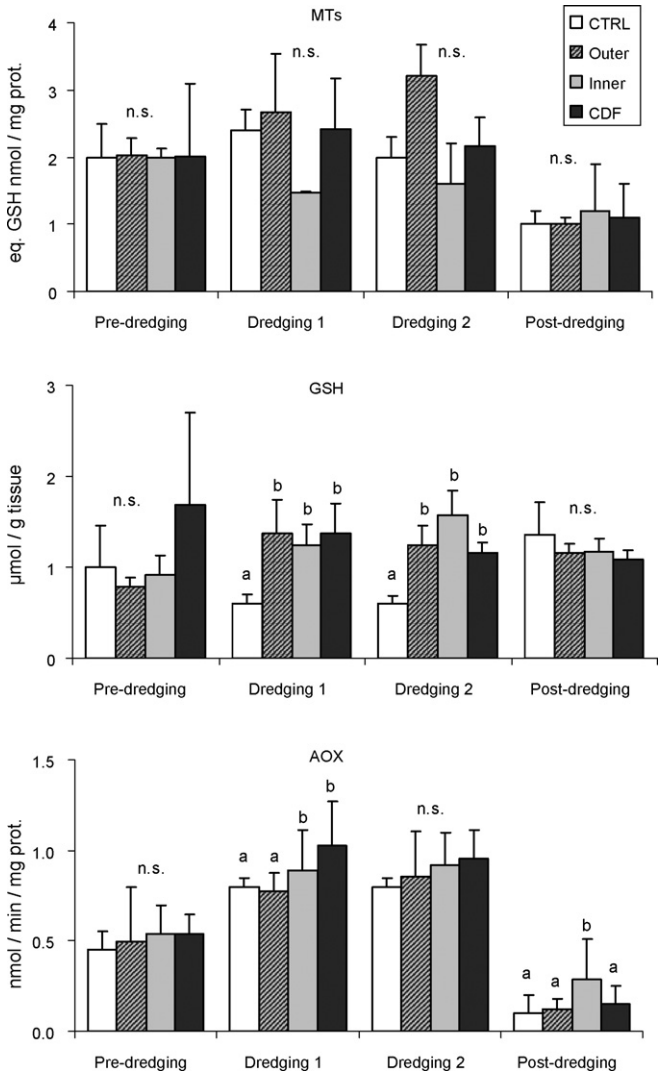

Fig. 3. Levels of metallothioneins (MTs), content of glutathione (GSH) and activity of acyl CoA oxidase (AOX) in reference and harbour-caged mussels. Mean values ± standard deviations ($n = 10$). Different letters indicate significant differences between groups of means (post hoc comparison using Newman-Keuls test); n.s., not significant.

tathione peroxidases were affected in the inner station resulting both lowered and enhanced activities in different periods (Fig. 4).

The analysis of total oxyradical scavenging capacity revealed a significantly lower capability to neutralize peroxyl radicals and hydroxyl radicals in mussels caged in various harbour areas compared to reference organisms (Fig. 4). However, such differences were observed also before the beginning of dredging and did not appear to be enhanced by these activities.

Lysosomal membrane stability confirmed the presence of significant differences between reference and harbour-caged mussels before the operations (Fig. 5); values of neutral red retention time were further lowered during dredging, remaining severely depressed after the end of activities in mussels caged in the inner area.

The genotoxic effects, measured as DNA strand breaks, were always more conspicuous in harbour-caged than in reference organisms (Fig. 5). In the pre-dredging period the highest damage was observed in mussels from the inner area, while during the operations similar values were measured for different harbour areas; a marked loss of DNA integrity was measured in organisms caged close to the CDF after the end of dredging.

Although the frequency of micronuclei was not measured before the dredging, during these activities harbour-caged mussels exhibited higher values than reference organisms (Fig. 5); in the post-dredging experiment the frequency of micronuclei remained significantly higher in the inner area and close to the CDF.

The principal component analysis of the overall results produced a two dimensional pattern explaining 62% of total variance. The factor loading showed that within the first axis concentrations of Pb, Hg, Zn and PAHs were positively correlated with levels of glutathione and genotoxic effects, negatively with the activity of glutathione reductase, total oxyradical scavenging capacity toward ROO• and •OH, lysosomal membrane stability; in axis 2 positive associations were obtained for catalase and glutathione *S*-transferase activities. The ordination plot discriminated the reference from all the harbour-caged mussels independent from the presence or phase of dredging activities (Fig. 6). Among the harbour samples, those obtained during the dredging were clearly separated from the others; post-dredging samples from the inner and CDF sites remained different from those of the pre-dredging period.

4. Discussion

The use of bioindicator organisms has been recently reported for various European harbours, such as Göteborg, Rotterdam, Leghorn, Genoa and Klaipeida (Baltic Sea), where the analyses of both bioaccumulation and biomarker responses in resident or translocated organisms allowed to evaluate the environmental impact and recovery associated with dredging and disposal of contaminated sediments (Stephensen et al., 2000; Regoli et al., 2002b, 2004; Stronkhorst et al., 2003; Frenzilli et al., 2004; Almroth et al., 2005; Sturve et al., 2005; Barsiene et al., 2006). In the present investigation, caged mussels, *M. galloprovincialis*, were chosen as widely validated sentinel organisms for monitoring anthropogenic activities in the Mediterranean (Regoli, 1992, 2000; Roméo et al., 2003; Regoli et al., 2004; Nigro et al., 2006; Damiens et al., 2007; Viarengo et al., 2007; Gorbi et al., 2008). Translocation experiments were performed in the Piombino harbour before, during and after the end of dredging and disposal operations, respectively, to reveal temporal variations in the environmental bioavailability and biological impact of pollutants during remobilization of sediments. The possibility to assess similar risks appears of particular concern for the Mediterranean, since approximately 5–10 million m^3 of harbour sediments are annually dredged and their disposal in artificial CDFs is increasingly being the preferred management option (ICRAM-APAT, 2007).

Before the beginning of dredging, a moderate bioaccumulation of polycyclic aromatic hydrocarbons was measured in organisms caged in the external location and in front of the CDF, indicating a general and diffuse influence of anthropogenic activities and maritime traffic on the whole harbour area. On the other hand, the marked increase of Pb and PAHs levels in mussels caged in the inner part of the harbour confirmed a more specific, localized impact of metallurgy, steel complex and carbon coke production, responsible for elevated concentrations of chemicals in sediments to be dredged from the inner area; the preliminary characterization of these sediments indicated values up to 3700 μg/g (dry weight) for Pb, 12000 μg/g for Zn, 10 μg/g for Hg, 25 μg/g for Cd, and 3000 μg/g for PAHs (ICRAM, 2006), often exceeding limits indicated by the Italian normative for remediation of contaminated sites (DM 471/99).

Concentrations of contaminants in sediments to be dredged do not fully reflect the potential threats arising during these activities when changes in oxic/anoxic conditions can greatly influence

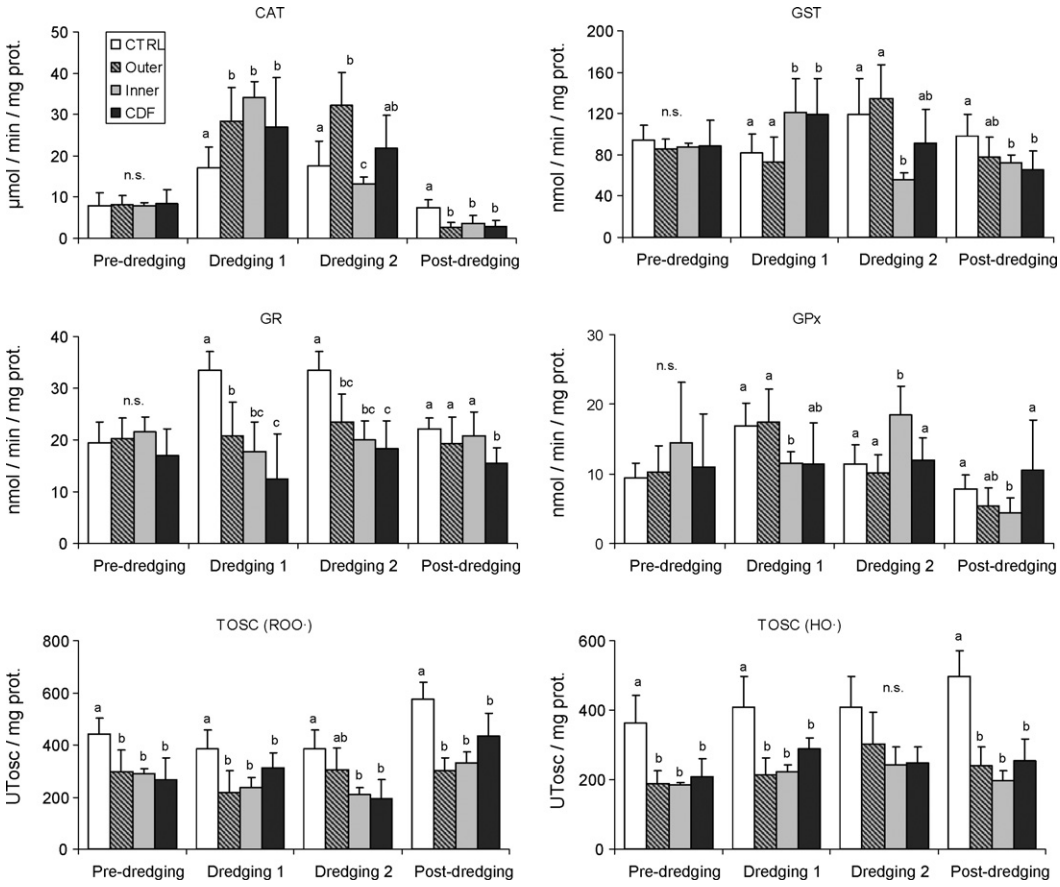

Fig. 4. Activities of catalase (CAT), glutathione *S*-transferases (GST), glutathione reductase (GR), sum of Se-dependent and Se-independent glutathione peroxidises (GPx) and total oxyradical scavenging capacity toward peroxyl radicals (TOSC-ROO•) and hydroxyl radicals (TOSC-HO•) in reference and harbour-caged mussels. Mean values ± standard deviations ($n = 10$). Different letters indicate significant differences between groups of means (post hoc comparison using Newman-Keuls test); n.s., not significant.

the resuspension of pollutants, their bioavailability and toxicological risks. Mobility of trace metals from dredged sediments has been shown in the Barcelona harbour, providing site-specific evidence for a significant remobilization especially for Cd, Zn, Pb and Cu (Guevara-Riba et al., 2004). Similarly, the distribution of PAHs between aqueous phase and sediments in the Brighton Marina (UK) indicated the remobilization of these chemicals from dredged sediments (King et al., 2004). General mechanism potentially involved in the release of chemicals from dredged sediments include the mixing of compounds entertained in pore waters, desorption due to increase of water content in the slurry and/or changes in redox conditions (Di Toro et al., 1991; Ankley et al., 1996).

The biological consequences of these physico-chemical processes were clearly demonstrated in our study by the significant increase of chemical bioavailability observed with the beginning of dredging and disposal of sediments. The more evident variations in tissue levels of trace metals and PAHs were measured in mussels caged in the inner site and, for PAHs, also in front of the CDF, the areas more influenced respectively by dredging and disposal operations. However, the significant accumulation of chemicals observed also in organisms translocated in the more external location, indicated a quite diffuse impact and transport of remobilized pollutants. Concentrations in caged organisms were particularly elevated for Pb (up to 40 μg/g) and PAHs (up to 2200 ng/g), revealing a severe impact on the bioavailability of these chemicals; levels of Zn, Cd and Hg increased to values comparable to those reported for other Mediterranean harbour areas (Zorita et al., 2007).

Bioavailability of trace metals and PAHs showed different time-course variations after the end of operations. While tissue levels of metals rapidly returned to the pre-dredging values, the accumulation of PAHs in caged mussels remained significantly elevated in all the harbour areas and, particularly, in the inner one. These results suggest that lipophilicity and sedimentation rate of chemicals can influence the duration of impact after their re-mobilization from contaminated sediments (Zhou et al., 1998). Interestingly, tissue levels of PAHs showed a change in the relative contribution of LMW and HMW compounds during different phases of activities. Before the dredging, the predominance of petrogenic LMW PAHs indicated a petroleum-derived source, quite typical for a harbour area where these hydrocarbons are present as dissolved, colloidal forms or loosely bound to suspended matter (Zhou et al., 1998). The re-mobilization of sediments markedly increased the bioavailability of pyrogenic HMW hydrocarbons, mostly originating from industrial activity and generally strongly bound to sediments (Zhou et al., 1998). Considering the higher toxicological relevance of HMW compared to LMW compounds, this effect, still evident in the inner area also after the end of operations, is worthy to be evaluated when assessing the risk of dredging and disposal operations.

The ecotoxicological approach, integrating chemical analyses with the use of a wide battery of biomarkers, provided further evidence on the biological and potentially harmful effects of re-mobilized contaminants from dredged harbour sediments. In the present investigation, levels of metallothionein-like proteins (MTs) and peroxisome proliferation were chosen as specific biomarkers toward trace metals and polycyclic aromatic hydrocarbons, variations of antioxidant defences were selected as general stress responses to pollutants, while lysosomal impairment and genotoxic

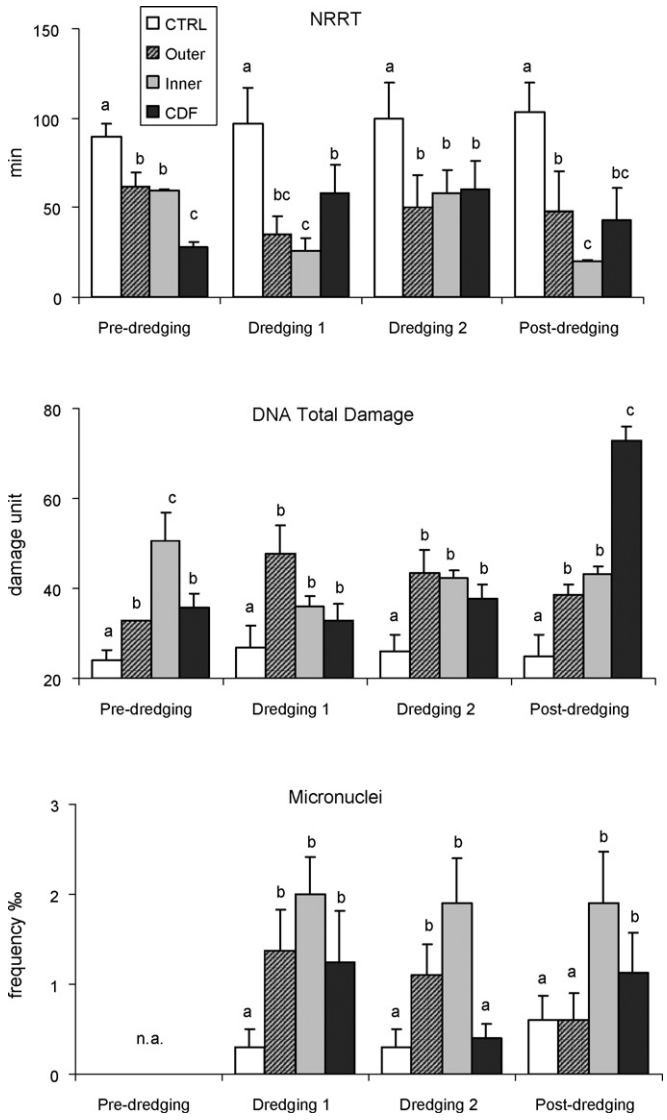

Fig. 5. Neutral red retention time (NRRT), DNA total damage and frequency of micronuclei in reference and harbour-caged mussels. Mean values ± standard deviations ($n = 10$). n.a.: not analysed. Different letters indicate significant differences between groups of means (post hoc comparison using Newman-Keuls test); n.s., not significant.

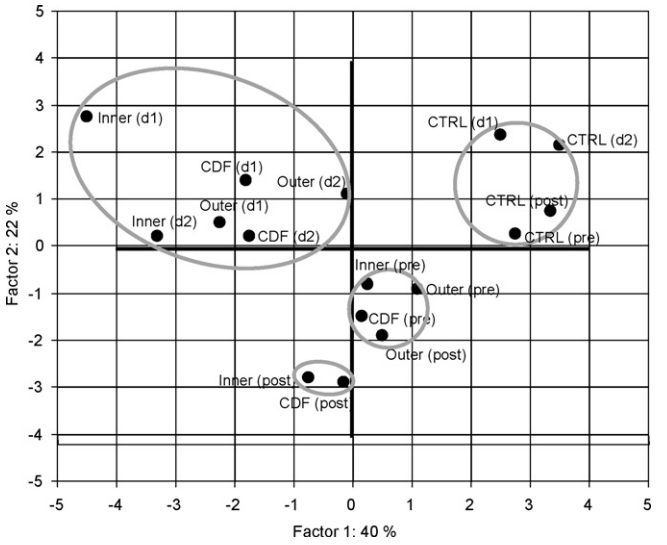

Fig. 6. Separation of sites and sampling periods obtained from PCA analyses on chemical parameters and biomarker data. Sites: CTRL, reference; Outer, the external side of the harbour; Inner, the area influenced by dredging activities, CDF, the site located in front of the confined disposal facility, where dredged sediments were disposed. Periods: pre, before the beginning of dredging; d1 and d2, during dredging activities; post, after the end of dredging activities.

alterations were expected to reveal the onset of cell damages in harbour-caged mussels.

Metallothioneins did not reveal any variation during different phases of dredging, despite the significant accumulation of trace metals in tissues of caged mussels. Several studies already reported conflicting results on MTs in field conditions where the natural variability and the influence of both environmental and biological factors have been shown to influence this response (Cajaraville et al., 2000; Petrovicĭ et al., 2001; Raspor et al., 2004; Bocchetti and Regoli, 2006; Zorita et al., 2007). The lack of MTs response observed in mussels caged in the Piombino harbour could be explained considering that the highest bioaccumulation was observed for Pb, which is not an inducer of MTs synthesis. Tissue concentrations of Cd, Zn and Hg in transplanted organisms exhibited more limited variations compared to those reported for MTs induction in laboratory exposures (Viarengo et al., 1997); values measured in this study were almost comparable to those observed along a pollution gradient in NW Mediterranean Sea, where mussels did not exhibit any difference in MTs levels (Zorita et al., 2007).

On the other hand, our results revealed that cellular availability of thiol groups in harbour-caged mussels was enhanced by the significant increase of glutathione during the dredging periods; values returned to control levels after the end of operations when also bioavailability of trace metals was similar to the pre-dredging phase. The temporary increase of GSH levels would thus provide promptly available –SH groups for both antioxidants and metal chelating functions, representing the first line of defence toward a wide spectrum of trace elements: a similar mechanism has already been described in marine organisms as an adaptive strategy to compensate for the limited or slower responsiveness of MTs after acute exposure to trace metals (Regoli et al., 2005).

Peroxisome proliferation has been reported in vertebrates and invertebrates species exposed to several organic xenobiotics such as water-accommodated fraction of crude and lubrificant oils, PAHs, polychlorinated biphenyls (PCBs), phthalate ester plasticizers and alkylphenols (Cajaraville et al., 2003). Some field studies have confirmed the occurrence of this phenomenon in wild populations of mussels from contaminated areas or in transplanted organisms (Cajaraville et al., 2003; Orbea and Cajaraville, 2006), but limited or contradictory results have also been reported (Zorita et al., 2007; Gorbi et al., 2008). In our investigation, the activity of AOX revealed some significant variations at the beginning of dredging and after the end of operations in the inner area, where bioavailability of PAHs remained elevated. Although these results would confirm a certain responsiveness of peroxisome proliferation to PAHs, the effect was of limited magnitude, thus indicating the need to further characterize mechanisms of peroxisomal responses to specific hydrocarbons or complex mixtures of chemicals. Trace metals have been widely documented to influence metabolism of organic xenobiotics through a complex pathway of interactions (Regoli et al., 2005), and their inhibitory effects on the activity of peroxisomal AOX have been suggested to be responsible for the limited variation of this enzyme (Zorita et al., 2007).

Prooxidant mechanisms are of primary importance in modulating toxicological effects of contaminants in marine organisms, and the measurement of oxidative stress biomarkers is thus useful from the early detection of a general disturbance contributing to later

impairment of health condition. In this study, the oxidative status of harbour-caged mussels was assessed by integrating the activity of some individual antioxidants with the total capability to neutralize specific oxyradicals. Catalase and glutathione *S*-transferases showed time-dependent and biphasic responses during dredging. After a significant increase observed in all harbour-caged mussels with the beginning of dredging, the activities of these enzymes were clearly lowered during the second part of operations in mussels from the inner area, which are more impacted by remobilization of contaminated sediments. Biphasic responses of catalase and glutathione *S*-transferases can be modulated by the intensity and duration of chemical disturbance (Stephensen et al., 2000; Regoli et al., 2002a, 2004, 2005): mussels caged in the harbour of Genoa exhibited no variation or induction of these antioxidants during the first 2 weeks of exposure, followed by a progressive decrease up to a significant inhibition after 4 weeks (Regoli et al., 2004). These findings confirm that counteractive responses to prooxidant challenge of environmental pollutants can be overwhelmed at longer periods or higher intensity of chemical exposure when oxidative perturbation exceeds the efficiency of specific antioxidant defences.

An oxidative stress disturbance during the dredging has been confirmed by the reduced activity of glutathione reductase in all the harbour-caged mussels and of glutathione peroxidases in those from the inner site. Glutathione reductase has often been described as very sensitive to environmental pollutants and useful as early warning signals in both fish and invertebrate species (Regoli and Principato, 1995; Stephensen et al., 2000; Regoli et al., 2004, 2005), while glutathione peroxidases appeared to respond to a higher intensity of oxidative disturbance.

When data on individual antioxidants were integrated with analysis of total oxyradical scavenging capacity, a reduced capability to neutralize peroxyl and hydroxyl radicals was always evident in mussels caged in various harbour areas before, during and after the end of dredging activities. The overall results on TOSC and oxidative stress responses indicate that a significant environmental disturbance, independent from sediments removal was already present in the harbour area and correlated to the pre-existing contaminated conditions; however, variations of individual antioxidants revealed an additional prooxidant challenge during various phases of activities. The reduced capability to neutralize oxyradicals has a fundamental role in oxidative toxicity of chemical pollutants, as widely demonstrated in field conditions by the increased rate of cellular alterations like lysosomal membrane destabilization and loss of DNA integrity (Regoli, 2000; Frenzilli et al., 2001; Gorbi and Regoli, 2003; Moore et al., 2004; Regoli et al., 2004, 2005).

These forms of cell damage were detected in mussels caged also in the pre-dredging period, confirming the environmental disturbance in the Piombino harbour area also before the beginning of works. Lysosomes have received considerable attention in ecotoxicological studies, being the target for a wide range of toxic chemicals which may affect these organelles directly and indirectly through the enhanced formation of oxyradicals (Moore, 1988; Cajaraville et al., 2000; Regoli, 2000; Regoli et al., 2004). The low levels of lysosomal membrane stability observed before the dredging, were further decreased with the beginning and, in the inner area, also after the end of operations indicating an additional impact caused by these activities. These results confirm the elevated sensitivity of lysosomal biomarkers as suitable tools for the early detection of adverse effects in marine organisms (Moore et al., 2004; Regoli et al., 2004; Viarengo et al., 2007).

Presence and time-course variations of biological disturbance in the harbour of Piombino were shown also by the high levels of DNA damage, measured in terms of strands breaks and frequency of micronuclei, which represent validated genotoxic biomarkers for Mediterranean mussels (Frenzilli et al., 2001; Bolognesi et al., 2004; Regoli et al., 2004; Nigro et al., 2006). Before the beginning of works, levels of DNA strand breaks were more elevated in the inner area, raised in all the sites during the dredging, remaining high also after the end of activities, particularly near the CDF. The elevated frequency of micronuclei in mussels caged in the inner site and close to the CDF confirmed the persistence of genotoxins released from dredged and disposed sediments. A genotoxic impact associated with extensive dredging and dumping activities was revealed by a multi-biomarker approach with *Mytilus edulis* also in the port of Klaipeida (Baltic Sea), where elevated levels of mutagenicity prevailed for almost 2 years after the end of operations (Barsiene et al., 2006). An oxidative pathway for such chemically induced effects is suggested by the more evident inhibition of antioxidant efficiency in organisms with highest levels of lysosomal destabilization and DNA alterations.

In conclusion, this study indicated the use of caged mussels as an important tool for improving the risk assessment and management of contaminated sediments during dredging and disposal operations in harbour areas. The integration of chemical analyses with a wide battery of biomarkers allow to detect whether the resuspension of sediments influence the mobility of pollutants, their bioavailability and potentially harmful effects. The overall results obtained in the harbour of Piombino indicate a general disturbance in the area already present before the beginning of operations; bioavailability of trace metals and PAHs markedly increased during dredging causing significant inhibition of antioxidant efficiency and the increase of oxidative damages in caged mussels. Although the impact was more evident close to dredging and disposal sites, the influence of operations was detected in the whole harbour area. Bioavailability of PAHs and biological effects of remobilized contaminants remained elevated also after the end of activities in the inner area and in front of CDF. The risk of dredging operations in harbour areas should thus be carefully monitored during and after the end of works, particularly when contaminated sediments are disposed in coastal CDFs.

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