Total thiol redox status as a potent biomarker of PAH-mediated effects on mussels

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A B S T R A C T

This laboratory study describes phenanthrene (Ph) and/or anthracene (An) ability to alter the total thiol redox status (TRS), via depletion of protein free thiols (PSH) and glutathione (GSH) levels, in gills of mussel Mytilus galloprovincialis, after a short-term (7 days) exposure period to each contaminant (at a final concentration of 0.1 mg L−1) or in a mixture (ration 1:1, at a final concentration of 0.2 mg L−1). A number of observable changes, like lysosomal membrane impairment (as detected via the neutral red retention time assay, primarily performed in haemocytes), enhancement of lipid peroxidation byproducts, increased nuclear abnormalities, inhibition of AChE and ALP activity, as well as a significant depletion of PSH and GSH were detected in gills of exposed mussels, in any case. Significant relationships occurred among TRS parameters with each change/stress indices measured in tissues of mussels, could reinforce the use of PSH as a potent biomarker.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), such as phenanthrene (Ph) and anthracene (An), are persistent chemicals of seawater, and their concentrations could range from undetectable to up to milligrams per litre in industrial effluents (Latimer and Zheng, 2003) and in heavily-polluted areas (Anyakora et al., 2005; Kilikidis et al., 1994; Zhou and Maskoui, 2003). These PAHs are able to readily accumulate in marine biota, in concentrations that are depended merely upon species ability to biotransform them (Guinan et al., 2001). Since there is a wide variation in the types and magnitudes of physiological responses, which determine PAHs bioavailability and toxic potency, a lot of studies have been focused on their effects on marine species, including mussels (Galgani et al., 2011; Giannapas et al., 2012; Hannam et al., 2009, 2010a, 2010b; Kaloyianni et al., 2009; Raftopoulou et al., 2006; Almroth et al., 2008).

Mussels, such as Mytilus sp., are systematically used as biological models for assessing the effects of pollutants on the marine biota, due to their well-known physiology and ability to respond rapidly to environmental stress (Livingstone, 1991). Moreover, in these organisms, PAH metabolic processes, such as biodegradation, bioaccumulation and biotransformation, are significantly less than PAH uptake, resulting in their bioaccumulation and toxic effects (Livingstone et al., 2000). In particular, PAHs undergoing biotransformation reactions, could lead to the production of reactive oxygen species (ROS), such as diol epoxides, free radical cations and reactive reducto o-quinones (Cavalieri and Rogan, 1995; Penning et al., 1996), which are able to attack cell membranes, thus leading to alterations in membrane fluidity and function of ionic pumps, oxidation of phospholipids, proteins and DNA, as well as alterations in antioxidant capability (Baussant et al., 2009; Hannam et al., 2009, 2010a, 2010b; Kaloyianis et al., 2009).

The early biological responses and prepathological alterations occurred before other disturbance as disease, mortality, or population changes occur, are measurable endpoints, commonly called stress-indices or biomarkers, and a wide battery of them is commonly used both in field and laboratory studies (for a review see Dailianis, 2011). In particular, the neutral red retention time (NRRT) assay is an early diagnostic low-cost biomarker, primarily performed in haemocytes of mussels for assessing lysosomal membrane stability, functional integrity impairment of cells (Livingstone et al., 2000) and the concomitant disturbance of the individual health status (Baussant et al., 2009; Domouhtsidou and Dimitriadi, 2001; Koukouzika and Dimitriadis, 2008). In addition, the micronucleus (MN) test represents a fast and sensitive indicator of genomic damage, due to both clastogenic effects and alterations of the mitotic spindle, and is widely performed in gills of mussels (for a review see Fang et al., 2009; Viarengo et al., 2007). Furthermore, the estimation of acetylcholinesterase (AChE) and to a less extend alkaline phosphatase (ALP) activity have been used as neurodegenerative and immunological biomarkers for both organic and inorganic...
substances (Dailianis et al., 2003; Day and Scott, 1990; Kalpaxis et al., 2004; Liu et al., 2010; Regoli and Principato, 1995).

Oxidative stress-related markers, such as glutathione (GSH) and lipid peroxidation (LPO) byproducts, are commonly used for the evaluation of PAH-mediated effects (Bausant et al., 2009; Halliwell and Gutteridge, 2007). Although GSH and GSSG (GSH oxidized form) levels are considered as a predispersing factor in adverse effects of oxidative damage, their estimation does not actually reflect the total thiol redox status (TRS). TRS is considered as an essential metabolic effector of oxidative stress (Dalle-Donne et al., 2006) and although other components, besides GSH, such as cysteine (CYS) and protein thiols (PSH), are also main targets of free radicals (Davies, 2005; McDonagh and Sheehan, 2007; Patsoukis and Georgiou, 2004, 2005), their investigation in tissues of mussels remains still unclear. Similarly, the LPO byproduct, malondialdehyde (MDA), is commonly measured in tissues of mussels, but early LPO intermediates, like lipid hydroperoxides (LOOH), have received less attention.

Since the entrance of waterborne pollutants across the gills of mussels commonly reflects the condition of the aquatic environment, as well their toxic potency (Vidal-Linan et al., 2010), the present study investigates for the first time TRS, in terms of PSH levels besides those of GSH and GSSG, in gills of mussel Mytilus galloprovincialis exposed to low molecular weight PAHs, such as Ph and An. In parallel, considering that PAH-mediated biological responses are not able to be assessed by an individual biomarker (Regoli et al., 2004), a wide battery of biomarkers was further used, thus allowing the investigation of PAH-mediated biological responses as well as whether there is any relationship among them with the TRS parameters tested. In specific, the NRRT assay was primarily performed in haemocytes of mussels for assessing their general health status, while LPO products (total and protein-bound MDA and LOOH), nuclear abnormalities (MN test), as well as AChE and ALP activity were measured in gills of mussels. Finally, linear correlation (Pearson test) analysis was performed among parameters tested, in order to investigate any relationship that could reinforce the usefulness of TRS, in terms of PSH, besides those of GSH, as a potent biomarker in mussels faced with organic contaminants, such as PAHs.

2. Materials and methods

2.1. Reagents

Phenanthrene, anthracene, bovine serum albumin (BSA), butyhydroxyl anisole (BHA), cumene hydroperoxide, GSH, GSSG, malonaldehyde bis(dimethyl acetal) (MDA), N-ethylmaleimide (NEM), o-phenthaldialdehyde (OPT), thiobarbituric acid (TBA), aldri-thiol (DPS) and xylenol orange were purchased from Sigma, St. Louis, MO, USA. All other reagents and solvents used were of the highest analytical grade and purity.

2.2. Mussel collection and handling

Mussels (5–6 cm length) were collected from a fish-mussel farm, located in the north side of Korinthiakos Gulf (Greece). Although Botsou and Hatzianestis (2012) reported that the current site receives the impact of human activities, the fish-mussel farm is located to a close gulf (Gulf of Kontinova) which is far away from any human activities and is characterized by negligible levels of inorganic and organic pollution (Banakou and Dailianis, 2010; Chatziarigou and Dailianis, 2010; Giannapas et al., 2012; Kalpaxis et al., 2004).

The collected mussels were transferred to the laboratory and maintained for 7 days in static tanks, containing re-circulated UV-sterilized and filtered artificial seawater (ASW; pH 7.9 ± 0.4, salinity 36 ± 1‰, temperature 15 ± 1 °C, dissolved oxygen 7 ± 0.5 mg L⁻¹), with a 14:10 h light: dark photoperiod, in order to be acclimated in laboratory conditions. During the acclimation period, mussels were maintained without food supply and water was changed daily (no mortality was observed throughout this period). After the end of the acclimation period and during the experimental period, mussels were fed daily with approximately 30 mg of dry microcapsules/mussel (Myspat, Inve Aquaculture NV, Belgium).

2.3. Experimental design and evaluation of NRRT and MN assays in tissues of mussels

After the acclimation period, 150 mussels were placed in 5 static tanks, containing re-circulated UV-sterilized and filtered ASW (30 mussels per tank, 1L/mussel). Three groups of mussels were exposed to nominal concentrations of An and/or Ph (at a final concentration of 0.1 mg L⁻¹ in each case) and a mixture of them (ration 1:1 at a final concentration of 0.2 mg L⁻¹) for 7 days, under conditions mentioned in the paragraph 2.2. PAH concentration used in the present study were within the range of their persistence in heavily-polluted coastal areas and harbours (Anyakora et al., 2005; Grundy et al., 1996; Kilkidis et al., 1994; Zhou and Maskoui, 2003), while similar concentrations were reported to enhance toxic effects on tissues of mussels, under laboratory conditions (Bellas et al., 2008; Giannapas et al., 2012; Hannam et al., 2010a, 2010b; Kaloyianni et al., 2009; Koukouzika and Dimitriadis, 2008; Raftopoulou et al., 2006). Stock PAH solutions were made by dissolving Ph and/or An in acetone, before diluting in ASW. In addition, acetone at the non-toxic concentration 0.05% v/v (Giannapas et al., 2012), was added to a fourth group (acetone-treated) and together with a fifth group (control) were maintained as reference group of mussels (PAH-free mussels). Although Ph and/or An concentrations into the seawater were not checked, it is well-known that these PAs are volatile, and a decrease of their concentration should be expected with time, at the respective temperature (15 °C). In order to avoid a significant reduction of each PAH level with time, the seawater renewed and spiked with new quantities of PAHs and food every 24 h. In addition, PAH absorption onto the surface of both organisms and exposure tank could contribute towards their reduction levels into the seawater, thus acting as a secondary supply of PAHs to the water column during the exposure period (for further details see Hannam et al., 2010a).

After the exposure period (no mortality was found in control and PAH-exposed mussels), haemolymph, withdrawn from the posterior adductor muscle, using a sterile 1 ml syringe with an 18G1/2” needle, containing equal volume of Alseve buffer (ALS buffer; 20.8 g L⁻¹ glucose, 8 g L⁻¹ sodium citrate, 3.36 g L⁻¹ EDTA and 22.5 g L⁻¹ NaCl, pH 7 and 1000 mOsm) and gills from 10 individuals per group of mussels were used for NRRT and MN assays in each case. For the other parameters tested (LPO products, TRS, AChE and ALP assays), gills dissected from 15 mussels per experimental group, were further divided in 3 subgroups (tissue of 5 mussels per group, which means 3 biological replicates per experimental group), cut into small pieces and portions of each subgroup were used for further analysis.

2.3.1. NRRT assay in haemocytes of mussels

A small portion of haemolymph suspension (40 μl) from each individual was spread on slides, transferred to a light-proof humidity chamber and allowed to attach. Afterwards, all slides were prepared for NRRT analysis (N = 10 in each case) with the use of the cationic probe neutral red (NR), according to the procedure
and criteria proposed by UNEP/RAMOGE (1999). In brief, the retention of NR within lysosomal compartment of cells over time, as indicated by analysis of each slide under a light microscope (Nikon, Eclipse E200) every 15 min, is determined as a measure of damage to the lysosomal membrane. At least 200 granular haemocytes were examined in each slide and the mean NRR time value corresponds to values obtained in each slide (N = 10) per experimental group. The aforementioned analysis was also performed in haemocytes of control-mussels before (0 days) and after the end of the exposure period (7 days), for assessing their health status.

2.3.2. MN test in gills of mussels

Gills dissected from 10 individuals from each tank, were cut into small pieces and a small portion was kept in 0.25% v/v trypsin (at 4 °C for 15 min), in order to obtain interphase cells (Arslan et al., 2010). Afterwards, the trypsin solution was removed by centrifugation at 150 × g (in duplicate), the cell pellet was spread on slides, fixed with ethanol–acetic acid (3:1), stained with Giemsa (3% v/v), and then mounted in Canada balsam. For MN assay all slides were counted under a light microscopy (100 × magnification with the use of immersion oil) for the presence of nuclear abnormalities, such as cells bearing one micronucleus (MN) and/or two micronuclei (2MN), micronucleus with a narrow nucleoplasmic connection to the main nucleus (nuclear bud/NB) and binucleated cells (BN) as suggested by Fenech et al. (2003). In addition, the total nuclear abnormalities (TNA, in terms of the sum of all abnormalities tested) were also estimated. At least 1000 cells were counted in each slide and the mean MN frequency value corresponds to values obtained in each slide per experimental group.

2.4. AChE activity in gills

A small portion of gills was homogenized in four volumes of 0.1 M TRIS buffer, pH 8 and centrifuged at 10,000 × g for 30 min. Crude supernatants were used for AChE activity determination, with the use of acetylthiocholine iodide as substrate (UNEP/ RAMOGE, 1993). Results (expressed as units/mg protein) are mean ± standard deviation (SD) from 3 measurements (each measurement was verified in triplicate).

2.5. Lipid peroxidation, TRS and ALP assays

For the estimation of TRS (PSH, GSH, GSSG), LPO products (MDA and pr-MDA), at least 0.15 ml of homogenate clear supernatant was brought to ammonium sulphate 90% w/v, incubated overnight at 0 °C and after centrifugation at 15,000 × g for 15 min at 4 °C, the protein pellet was solubilized in water and used for ALP determination assay.

2.5.1. LOOH assay

LOOH were determined by a modification of the ferrous oxidation-xylene orange (FOX) assay (Wolff, 1994). In brief, homogenates were extracted twice with equal volume of chloroform:methanol (2:1), centrifuged at 15,000 × g and the collected bottom organic layer was vacuum-dried. Afterwards, the lipid pellet was dissolved in absolute ethanol and mixed with FOX assay reagent (2 mM xylene orange, 2 M sorbitol, 0.25 M H2SO4), with either the presence of Fe2+ (in terms of 8 mM ammonium ferrous sulphate) for sample or the absence of Fe2+ for sample blank. After incubation for 30 min at room temperature, samples were measured at 560 nm (Shimadzu UV-1201 UV–VIS spectrophotometer) and the absorbance value was converted to nanomole of cumene hydroperoxide equivalents/mg protein from a cumene hydroperoxide standard curve (0.25–4 μM).

2.5.2. MDA assay

Homogenate samples were assayed by a modified thiobarbituric acid (TBA)-based method (Girotti et al., 1991). For total MDA (free and pr-MDA), at least 0.15 ml of homogenate clear supernatant was mixed with equal volume of TBA (TBA 0.5% w/v in TCA 20% v/v − 0.33 N HCl) and BHA 0.02% w/v. After incubation at 100 °C for 15 min, samples were brought to room temperature and thereafter, 0.3 ml isobutanol was added, mixed by vigorous vortexing and centrifuged at 15,000 × g for 3 min. Fluorescence of the upper butanol layer was measured (excitation 535 nm, emission 550 nm, Shimadzu RF-1501) against butanol-treated sample and reagent blanks. Emission fluorescence was converted to nmol MDA/mg total protein from a malonaldehyde bis(dimethyl acetal) standard curve (0.2–2 μM).

For pr-MDA, TCA (5% w/v)-precipitated protein pellets were solubilized in 0.1 N NaOH and incubated at 70 °C for 30 min, in order to alkaline-hydrolyse protein and release bound MDA (Carbonneau et al., 1991). Thereafter, the same procedure as that of total MDA (using 0.1 N NaOH as reagent blank) was followed. Results are expressed in nmol MDA/mg of protein.

2.5.3. TRS assay

For TRS determination, the method by Patsoukis and Georgiou (2005) was performed with minor modifications. In brief, the protein-free homogenate supernatants (after TCA 5% w/v protein precipitation) were washed 3 times with equal volume of diethyl ether and the lower aqueous phase was collected. GSH was quantified fluorometrically (excitation 340 nm and emission 420 nm) via its reaction with o-phthalaldehyde (OPT 20 μg ml−1) at pH 8.0, while GSSG was quantified via its reaction with OPT at pH 12, preincubated with 300 μM N-ethylmaleimide (NEM) to alkylate any GSH presence in the sample. Emission fluorescence was converted to GSH or GSSG equivalents from a corresponding standard curve. The results were expressed as nmol GSH or GSSG equivalents/mg total protein.

For PSH determination, TCA (5% w/v)-precipitated protein pellets were solubilized in 12 M formamide–10 mM citric acid solution, pH 4.5 using a mortar. From the solubilize a portion was kept for protein determination, while the remaining portion was diluted appropriately and mixed with DPS (150 μM DPS in DMSO 0.5% w/v and 200 mM acetic acid). As a sample blank an equal portion of protein solubilize was mixed with DMSO and acetic acid. A reagent blank with citric acid instead of protein solubilize was used. After 10 min incubation at room temperature the absorbance was measured at 325 nm and the net value was converted to nanomole GSH equivalents/mg protein from a GSH standard curve (1–15 μM).

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2.5.4. ALP assay

The determination of ALP is based on the conversion of p-nitrophenyl phosphate (pNP) to p-nitrophenol (pN), catalysed by ALP at pH 9.8 (Principato et al., 1985). Specifically, enzyme solubilize diluted appropriately with 100 mM boric acid, pH 9.8, was mixed with 1 mM pNP, incubated for 20 min at room temperature and the reaction was finally stopped by the addition of 0.4 M NaOH. Then samples were measured at 405 nm, the net absorbance was corrected per minute and converted to units of ALP activity/mg of protein (from 18.2 × 10⁷ M⁻¹ cm⁻¹ 1 mol extinction coefficient and with 1 Unit being equal to the quantity of enzyme required for the formation of 1 μmol of pN min⁻¹ 1 ml⁻¹).

2.6. Protein concentration assay

Protein was determined by an ultrasensitive hydrophobic method (Georgiou et al., 2008), based on Coomassie Brilliant Blue-G assay, with the use of known concentrations of bovine serum albumin (BSA).

2.7. Statistical analysis

Values are means ± SD from 3 different measurements in each case, while NRR and MN values represent the mean values ± SD obtained after analysis of samples derived from 10 individuals per tank, in each case. After checking for equality of error variances (Levene’s test, SPSS Inc. 16), one-way ANOVA analysis of variance was applied in order to identify significant differences between values of parameters obtained in gills of control and PAH-exposed mussels. Post-hoc multiple comparison (Bonferroni test, p < 0.05) was conducted for NRR, MN frequency, MDA, AChE and ALP activity, while differences between values of the other parameters tested were performed non-parametrically with the use of Mann–Whitney U test (p < 0.05). Simple linear correlation (Pearson test, p < 0.05) analysis was conducted with the mean values of each parameter tested, in order to investigate significant relationships between the biological responses.

3. Results

3.1. NRRT assay in haemocytes of mussels

Prior analysis showed no significant variations between NRR time values obtained in haemocytes of control group of mussels, before (0 days) and after the end (7 days) of the exposure period (101.2 ± 20.83 min and 90 ± 19.63 min respectively), thus revealing no time-dependent and inter-tank variations. Similarly, there were no significant variations among each of the parameter tested (NRR, MN, TRS, AChE, ALP and LPO products) in tissues of control and acetone-treated group of mussels (PAH-free mussels), thus excluding the interference of acetone with PAH-mediated effects described below. On the other hand, significantly lower NRR time values were observed in PAH-exposed mussels, compared with those occurred in PAH-free mussels, while NRR time values obtained in haemocytes of mussels exposed to 0.1 mg L⁻¹ of either Ph or An were almost similar (p > 0.05) to those observed in haemocytes of mussels exposed to 0.2 mg L⁻¹ of their mixture (Fig. 1).

3.2. MN test in gills of mussels

Regarding MN test, PAH-exposed mussels showed significantly higher MN and BIN frequencies than those occurred in PAH-free mussels in any case (Table 1). According to TNA frequency, there was a significant increase in gills of PAH-exposed mussels (Fig. 2), while TNA values obtained in gills of mussels exposed to 0.1 mg L⁻¹ of either Ph or An were not significantly different from those obtained in mussels exposed to 0.2 mg L⁻¹ of their mixture (Bonferroni test, p > 0.05).

Fig. 1. NRRT values in haemocytes of PAH-exposed (Ph and An alone or in mixture) and PAH-free mussels (control and acetone-treated). Values for NRRT (expressed in min) are the mean value ± SD obtained by the analysis of each slide (N = 10) for each group of mussels in each case. *Indicates significant difference from control, †Indicates significant difference from acetone-treated mussels (Bonferroni test, p < 0.05). No significant alterations occurred among NRRT values obtained in haemocytes of control mussels before (0 days) and after the end (7 days) of the exposure period.

3.3. ALP and AChE activity in gills of mussels

A significant inhibition of ALP and/or AChE activity was observed in gills of PAH-exposed mussels, compared with those occurred in PAH-free mussels (Fig. 3A, B). ALP levels measured in gills of mussels exposed to 0.1 mg L⁻¹ of either Ph or An were almost similar to those obtained in tissues of mussels exposed to PAH mixture (Fig. 3A), while there were significant variations of AChE levels measured in gills of mussels exposed to each PAH alone and their mixture (Fig. 3B).

3.4. PAH-mediated oxidative stress, via determination of LPO products

According to the results of the present study, MDA content (in terms of total and protein-bound MDA) measured in gills of PAH-exposed mussels, showed significant increased levels, compared to those occurred in PAH-free mussels in any case (Fig. 4A, B). Similarly, increased levels of LOOH were measured in gills of...
Mussels exposed to each PAH alone and/or their mixture (at final concentrations of 0.1 and 0.2 mg L\(^{-1}\) respectively) showed significantly increased levels of GSSG, with a concomitant reduction of GSH levels and GSH/GSSG ratio, compared with those measured in gills of PAH-free mussels in any case (Fig. 5A–C). Moreover, PSH levels measured in gills of PAH-exposed mussels were significantly lower than those observed in tissues of PAH-free mussels in any case (Fig. 5D). No significant differences were obtained between all parameters measured in gills of mussels exposed to each PAH alone and/or their mixture.

### 3.5. Evaluation of TRS, in terms of GSH, GSH/GSSG ratio and PSH, in gills of mussels

In order to elucidate any relationship among biological responses, linear correlation analysis (Pearson test, \(p < 0.05, N = 5\)) was performed (Table 2). According to the results, general (NRRT) and genotoxic-related biomarkers (in terms of TNA) seemed to be correlated negatively (\(r = -0.90\)), while there was a significant relationship among both of them with LPO products in all cases (Table 2a). In specific, a strong negative correlation was observed among NRRT with total MDA (\(r = -0.96\)), protein-bound MDA (\(r = -0.88\)) and LOOH (\(r = -0.98\)), while TNA values showed a strong positive correlation with the protein-bound MDA (\(r = 0.88\)). Similarly, NRRT seemed to be correlated positively with both neurotoxic- and immune-related biomarkers (\(r = 0.90\) and 0.99 for AChE and ALP respectively), while a significantly negative correlation was obtained among TNA with ALP (\(r = -0.95\)). Moreover, NRRT and TNA showed a significant correlation with TRS products in almost all cases. In specific, there was a strong positive correlation between NRRT and GSH/GSSG ratio, GSH and PSH (\(r = 0.98, 0.98\) and 0.99 in each case), as well as a negative correlation with GSSG (\(r = -0.92\)), while TNA seemed to be correlated negatively with GSH/GSSG ratio, GSH and PSH (\(r = -0.91, -0.92\) and \(-0.93\) in each case) (Table 2a). Regarding the potential relationship among TRS with LPO byproducts, the results showed a strong positive correlation among PSH with both GSH/GSSG ratio (\(r = 0.94\)) and GSH (\(r = 0.95\)), as well as a strong negative correlation with each of the LPO byproducts (\(r = -0.98, -0.96\) and \(-0.93\) for total MDA, protein-bound MDA and LOOH respectively). On the other hand, GSH/GSSG ratio seemed to be correlated negatively with total MDA (\(r = -0.92\)) and LOOH (\(r = -0.97\)), while there was no significant correlation among GSH and/or GSSG with each of the LPO byproducts (Table 2b). Similarly, both PSH and GSH/GSSG ratio showed a strong positive correlation with ALP (\(r = 0.98\) and 0.97 respectively), while only GSH/GSSG ratio seemed to be correlated positively with AChE (\(r = 0.90\)).

### 4. Discussion

The current laboratory study showed for the first time PSH alterations, besides those of GSH and GSSG, with a concomitant enhancement of a battery of PAH-mediated biological responses in gills of mussels exposed for 7 days to Ph and/or An as well as in mixture of them. Although there is no current data, regarding PAH-mediated effects at intermediate times of exposure, previous studies showed PAH ability to promote both oxidative (in terms of cellular antioxidant efficiency against PAH-mediated oxidative effects, ROS generation and MDA enhancement) and genotoxic (in terms of nuclear abnormalities) effects, as well as different kind of biological responses within an exposure period of 7 days (for further details see Giannapas et al., 2012; Kaloyianni et al., 2009). In addition, the increased levels of GSH oxidation obtained in gills of...
PAH-exposed mussels within an exposure period of 7 days, are in accordance with previous studies which reported a significant GSH depletion after 2 days of exposure to PAHs, and its recovery to control levels only after 14 days of exposure in tissues of scallops *Pecten maximus* and *Chlamys islandica* (Hannam et al., 2010a, 2010b).

In case of PAHs in mixture, no additive effects were observed, regarding biological responses currently estimated, a fact that could be related to an adaptation of exposed mussels at the tested high/nominal concentration of PAHs or to the prolonged exposure (Baussant et al., 2009; Binelli et al., 2010).

4.1. Evaluation of PAH-mediated oxidative stress, via estimation of TRS

Since TRS is considered a marker of non-enzymatic antioxidant status, the present study showed not only a significant depletion of GSH, but PSH as well, in gills of PAHs-exposed mussels. In fact, GSH depletion and the concomitant decrease of GSH/GSSG ratio, could be due to PAH-mediated oxidative effects, as reported by a lot of previous findings in fish, scallops, crustaceans and bivalves mollusks exposed to PAHs (Hannam et al., 2010a; Regoli et al., 1998, 2004; Sureda et al., 2011; Yin et al., 2007).

It is possible that PAH oxidative intermediates could lead to TRS depletion in tissues of mussels, which in turn could lead to a wide range of physiological alterations. In fact, in the strongly reducing intracellular environment, protein cysteines, mainly in reduced state, are susceptible to oxidation and the concomitant transient formation of intramolecular or intermolecular disulfides with GSH (Ghezzi, 2005; McDonagh and Sheehan, 2007). Similarly, Dailianis et al. (2009) reported the formation of reversible mixed disulfides between GSH and cysteine in haemocytes of mussels faced with pro-oxidants after 6 days of exposure, possibly to mask sulfhydryl groups until oxidative stress is overcome. According to the latter, the significant depletion of both PSH and GSH in gills of PAH-exposed mussels, as well as the significant correlation obtained among the aforementioned parameters, reveals that thiol modifications due to protein misfolding and disulfide bond formation could be associated with PAH-mediated oxidative stress, thus providing a valuable tool in understanding the cellular redox status.

4.2. Stress-indices alterations in tissues of PAH-exposed mussels as a result of TRS depletion

The present study showed that PAH-mediated toxic effects are primary reflected by the destabilization of lysosomal membranes Following the analysis of lipid peroxidation (LPO) products, it was observed that PAH-exposed mussels suffered from an increased formation of lipid hydroperoxides (LOOH) and MDA. These findings indicate that PAH exposure led to oxidative stress in mussels, as evidenced by the significant increase in lipid peroxidation products in gill tissues compared to control and acetone-treated mussels (Bonferroni test, p < 0.05). It is worth mentioning that the formation of lipid hydroperoxides and MDA is a result of oxidative stress induced by PAHs, which in turn leads to the destabilization of lysosomal membranes. This finding underscores the potential role of PAHs in modulating the cellular redox status and oxidative stress in mussels.
and the enhancement of genomic damage, thus confirming PAH-mediated cytotoxic and genotoxic effects (Binelli et al., 2010; Domouhtsidou and Dimitriadis, 2001; Giannapas et al., 2012; Koukouzika and Dimitriadis, 2008). Since lysosomes play an important role in several biological pathways, including the immunological defence system, while there is evidence for a link between their membrane impairment with the enhancement of oxidative and genotoxic effects, as well as with changes occur in several fitness parameters (Moore et al., 2004), it could be suggested that the presence of ROS and PAH oxidative derivatives, such as anthraquinones, are responsible for the induction of PAH effects. In particular, these radicals and derivatives, undergoing redox cycling within cells and especially lysosomes, could lead to TRS depletion (in terms of PSH and GSH), the enhancement of lipid peroxidation, lysosomal destabilization and a concomitant disturbance of DNA integrity (Baussant et al., 2009; Franco et al., 2009).

![Fig. 5. Thiol redox status (TRS), in terms of (A) GSH, (B) GSSG, (C) GSH/GSSG and (D) PSH, in gills of PAH-exposed (Ph and An alone or in mixture) and PAH-free mussels (control and acetone-treated).](image)

### Table 2

Correlation coefficients between biological parameters measured in gills of PAH-exposed (Ph and An alone or in mixture) and PAH-free mussels (control and acetone-treated).

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<td>PSH</td>
<td>1</td>
<td>0.94*</td>
<td>0.95*</td>
</tr>
<tr>
<td>GSH</td>
<td>1</td>
<td>0.94*</td>
<td>0.80</td>
</tr>
<tr>
<td>GSSG</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NRRT: neutral red retention time assay; TNA: total nuclear abnormalities; t-MDA: total malondialdehyde; pr-MDA: protein-bound malondialdehyde; LOOH: lipid hydroperoxides; GSH/GSSG: the ratio between the reduced (GSH) and oxidized form (GSSG) of glutathione; GSH: glutathione; GSSG: oxidized form of GSH; PSH: protein free thiols; AChE: acetylcholinesterase; ALP: alkaline phosphatase.

Pearson’s correlation test (N = 5).

*Significant at the 0.05 level.

**Significant at the 0.01 level.
The increased levels of MDA, measured in gills of PAH-exposed mussels, are in accordance with previous studies (Frouin et al., 2007; Giannapas et al., 2012; Kopecka-Pilarczyk and Correia, 2009), thus indicating PAH-mediated oxidative effects. However, although MDA could reflect a valuable marker of oxidative stress, its short-half life could lead to incorrect results, concerning pro-oxidants ability to enhance oxidative damage within cells (Siu and Draper, 1982). According to the latter, the investigation of an additional LPO early byproduct, such as LOOH, could possess a reliable tool for evaluating PAH-mediated oxidative effects. Indeed, the enhanced LOOH levels measured in gills of PAH-exposed mussels reveal for the first time their potient ability to participate in redox reactions and exacerbate peroxidative cell injury, probably via its further oxidation to reactive aldehyde products, such as MDA, which could promote membrane, genome and protein oxidative alterations (Halliwell and Gutteridge, 2007; Richardson et al., 2008).

Since neurotoxicity is commonly associated with the presence of free radicals (Junqueira et al., 2004), inhibition of AChE activity in gills of mussels could be an indirect effect of PAHs, probably due to the enhancement of PAH-mediated oxidative derivatives within cells. The presence of these intermediates could affect the synthetic pathway of AChE, which in turn could lead to necrotic cell death and some neuronal degenerative diseases as recently mentioned (Danelakis et al., 2011; Eckert et al., 2003; Ricciardi et al., 2010).

Similarly, although ALP seemed to play an important role in immune defence, cell differentiation and shell formation in mollusks (Wootten et al., 2003) and is considered as a good immunological biomarker because of its sensitivity to metallic salts (Regoli and Principato, 1995) and organophosphates (Rao, 2006), little is known about ALP in gills of mussels faced with organic contaminants, such as PAHs. According to the results of the present study, the significant inhibition of ALP measured in gills of PAH-exposed mussels, could be due to the presence of PAH-mediated oxidative derivatives, which are able to attack sulfhydryls within catalytic ALP cysteine protein groups, thus promoting ALP inhibition (Atti and Canli, 2007). Although more field and laboratory studies are needed for understanding the role of ALP in tissues of mussels faced with PAHs, the present study gives rise to its usefulness as biomarker in ecotoxicology, a fact that is further reinforced by the significant correlations obtained among ALP with TRS, as well as between ALP and LPO byproducts.

5. Conclusion

The present study describes for the first time total thiol redox status (TRS), in terms of PSH, besides those of GSH and GSH/GSSG ratio, in mussels faced with organic contaminants, such as Ph and An. PAH-ability to induce oxidative alterations in TRS, not only via the depletion of GSH but also PSH, could lead to the enhancement of physiological alterations in gills of mussels. In fact, TRS depletion seemed to be related with immune depression and the concomi- tant induction of oxidative damage, via the enhancement of LPO byproducts, such as MDA and LOOH. Despite the fact that further field studies is crucial for estimating the role of PSH as biomarker, the fact that PSH is significantly correlated either with GSH and GSH/GSSG ratio, or with LPO byproducts, could give rise to the importance of PSH as a potent biomarker of PAH-mediated oxidative effects on tissues of marine bivalves. Furthermore, since PAH-mediated TRS depletion is significantly related with a battery of well-known genotoxic- and neurotoxic-related stress indices, such as MN frequencies and AChE activity, the present study gives encouraged data for the usefulness of TRS in future field and laboratory studies.

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References


Baussant, T., Bechmann, R.K., Taban, I.C., 2009. Environmental impact of anthropogenic activities: the use of total thiol redox status as a potent biomarker in ecotoxicology, a fact that is further reinforced by the significant correlations obtained among ALP with TRS, as well as between ALP and LPO byproducts. Marine Environmental Research xxx (2012) 1–9


