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Impact of toxicant exposure on the proteomic response to intertidal condition in *Mytilus edulis*

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ABSTRACT

Intertidal blue mussels display physiological adaptations to emersion-submersion cycle that can be impacted by response to chemicals. In order to study the interference of cellular response to pollutants on intertidal physiology, we analysed proteomic (2-DE) responses in gills of mussels exposed for 14 days to regular emersion (intertidal condition) or continuous submersion (subtidal condition) and to a mixture (B[a]P/phenantrene) of polycyclic aromatic hydrocarbons (PAHs). Antioxidant activities were measured as general stress markers. In clean context, emersion generated several over-expressions of proteins mainly involved in cytoskeleton, chaperoning, energetic metabolism and transcription regulation. Mussels exposed to PAHs showed equivalent accumulation levels of contaminants in both physiological conditions but an increased GST activity specifically in intertidal context, highlighting the high degree of stress underwent in this group, as well as over-expressions of Cu/Zn SOD and stress proteins in subtidal context. Presence of contaminants partly impacted the response to emersion: cytoskeletal rearrangements and energetic adjustments were mostly maintained whereas stress response was dramatically altered. These findings highlight the potential adverse effects of toxicants on physiological adjustments linked to air-exposure, thus suggesting to take into account in the evaluation of environmental risk the multiplicity of stresses that wild animals are likely to encounter.

1. Introduction

Organisms inhabiting intertidal zone undergo regular fluctuations of abiotic parameters, the main being water deprivation and consequent arrest of gas exchanges, osmoregulation and feeding. Cyclic alternation of emersion-submersion generates in particular a succession of tissular anoxia-normoxia resulting in repeated oxidative and energetic stresses for water-breathing animals. Blue mussels (*Mytilus edulis*) are distributed from subtidal to high intertidal heights and exhibit remarkable physiological plasticity allowing a great tolerance to this situation. They indeed developed adaptations to endure air exposure, which are: i) maintenance of large reserves of fermentable fuels (e.g. glycogen, aspartate) in all tissues (De Zwaan and Wijsman, 1976); ii) metabolic rate depression during the emersion period with production of succinate, alanine and volatile fatty acids (e.g. acetate and propionate) as alternative end products of glycolysis instead of lactate, that maximise ATP generation by the fermentative metabolism and minimise metabolic acidosis (Hochachka, 1986; Storey and Storey, 1990; De Zwaan, 1991), and iii) modification of the redox status (Letendre et al., 2008). These mechanisms limit the harmful consequences of emersion but involve recurrent and relatively energy-consuming adjustments while their energetic resources are restricted.

In addition, human activities can generate chemical contamination of water, suspended matters and sediments in coastal areas. In shore close to highly inhabited zones, blue mussels are particularly exposed to pollutants because of their sessility, their filtering feeding and their high capacity of bioaccumulation. Besides, these features make them widely used sentinel organisms in monitoring programs (Goldberg et al., 1975; Goldberg and Bertine, 2000). Cellular response to contaminants is likely to interfere whith physiological adjustments linked to regular emersion and to disrupt processes that would be crucial for withstanding air-exposure. Furthermore, the chemical stress represents a supplementary insult for intertidal animals imposing them to deal with multiples stresses, which are likely to overwhelm their energetic capacities and potentially threaten their survival.

To study the interference of the cellular response to xenobiotics on intertidal physiology in blue mussels, we analysed the interactive effects of regular emersion (intertidal context, 6 h emersion /12 h) and exposure to a mixture of two polycyclic aromatic hydrocarbons (PAHs) on gill 2-DE proteomic profile. Cell response to stress mainly

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consists in regulation of proteins expression and metabolism, thus open approach such as proteomics constitute powerful tools to understand sub-lethal effects of ecophysiological and chemical stresses (Möller et al., 2001; Manduzio et al., 2005; Olsson, 2005; Lopez, 2007; Monsinjon and Knigge, 2007; Tomanek and Zuzow, 2010). By studying the proteomic response to repeated air exposure and simultaneous chemical pollution, we aim at i) gaining insight into physiological adjustments developed to face intertidal condition and ii) better understanding interactive effects of chemical and ecophysiological stresses. PAHs were chosen for chemical treatment because they are ubiquitously distributed in the environment, present at elevated concentrations, highly toxic and therefore considered as major pollutants (Samanta et al., 2002). Benzo[a]pyrene (B[a]P) is largely found in biota mainly due to extraction and use of gas and oil and represents a particularly noxious molecule that becomes mutagene and carcinogene after activation by biotransformation systems of living organisms (Livingstone et al., 1990; Motelay-Massei et al., 2004; Skupinska et al., 2004). Phenanthrene is a low molecular weight PAH used in the synthesis of different organic substances composing pesticides, fungicides, detergents and dyes, and constitutes the predominant fraction of PAH contamination in estuaries such as the Seine basin (Motelay-Massei et al., 2004). These contaminants were delivered in continuous flow at environmental concentrations during 14 days and their levels in tissues followed in the course of the treatment. Levels of antioxidant activities (catalase, Cu/Zn superoxide dismutase, glutathione peroxidase and glutathione transferase) were measured at the end of the experiment as indicator of general stress.

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2. Materials and methods

2.1. Experimental exposure to PAHs

Specimens of adult blue mussels, *M. edulis*, of 4–5 cm shell length, were collected from a natural intertidal location in a clean area, Yport, located in the north of France (49°44′N, 0°18′E). This site is routinely used as a reference site although background PAHs levels are regitered in mussels (around 250 ng g^{-1} d.w. of total PAHs). The mussels were placed in an artificial open-air stalling system. Half of the mussels were submitted to an artificial tidal cycle (2 cycles a day of 6 h immersion and 6 h emersion) and the other half was placed in continuous immersion. Before exposure to xenobiotics, mussels were acclimatised to their new environment for a week. Within these two physiological conditions, mussels were separated in two groups: one exposed to PAHs (phenanthrene 5 μg L^{-1} and benzo[a]pyrene 500 ng L^{-1}) diluted in dimethyl sulfoxide 90% v/v (DMSO), and the other one not exposed (control group). Concentrations were chosen in regard to environmental contamination and are in the same order of magnitude as maximal values obtained for the Seine bay (Tronczynski et al., 1999). Table 1 summarises the different tested conditions (SX: subtidal exposed mussels; SC: subtidal controls; IX: intertidal exposed mussels; IC: intertidal controls). DMSO containing PAHs was delivered in a mixing tank through a peristaltic pump at 100 μl/min (final dilution: 200 μL L^{-1}) and resulting contaminated water was transferred in 60 L-exposure tanks, providing continuous and regular amount of pollutant in water. Mussels were exposed to this system during 14 days in order to have the most ecologically relevant picture as possible and to bring out sublethal effects but not lethal ones. During all the experiment mussels were fed with a mix of *Skeletonema costatum* and *Tetraselmis suecica* (respectively 2/3 and 1/3 v/v, 10,000 cells L^{-1} continuously delivered during 2 h twice a day). 2 to 5% of mortality was recorded at the end of the experiment whatever the condition. For chemical analysis, 5 points were performed (day 0 and 1, 4, 7, 14). For each of them, soft tissues of 25 mussels per condition were used and immediately deep frozen in liquid nitrogen. For enzymatic and proteomic analyses, sixteen mussels per condition were dissected at day 14, their gills removed and frozen in liquid nitrogen.

Table 1
Cross-exposure to PAHs and intertidal regime. Mussels were distributed in 4 different conditions (SX: subtidal exposed; IX: intertidal exposed; SC subtidal control; IC: intertidal control).

	Subtidal 0 h emersion /12 h	Intertidal 6 h emersion /12 h
Exposed (phenanthrene 5 μg mL^{-1} B[a]P 500 ng mL^{-1})	SX	IX
Control	SC	IC

2.2. Chemical analyses

2.2.1. Sample preparation

Freeze-dried mussels (0.5 g to 1 g d.w.) were extracted by microwave-assisted extraction with dichloromethane (30 W; 10 min) (as presented in Cailleaud et al., 2007). The mixing of the freeze-dried mussel tissues makes the matrices homogeneous (Baumard et al., 1997; Letellier et al., 1997). Therefore, a single analysis was performed per sample.

The internal standards (Phed10 and BaPd12; Promochem) were gravimetrically added prior to the extraction and one extraction blank was performed with each series of extraction. For PAH analysis (Baumard et al., 1999), the extract was directly reconcentrated with a rotary evaporator. A purification step on alumina and silica microcolumns was performed. The extract was passed through the alumina column and PAHs were further eluted with dichloromethane. The sample was then loaded onto the silica column. The aliphatic fraction eluted with pentane was discarded and PAHs were then eluted with a mixture of pentane/dichloromethane (65/35, v/v). The final extract was reconcentrated in isooctane and analysed by gas chromatography/mass spectrometry (GC–MS). Pyrene d10 was added prior to the injection to serve as syringue standard to quantify internal standards and control the recoveries.

To test the accuracy and the validity of the quantification method, standard solutions of compounds to be quantified mixed with the related internal standards are regularly run on the GC/MS system. These solutions are used one hand to calculate the response factors. On the other hand other independent solutions are used to test the precision of the quantification which has been evaluated to be comprised between 90% and 110% depending on the compounds with reproducibilities comprised between 1% to 4%.

Moreover, the entire analytical procedure was applied several times to the certified mussel tissue, SRM 2974 (NIST, Gaithersburg, MD, USA). The recoveries for five replicates on this SRM were between 70% and 110% with repoducibilities ranging from 7% to 17% depending on the compounds (Rocher et al., 2006).

2.2.2. GC-MS analyses

The aromatic fraction was analysed by gas chromatography coupled to mass spectrometry. An HP 5890 series II GC (Hewlett-Packard, Palo Alto, CA) equipped with a split/splitless injector was used (Splitless time: 1 min, flow 60 mL min^{-1}). The injector temperature was maintained at 270 °C. The GC temperature program was: from 50 °C (2 min) to 290 °C (20 min) at 5 °C/min. The carrier gas was helium at a constant flow rate of 1 mL min^{-1}. The capillary column used was an HP-5 (Hewlett-Packard, Palo Alto, CA): 60 m × 0.25 mm I.D. × 0.25 μm film thickness.

The GC was coupled to an HP 5972 mass selective detector (MSD). The MS was operated under the selected ion monitoring mode using the molecular ions of the studied PAHs (Electron impact at 70 eV,

2000 V, 1.4 scan s^{-1}, dwell time: 40 ms). The interface temperature was 290 °C. The PAHs were quantified relative to perdeuterated PAHs added to the matrix prior to the extraction (Quilliam et al., 1994; Baumard and Budzinski, 1997). One deuterated internal standard per class of aromaticity of the studied PAHs was used. The response factors of the different compounds were measured by injecting SRM 2260 (24 Aromatic Hydrocarbons in toluene; NIST) solution spiked with the same solution containing the perdeuterated PAHs as the one used for spiking the mussels and the sediments.

B[a]P and phenanthrene concentrations in mussel tissues are given on a dry weight basis. The detection limits for all the PAHs were about 300 pg injected, which means about 50 pg g^{-1}. Protocol was validated by the use of certified materials. For a certified mussel tissue SRM 1974a, the PAH recovery is >60% and the variation coefficient is also low: 8.8% (Baumard et al., 1997).

2.3. Enzymatic assays

2.3.1. Sample preparation

The gills were homogenised on ice in 10 mM Tris, 10 mM EDTA, 5 mM 2-β mercaptoethanol and 1% Triton, pH 7.5 (1:3 volume) using an electric homogeniser (Ultra Turrax). Homogenates were treated using ultrasonics at 20 kHz for 2×10s periods and centrifuged for 15 min at 10,000 g and 4 °C. Supernatants were kept on ice and used immediately for enzymatic assays. Protein concentration of supernatants was measured according to Bradford (1976) with bovine serum albumine as standard.

2.3.2. Catalase (CAT, EC 1.11.1.6)

CAT activity was measured by the adapted method of Clairbone (1985) according to Livingstone et al. (1990). H_2O_2 disappearance was recorded at 240 nm. Activity was expressed as micromoles of H_2O_2 per min and per mg of protein, or $U.mg^{-1}$prot.

2.3.3. Glutathione peroxidase (GPX, EC 1.11.1.9)

Total GPX activity was measured using Bioxytech GPx-340TM kit (Oxys International Inc.) adapted for mussel extracts by Manduzio et al. (2004). Briefly, the enzymatic assay was based on a coupled enzyme system, where GSSG (glutathione oxidized form) formed in the GPX reaction with a tert-butyl substrate was converted to the reduced form GSH by GRD (Lawrence and Burk, 1976). The consumption of NADPH was monitored at 340 nm and directly proportional to the GPX activity in the sample. Activities were calculated with the extinction coefficient of NADPH ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$; Dawson et al., 1989). Results were expressed as nanomoles of NADPH per min per mg of protein or mU mg^{-1} prot.

2.3.4. Cu/Zn superoxide dismutase (EC 1.15.1.1)

Cu/Zn SOD activity was determined after migration of native proteins and specific photochemical revelation. Isoelectric focusing on high resolution gels (pH 4.0–5.0) was performed in a Multiphor II apparatus (Amersham Pharmacia Biotech, France). For each extract, a volume corresponding to 50 μg of total protein content was loaded on the gel. Staining of SOD activity was performed immediately after the completion of electrophoresis, using the photochemical method of Beauchamp and Fridovich. (Beauchamp and Fridovich, 1971). Gels were first soaked in 2.5 mM nitrobluetetrazolium (NBT) for 30 min in darkness under gentle agitation. They were then incubated in a solution of 1 M phosphate buffer (pH 7.8) containing 0.04 mM riboflavine and 0.5% TEMED for 30 min in the dark and under gentle agitation. Detection of SOD isoforms and associated activity was carried out by soaking gels in 10 mM phosphate buffer (pH 7.8) under light conditions. In mussel extracts, using IEF, three isoforms of SOD with acidic isoelectric points were detected: 4.7 for the major band, SOD-1, 4.6 for SOD-2 and 4.55 for the minor one, named SOD-3 (Manduzio et al., 2003).

Gels were scanned and subsequently analysed using the ImageMaster TotalLab software from Amersham Pharmacia Biotech (France). The activity of each SOD band was determined from the pixel intensity of its volume. The total activity of SOD was compared to a standard of Cu/Zn-SOD of bovine erythrocyte and expressed in U mg^{-1} prot.

2.3.5. Glutathione transferase (EC 2.5.1.18)

GST activity was measured according to the method of Habig et al. (1974) using CDNB (1-chloro-2,4-dinitrobenzene) and GSH (glutathione reduced form) as substrates (Habig et al., 1974). Absorbance was read at 340 nm and activities were expressed as nanomoles of conjugated product formed per min and per mg of protein or mU mg^{-1} prot.

2.3.6. Statistical analysis

Values were expressed as mean values ± SEM of ten independent individual values (five individuals concerning Cu/Zn SOD activity). Statistically significant differences between samples were determined with SigmaStat (SPPS, Chicago, USA) using the Student's *t* test. $p<0.05$ was accepted as significant.

2.4. Proteomic analyses

2.4.1. Preparation of protein extracts

Individual gills were homogenized with an electric homogeniser (Ultra Turrax) during 15 sec in buffer containing 50 mM Tris pH 7.5, 9 M urea, 0.5% chaps, 2% β-mercaptoethanol, 0.8% pharmalytes pH 3–10 and 20 μg mL^{-1} aprotinin. The homogenates were twice sonicated briefly (10s) and then centrifuged at 9000 g, at 4 °C for 15 min. After centrifugation, supernatants were collected and protein contents were measured according to the method of Bradford with bovine serum albumin as a standard (Bradford, 1976). Total protein concentrations were similar between the different conditions throughout the experiment (data not shown).

2.4.2. 2D-gel electrophoresis

The IEF step was carried out on nonlinear wide-range immobilized pH gradient gels (IPG Strips, pH 3–10NL 18 cm, Amersham Pharmacia Biotech, Uppsala, Sweden) with horizontal electrophoresis apparatus (Multiphor; Amersham Pharmacia Biotech). The dry strips were rehydrated overnight directly with 750 μg proteins of sample solution (in-gel rehydration). Strips were then subjected to isoelectrofocusing (20 °C, 83 μA/strip) with the following parameters for imposed electric field according to manufacturer's instructions: 1Vh during the first minute (gradient), then 2500 Vh during 5 h, 10,000 Vh during the following 5 h (gradient) and 33250 Vh during 9 h30. The second dimension was carried out on 12 % SDS-PAGE gels (20 cm×20 cm×1.5 cm) at 15 °C. Gels were prepared in MultiCasting Chambers (BIO-RAD) and electrophoreses were performed in parallel for the 24 gels (6 gels representing 6 individual extracts for each condition) in Protean plus Dodeca-Cells (BIO-RAD). After migration, proteins were visualised by colloidal Coomassie blue staining.

2.4.3. Gel analysis

The 24 gels were scanned using the ImageScanner (Amersham Pharmacia Biotech) and analysed using Melanie software (ImageMaster™ 2D Platinium version 5.0; Amersham). The relative volume of each spot was calculated as percentages of the total volume over all the spots in the gel. With that normalisation, variations due to protein loading and staining between gels were taken into account. Spots that were equivalent between gels were matched by the software to form groups. Groups in which spots were expressed in less than 2/3 of the replicates for both compared conditions were excluded from statistical analysis.

2.4.4. Statistical analysis

The differences in protein expression were statistically analysed by STATISTICA Software v7.0 (Statsoft. Inc., 2004). Mann–Whitney U tests were used to evaluate whether the outcome of two conditions was different. In all cases, the level of significance (p) was set to 0.05. Data were also submitted to a multivariate analysis (PCA) to extract global information. Data (vol.%) were first normalised with the following equation : Nvol.% = Ln (vol.% + 1), where Nvol.% is the normalised vol.% in order to increase the efficiency of PCA by improving data linearity (Quinn and Keough, 2002; Apraiz et al., 2009). Then, data were filtered according to the coefficient of variation (CV) of replicates of each condition so as to minimise the inter-replicate variability. Only groups with CV<40% in all conditions were kept for the subsequent analysis (172 spots).

2.4.5. Trypsin digestion: automated in gel-digestion

Spots were excised manually from the polyacrylamide gels and sliced into small pieces using a sterile scalpel when necessary. Spot were stored in twin.tec 96-well polypropylene PCR plate (Eppendorf, france) and automatically digested on the Biomek® 3000 workstation (Beckman Coulter™). The gels pieces were alternatively rinsed and destained three times 15 min at 750 rmp with ultrapure water and 15 min at 750 rmp with 50 mM $NH_3H_2CO_3$ 20 mM/acetonitrile (1:1). The spots were evaporate to dryness on speedvac® (Thermo Electron Corporation) and were digested in 30 µL at 6 ng µL^{-1} in 50 mM ammonium bicarbonate of Sequencing Grade Modified Trypsin (Promega) overnight at 37 °C. After digestion, the peptides were extracted twice with water/acetonitrile/TFA mixture (50:50:0.1), dried on speedvac and reconstituted in 3% acetonitrile and 0.1% TFA.

2.4.6. Mass spectrometry: nanoLC-ESI-MS/MS

All analyses were performed using a NanoLC1200 system coupled to a 6340 Ion Trap mass spectrometer with HPLC-chip cube interface (Agilent Technologies). Peptides were separated on Agilent HPLC-chip (40 nL enrichment column; LC separation channel of 43-mm-long × 75-µm-inner diameter) which is packed with C18 reversed phase matrix (zorbax 300-Ä pore size, 5-µm particle size) by applying a 17-min binary gradient (solvent A: 0.1% FA; solvent B: 0.1% FA, 90% ACN) ranging from 3% to 80% solvent B at a flow rate of 300 nL/min. Separated peptides were submitted to electrospray ionisation and analysed in mass spectrometer. Full MS scans from 300 to 2200 *m/z* were recorded, and the five most intensive peaks were subjected to MS/MS taking into account a dynamic exclusion after 2 spectra. Single charged ions were excluded from CID selection.

2.4.7. Database searches

Proteins were identified from the peptide mass fingerprint obtained by NanoLC-ESI-MS/MS using MASCOT software. Peak lists for database searching, containing mass and intensity paired information of multiple peptides issued from one protein, were created using DataAnalysis (version 6.1, Agilent Technologies). Peak lists were submitted to the NCBInr database in MASCOT MS/MS Ion search allowing up to 1 missed trypsic cleavage, with carmabodomethyl of cysteins as fixed modification and oxidation of methionins as variable one, and peptide mass values from 0.6 to 1.6 Da. Positive match was considered when it ranked among first positions and presented a score with significance threshold p>0.05 and with a low false discovery rate.

3. Results

3.1. Accumulation of B[a]P and phenanthrene

Mussels were exposed to a mix of benzo[a]pyrene (B[a]P; 500 ng L^{-1}) and phenanthrene (5 µg L^{-1}) during 14 days in two physiological conditions (subtidal or intertidal). Kinetic of B[a]P and phenanthrene accumulation in the whole animals are shown in Fig. 1. In controls, only traces of these PAHs were measured (B[a]P<5 ng g^{-1} d.w; phenanthrene<13 ng g^{-1} d.w.; Table 2). In exposed mussels, levels of accumulated B[a]P were comparable between the two ecophysiological conditions (XS: 14048 ng g^{-1} d.w. ; XI:12,900 ng g^{-1} d.w.; Fig. 1A, Table 2) at the end of the treatment, so were the levels of phenanthrene (XS: 24,128 ng g^{-1} d.w; XI: 20,037 ng g^{-1} d.w.; Fig. 1B, Table 2). Kinetic of B[a]P accumulation present a different trend according to the tidal regime of animals, with lower levels in intertidal than in subtidal individuals in the course of the exposure. Phenanthrene accumulation reached a plateau at day 7.

3.2. Antioxidant activities

Levels of catalase, glutathione peroxidase, Cu/Zn superoxide dismutase and glutathione transferase activities measured in gills of mussels are reported in Fig. 2. A strong decrease of Cu/Zn SOD activity was noticed in subtidal exposed compared to subtidal controls ($p = 2.4\ 10^{-6}$; Fig. 2A) On the other hand, an increase in GST activity was observed (Fig. 2D); this increase was just a trend in the subtidal context (p = 0.0509) but was very significant in intertidal context (p = 0.00026). Other antioxidant enzymes did not show any differences after 14 days of experiment (Fig. 2B, C).

3.3. 2DE image patterns

Gels of the four conditions (SC, SX, IC, IX) were treated within a single computer analysis, with 4 to 6 gels per condition. The proteomic map of mussel gill samples are presented in Fig. 3. Software detection process allowed us to detect 970 to 1308 spots for subtidal control condition (SC), 1129 to 2581 spots for intertidal control condition (IC), 887 to 1156 spots for subtidal exposed condition (SX) and 1288 to 2042 spots in intertidal exposed condition (IX). Further to matching

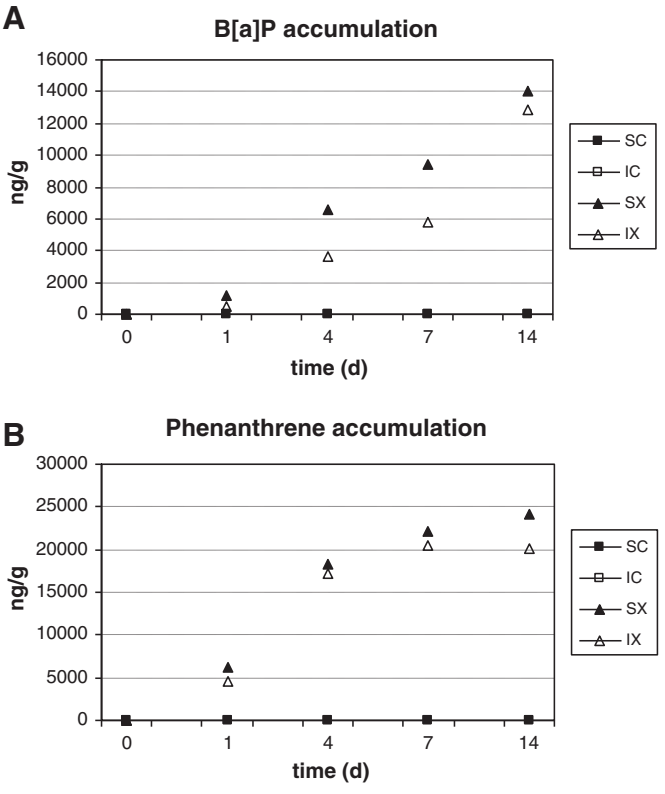


Fig. 1. Kinetics of accumulation of benzo[a]pyrene (A) and phenanthrene (B) in whole tissues of mussels after 0, 1, 4, 7 and 14 days of exposure to 500 ng L^{-1} of B[a]P and 5 µg L^{-1} phenanthrene in continuous flow. SX: subtidal exposed mussels; SC: subtidal controls; IX: intertidal exposed mussels; IC: intertidal controls.

Table 2
Body burdens (ng g^{-1}) of benzo[a]pyrene (a) and phenanthrene (b) in whole tissues of mussels after 0, 1, 4, 7 and 14 days of exposure to 500 ng.L^{-1} of B[a]P and 5 μg L^{-1} phenanthrene in continuous flow.

Time (days)	SC	IC	SX	IX
(a)				
0	4	4	5	5
1	4	3	1195	452
4	5	3	6575	3600
7	6	4	9380	5801
14	5	5	14,048	12,900
(b)				
0	15	15	7	17
1	16	7	6148	4548
4	13	12	18,264	17,279
7	9	12	22,051	20,459
14	11	13	24,128	20,037

process, precisely 900 groups were established. Groups which spots were expressed in less than 2/3 of the replicates in both compared conditions were excluded from statistical analysis.

Intertidal condition gave raise to changes in expression level of 58 spots in non-contaminated mussels (IC/SC): 48 spots were over-expressed whereas only 10 spots were under-expressed or even disappeared (Fig. 3A). PAHs exposure (SX/SC) generated 27 modifications of which 13 over-expressions and 14 under-expressions (Fig. 3B). When mussels were exposed simultaneously to intertidal condition and PAHs contamination (IX/SC), 39 spots were affected in comparison to control, with 27 over-expressions and 12 under-expressions (Fig. 3C).

Covering of the sets of spots of interest between the different treatments are represented in a Venn diagram (Fig. 4). 4 spots (n°16, 30, 53, 93) were affected by intertidal condition alone or contamination alone but not by the combination of the two stresses. 7 spots affected by intertidal condition are found in clean and contaminated context at the same time (n°35, 41, 57, 64, 83, 95, 96); those are not altered by PAHs in subtidal condition. 4 spots were affected by PAHs contamination whatever the tidal condition (n°39, 54, 62, 97). 3 spots have their expression levels modified by every treatment in comparison to controls (n°72, 84, 92).

3.4. Identification of differentially expressed protein spots

All spots of interest were excised in order to be identified. Analysis of digested peptides by mass spectrometry (Nano-LC-chip-MS) and search of sequence similarity on MASCOT MS/MS gave identification of only 41 of 102 spots of interest (Table 3) due to difficulties in matching with available sequence data.

3.5. Proteomic response to emersion in clean context

Among the proteins over-expressed by clean emersed mussels, we identified several molecular chaperones (spots n°7, 9 and 10: HSP/HSC70; spot n°19: TCP1; spot n°25: protein disulfide isomerase; spots n°45 and 53: calumenin precursor) as well as proteins involved in protein synthesis (spot n° 31: translation elongation factor 1α) and degradation (spot n°37: 26S protease regulatory subunit 6A), cytoskeleton components (spot n°29: α tubulin; spot n°35: tektin; spot n°38: β tubulin; spots n°48 and 55: β actin; spot n°49: actin-like protein; spot n°60: similar to H2-α tubulin; spots n°74 and 75: tropomyosin) and regulators (spot n°41: gelsolin; spot n°92: destrin), enzymes of energetic metabolism (spot n°50: phosphoglycerate kinase; spots n°64 & 84: fructose bisphosphate aldolase; spot n°5: long chain acylCoA dehydrogenase), proteins involved in DNA expression, replication or repair (spot n°69: pur-alpha protein; spot n°88: arginine/serine-rich 2 splicing factor; spot n°95: histone H4) and C isoform of the multifunctional regulatory molecule 14-3-3 epsilon (spot n°78). Among the proteins under-expressed in clean emersed mussels, a HSP70 (spot n°16), a 40S ribosomal prot SA (p 40) (spot n°57), a peptidyl-prolyl cis-trans isomerase (spot n°93) and a histone H2 (spot n°96) has been identified.

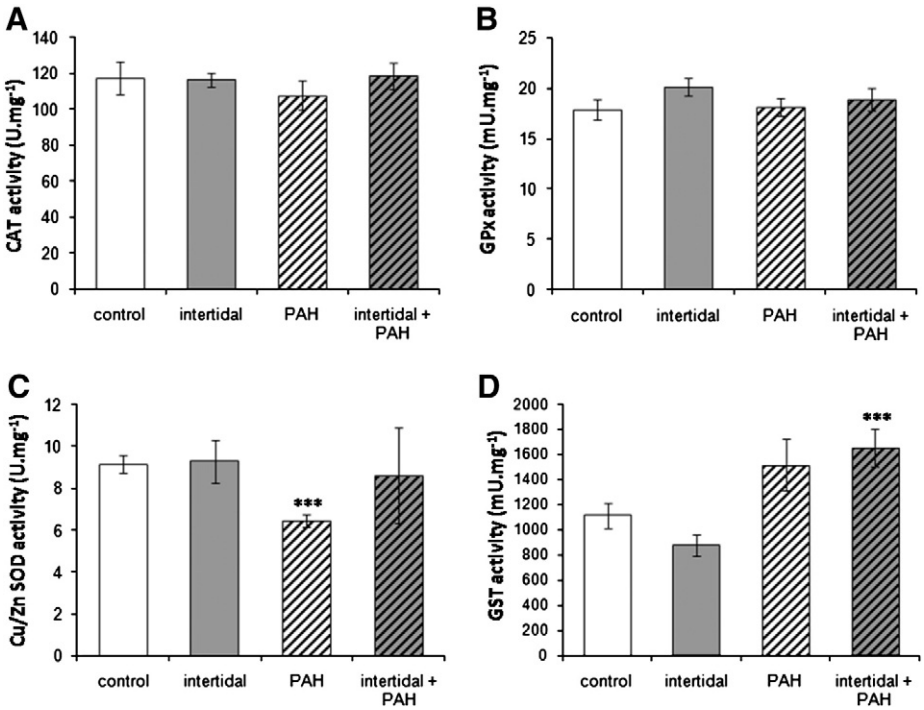
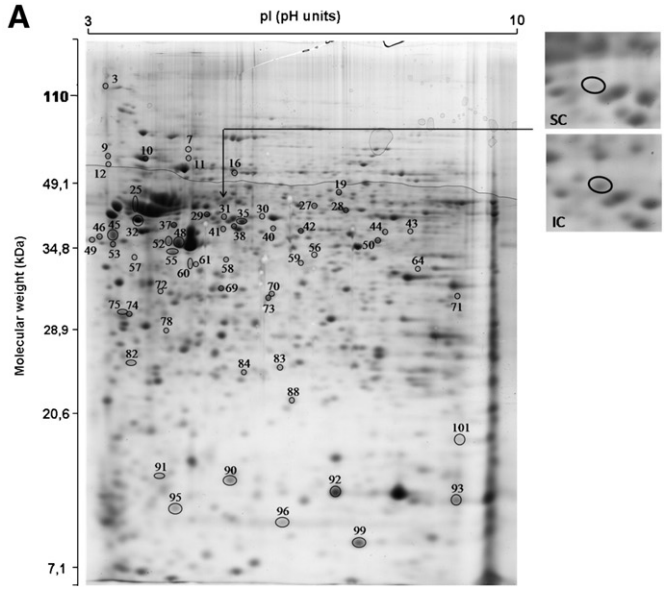
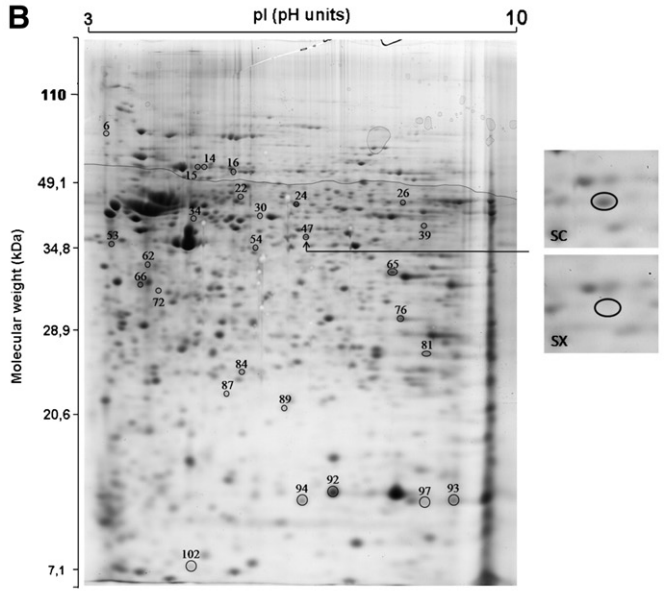
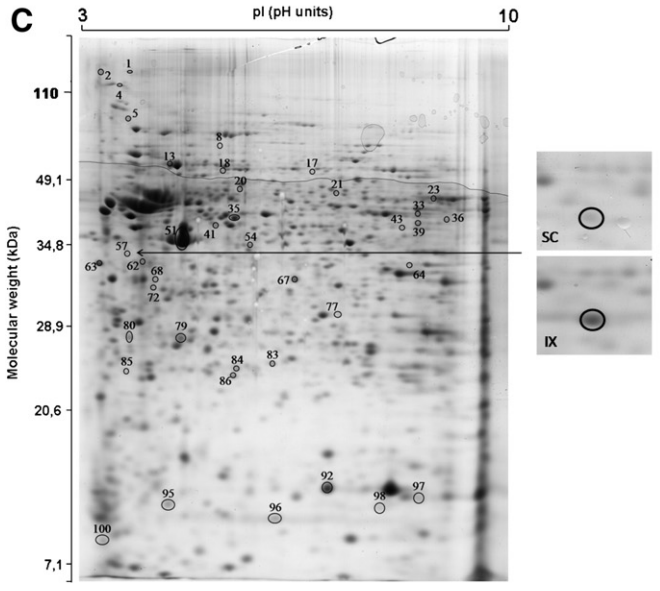


Fig. 2. Levels of enzymatic antioxidant activities (CAT, catalase, A; GPX, glutathione peroxidase, B; Cu/Zn SOD, C; GST, glutathione S-transferase, D) in gills of intertidal and subtidal mussels exposed to PAHs during 14 days. Activities are expressed as mean ± SEM (n = 10) of levels measured at the 14th day of exposure except for Cu/Zn SOD which values are the mean ± SEM of 5 individuals. Differences of values between exposed and controls were considered as significant from $p<0.05$ (*), $p<0.01$ (**), $p<0.001$(***).







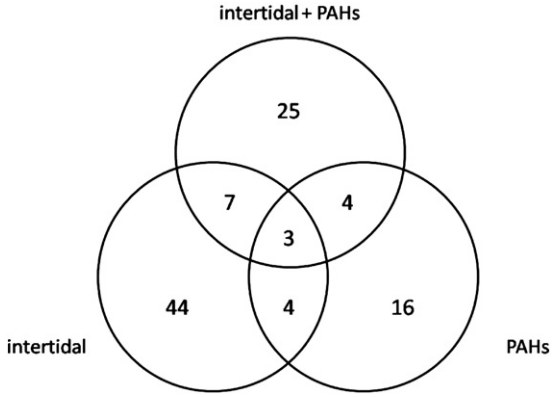


Fig. 4. Venn diagramm representing differentially expressed spots common between the different conditions tested. 3 spots were common to the 3 treatments (intertidal condition, PAHs exposure, intertidal condition + PAHs exposure). 7 spots were affected by intertidal exposure whatever the contamination context. 4 spots were affected by PAHs contamination whatever the tidal condition. 4 spots were common to intertidal condition and PAHs exposure but not found in the response to the combination of intertidal condition and PAHs exposure.

3.6. Proteomic response to PAHs exposure in subtidal context

Among over-expressed proteins in response to PAHs exposure of subtidal mussels, two HSC70 (spots n°14 and 15) has been identified as well as actors of cytoskeleton (spot n°54: actin; spot n°92: destrin), protein folding (spot n°53: calumenin precursor; spot n°93: peptidyl-prolyl cis-trans isomerase A; spot n°89: peptidyl-prolyl cis-trans isomerase B), glycolysis (spot n°84: fructose bisphosphate aldolase) and defences (spot n°94: Cu/Zn superoxide dismutase). Among under-expressed proteins, we identified chaperones (spot n°16: HSP70; spot n°26: TCP1 δ subunit), a 3-oxoacid coA transferase (spot n°24), an arginine kinase (spot n°65), a heavy metal binding protein (spot n°66) and a receptor of activated kinase C1 (RACK1; spot n°76).

3.7. Proteomic response to emersion and PAHs exposure

Among over-expressed proteins detected in the response to combination of intertidal condition and PAHs contamination, only HSP90 (spot n°8) has been identified as a chaperone. As over-expressed proteins we also identified structural proteins and polymerization regulators (spot n°35: tektin; spot n°41: gelsolin; spot n°51: β actin; spot n°54: actin; spot n°92: destrin), actors of energy production (spot n°36: NADH ubiquinone oxidoreductase; spots n°64 & 84: fructose bisphosphate aldolase; spot n°67: cytosolic malate dehydrogenase) as well as a phosphatase 2A inhibitor (spot n°63), a proteasome α subunit (spot n°80) and a histone H4 (spot n°92). Among under-expressed proteins, we identified a 40S ribosomal prot SA (p 40) (spot n°57), a ribosomal protein S2 (spot n°77) and a histone H2 (spot n°96).

3.8. PCA

Data were submitted to a reduction through Principal Components Analysis in order to reveal global differences between conditions.

Fig. 3. Representative two-dimensional electrophoresis (2-DE) maps from subtidal control sample showing proteins of mussel gills (non-linear pH 3–10 range; 12% acrylamide; colloidal blue staining). Numbers refers to proteins in other figures and tables. A: Signature of exposure to intertidal condition; labelled spots depict proteins with statistically significant differences of expression between intertidal controls (IC) and subtidal controls (SC). B: Signature of exposure to B[a]P/phenanthrene; labelled spots depict proteins with statistically significant differences of expression between subtidal exposed (SX) and subtidal controls (SC). C: Signature of exposure to intertidal condition and B[a]P/phenanthrene; labelled spots depict proteins with statistically significant differences of expression between intertidal exposed (IX) and subtidal controls (SC).

After reducing the inter-replicate variability by filtering normalised data according to the coefficient of variation (CV) values, a correlation matrix of 172 variables (or spots) and 18 samples (or gels) was calculated in PCA. We obtained therefore linear and uncorrelated combinations of original variables called principal components, of which the first explains the largest variability of the data. Eigen values, that are expressed here as percentage values, represented the amount of original variance explained by each component. In the present analysis, two principal components explained 29.20% of the total variation and three components 39.25% (Table 4). The two first components are represented in a bidimensional graph, where each axis corresponds to each component and plotted objects to samples (Fig. 5). The distance between the latter represents dissimilarity in the protein expression pattern of samples (Quinn and Keough, 2002). Group discrimination by PC1 is not clear while PC2 clearly separates intertidal exposed group from subtidal exposed one.

4. Discussion

4.1. Effect of intertidal condition on antioxidant activities and protein expression

4.1.1. Antioxidant activities

Intertidal mussels were subjected twice a day to 6 h-air exposure for two weeks in order to mimic tidal cycle. Sampling was performed at day 14 when mussels were in emersion since 4 h, so that oxygen tension and redox status in tissues should theoritically be modified in comparison to "normal" submersion state (De Zwaan, 1991; Letendre et al., 2011). However, no differences between intertidal and subtidal conditions were recorded at the level of the main antioxidant activities, *i.e.* catalase, superoxide dismutase, glutathione peroxidase and glutathione transferase. These results are not consistent with those obtained in other studies showing variations of antioxidant activities in the course of emersion in different mussel species. Air-exposure induced a general increase in antioxidant activities in gills of blue mussels subjected to 6 h/12 h emersion-submersion cycles during 9 days (Letendre, 2009). In this study, levels remained all the same lower than those of subtidal control mussels during anoxia for CAT activity. In the brown mussel *Perna perna*, a 4-hours air exposure generated a reversible increase in SOD activity in digestive glands (Almeida and Bainy, 2006). These works show nonetheless that antioxidant response to emersion is labile and not univocal: the nature of involved antioxidant defence and the moment of hypoxic stress at which it is induced vary according to experimental conditions and model. Moreover, only a part of enzymatic component of antioxidant forces were measured here and the contribution of molecular antioxidant such as glutathione cannot be excluded.

4.1.2. Proteomic response

Several modifications of protein expression are registered in 2-DE experiment in response to repetitive emersion. It must be noticed that given the error percentage of the statistical test used to detect differences between conditions, a part of the spots of interest could be false positives and improperly proposed as differentially expressed proteins. However, such selection criterion reduces at the same time the proportion of false negatives. This increases the chances to detect weak but real differences of expression, which is important in the explanatory context of this study. To deal with this dilemma, a subset of highly significant changes in protein expression (below 0.01) is highlighted in the table and in the text. They form a pool of proteins where the proportion of potential false positives are reduced, which means that their regulation in the face of emersion is not more important but rather more confident than with a 0.05 threshold value. Interestingly, no spot of interest detected in the responses to PAHs treatment in combination or not with intertidal condition had a significance probability for expression change lower than 0.01.

Anyway, the proportion of changes due to emersion is twice higher than in the response to PAHs treatment, which highlights the importance of the regulations that are set up to face water deprivation. Noticeably, the majority of spots of interest are over-expressed.

4.1.3. Cytoskeletal proteins

Levels of several structural proteins and regulators are particularly enhanced in intertidal mussels during air exposure. This statement is reinforced by high statistical significance (below 0.01) observed for the over-expression of several spots corresponding to β tubulin isoforms, which are responsible for microtubule polymerisation, and to tropomyosin isoforms. Although changes in the levels of such proteins are very often detected in proteomics whatever biological question (Petrak et al., 2008), their univocal and large response here is remarkable. Over-expression of actin subunit, tropomyosin and other structural proteins have already been shown in *Caenorhabditis elegans* subjected to 4 h-hypoxia (Li et al., 2010); these changes were attributed to maintenance of cell shape and functions during low O_2 challenge. Besides, cytoskeleton components are very sensitive to post-translational modifications (PTM) and actin constitutes a major target of oxidative alterations like carbonylation or glutathionylations (Dalle-Donne et al., 2001; McDonagh et al., 2005; Sheehan and McDonagh, 2008). Actin and tubulin subunits correspond to multiple spots on gels and observed changes could be due to such structural modifications and consequent changes in pI or MW location on gels. Some of these modifications, *i.e.* glutathionylation, can play functional roles in various pathways of cellular signalling (Dalle-Donne et al., 2009). Moreover, oxidative modifications of actin alter microfilaments structure and consequently cell morphology (Dalle-Donne et al., 2001). These modifications are dramatic and can generate membrane blebbing if the insult is to high, but they probably increase cell resistance in situation of mild stress. The importance of actin cytoskeleton dynamics in stress response is underlined by the regulation of microfilament stability by stress-induced activation of p38MAPK and downstream MAPKAP - HSP27 phosphorylation cascade (Dalle-Donne et al., 2001), the existence of which has been partially demonstrated in the intertidal anoxia-tolerant mollusc *Littorina littorea* (Larsen et al., 1997; Larade and Storey, 2006). Variations of the expression levels of two actin polymerisation regulators in intertidal mussels, *i.e.* gelsolin, which regulation is particularly significant ($p<0.01$), and destrin, support the hypothesis of cytoskeleton rearrangement in the face of water-deprivation that induces hypoxic stress and potentially osmotic and thermal stresses.

4.1.4. Molecular chaperones

Levels of molecular chaperones are also affected during emersion in intertidal mussels. In clean mussels, several spots corresponding to chaperones like HSC70 and TCP1 subunit are over-expressed as well as a protein similar to HSP70 during emersion, whereas another form of HSP70 is strongly under-expressed at the same time. The majority of these regulations are statistically very significant (below 0.01), showing the reliability of these observations. Molecular chaperones heat shock cognates are essential for protein folding and trafficking and represent the constitutive forms of HSP *l.s.*, but their levels can also be induced by chronic stress (Franzellitti and Fabbri, 2005). Conversely, HSP70 are stress proteins which gene expression is induced by acute stress but is potentially inhibited if treatment is prolonged (Franzellitti and Fabbri, 2005). Induction of chaperones during emersion probably increases protection against protein misfolding and damages considering the adverse conditions due to air-exposure. HSP response is an important component of hypometabolic state that could help to extend protein life-span insofar as both synthesis and degradation, which are energy-consuming processes, are reduced in this situation (Storey and Storey, 2007). Anestis et al. recently studied HSP response of *M. galloprovincialis* to air exposure and showed significant variation of levels of HSP72 and HSP73 forms in mantle only

Table 3
Mytilus edulis. List of spots of interest with identified proteins (labelled in Fig. 3). Ratios in bold depict proteins which expression change present a statistical significance below 0.01 between the two conditions. Data were obtained from the peptide mass fingerprint analysis of proteins (nano-LC-chip-MS). SX: subtidal exposed mussels; SC: subtidal controls; IX: intertidal exposed mussels; IC: intertidal controls.

n°	Mr	pi	Protein name	Ratio	Ratio	Ratio	Accession number	Species	Score	Queries	%	Mr	pI
	obs.	obs.		IC/SC	SX/SC	IX/SC				match.	coverage	th.	th.
1	142	4,7				5,65							
2	140	4,5				5,83							
3	130	4,5		3,83									
4	128	4,6				2,12							
5	110	4,7				3,92							
6	100	4,5			3,06								
7	81	5.4	Heat shock cognate 71	**8,12**			gi|76780612	*Mytilus galloprovincialis*	410	9	17	71.51	5.29
8	81	5.6	Heat shock protein 90			13,67	gi|205362524	*Mytilus galloprovincialis*	69	4	7	83.35	4.85
9	75	4.5	Similar to heat shock protein 70	**2,56**			gi|91087711	*Tribolium castaneum*	100	2	4	71.15	5.55
10	75	4,8	Heat shock cognate 71	**1,72**			gi|76780612	*Mytilus galloprovincialis*				71.51	5.29
11	75	5,3		2,83									
12	73	4,2		**2,72**									
13	72	5.2				1,496							
14	71	5.55	Heat shock cognate 71		1,25		gi|76780612	*Mytilus galloprovincialis*	663	51	46	71.51	5.29
15	71	5.55	Heat shock cognate 71		1,87		gi|76780612	*Mytilus galloprovincialis*	663	51	46	71.51	5.29
16	70	5.7	Heat shock protein 70	**−7,14**	−4		gi|193999217	*Pinctada fucata*	130	6	14	71.61	5.26
17	70	6,1				9,49							
18	70	5,6				2,56							
19	56	6,3	T-complex polypeptide 1 (TCP1)	2			Q9PW76_BRARE	*Danio rerio*	252	11	16	58.40	5.96
20	56	5,8				2,08							
21	54	5,3				2,68							
22	54	5,7			dis.								
23	52	8,5				8,57							
24	48	6.2	3-oxoacid coA transferase		−1,69		gi|241118969	*Ixodes scapularis*				44.51	5.56
25	50	4.6	Protein disulfide isomerase	1,28			gi|126697420	*Haliotis discus discus*				55.49	4.56
26	51	7.6	T-complex protein 1 δ SU		−2,08		gi|170579911	*Brugia malayi*	60	2	4	57.33	6.31
27	50	6,2		2,04									
28	49	6,3		−3,23									
29	48	5.6	α tubulin	1,82			gi|56603668	*Crassostrea gigas*	92	5	13	50.78	4.97
30	48	5,9		**4,63**	5,62								
31	48	5.7	Translation elongation factor 1 α	**3,86**			gi|46909383	*Mytilus edulis*	72	2	4	35.25	6.28
32	48	4,8		1,34									
33	48	8				−7,69							
34	47	5.3			dis.								
35	47	5,7	Tektin A1	1,28		1,34	gi|211998646	*Haliotis asinina*	60	6	5	52.28	5.5
36	48	8.3	NADH ubiquinone oxidoreductase SU			5,8	gi|242001646	*Ixodes scapularis*	74	3	7	52.86	8.69
37	47	5,2	26S protease regulatory subunit 6A	1,62			gi|225712126	*Lepeophtheirus salmonis*	180	7	13	48.13	4.99
38	47	5,6	β tubulin	**1,53**			Q5FYA5_SCHHA	*Schistosoma haematobium*	171	10	19	50.13	4.79
39	45	8.5			dis.	−20							
40	45	5,9		1,58									
41	45	5,6	Gelsolin	**2,07**		1,92	gi|126697490	*Haliotis discus discus*	70	4	9	23.41	5.24
42	44	6		**1,53**									
43	44	8		**−30**		dis.							
44	44	7.5		**−20,2**									
45	44	4,6	Calumenin precursor, putative	1,29			gi|242005220	*Pediculus humanus corporis*	65	2	3	37.88	4.61
46	43	4		4,75									
47	43	6,1			−5,56								
48	42	5,3	β tubulin	**1,46**			gi|18565104	*Crassostrea gigas*	184	16	47	42.00	5.3
49	42	4	Hypothetical actin like protein	**14,87**			Q5DFP8_SCHJA	*Schistosoma japonicum*	110	6	22	40.4	5.66
50	42	7	Phosphoglycerate kinase	**1,7**			Q8WQL0_AEDAE	*Aedes aegypti*	126	3	9	44.06	7.01
51	42	5.6	β tubulin			1,31	gi|18565104	*Crassostrea gigas*	184	16	47	42.01	5.3
52	42	5,2		1,76									
53	42	4.57	Calumenin precursor, putative	2,8	2,93		gi|242005220	*Pediculus humanus corporis*	65	2	3	37.89	4.61
54	41	5.9	Actin		2,27	1,86	gi|5114428	*Mytilus galloprovincialis*	163	5	18	42.12	5.46
55	40	5,2	β tubulin	1,5			gi|159507454	*Crassostrea ariakensis*	165	20	42	42.05	5.3
56	40	6.3	Long chain acylCoA dehydrogenase	**1,81**			gi|223590148	*Homo sapiens*	103	2	6	48.05	7.28
57	42	4.65	40S ribosomal prot SA (p 40)	**dis.**		−7,69	gi|2298916	*Pinctada fucata*	78	5	17	33.7	5.24
58	39	5,6		1,6									
59	37	6,1		1,77									
60	37	5,3	Similar to human α tubulin (H2-ALPHA)	1,65			Q4R4C4_MACFA	*Macaca fascicularis*	231	5	14	50.65	4.97
61	37	5,4		1,38									
62	37	4,9			dis.	dis.							
63	35	4.3	Phosphatase 2A inhibitor			2,69	gi|226477562	*Schistosoma japonicum*	60	1	4	28.07	4.24
64	35	8	Fructose-bisphosphate aldolase	2,32		2,58	gi|46909221	*Mytilus edulis*	103	7	35	21.78	5.86
65	34	7.45	Arginine kinase		−1,82		gi|227955315	*Pholas orientalis*	76	2	3	81.95	6.66
66	33	4,8	Heavy metal-binding protein HIP		−2,78		gi|46395578	*Mytilus edulis*	165	7	45	24.39	5.09
67	33	6	Cytosolic malate dehydrogenase			1,57	Q3S892_MYTGA	*Mytilus edulis*	446	14	33	36.63	6.02
68	33	5,1				−3,13							
69	33	5,6	Pur-alpha, putative	1,37			gi|242046488	*Ixodes scapularis*	111	2	11	26.67	9.41
70	31	6.1		1,272									

Table 3 (*continued*)

n°	Mr obs.	pi obs.	Protein name	Ratio IC/SC	Ratio SX/SC	Ratio IX/SC	Accession number	Species	Score	Queries match.	% coverage	Mr th.	pI th.
71	31	9,1		−7,14									
72	31	5,1		**−6,25**	−12,5	−5,88							
73	31	5,8		1,54									
74	30	4,6	Tropomyosin	**1,52**			gi|6647862	*Mytilus galloprovincialis*	88	5	8	32.81	4.62
75	30	4,5	Tropomyosin	**2,03**			gi|6647862	*Mytilus galloprovincialis*	139	3	11	32.81	4.62
76	30	7.6	Receptor of activated kinase C 1		−1,61		gi|115501910	*Mya arenaria*	196	25	58	35.53	6.74
77	30	6.62	Ribosomal protein S2			−5,26	gi|22203717	*Chlamys farreri*	147	5	26	27.08	10.49
78	29	5.2	14-3-3 CG31196-PC, isoform C	**1,79**			gi|48096523	*Apis mellifera*	62	2	8	29.26	4.78
79	29	5,3				dis.							
80	29	4.8	Proteasome α SU			1,54	gi|41352543	*Ornithodoros moubata*	151	5	25	26.67	4.99
81	27	8,2			−2,38								
82	27	4,6		1,46									
83	27	6.3		2,07		1,861							
84	25	5.7	Fructose-bisphosphate aldolase	1,74	1,93	1,6	gi|256010152	*Glottidia species*	104	2	12	22.00	5.37
85	25	4,6				dis.							
86	25	5.9				1,773							
87	24	5,6			1,55								
88	23	6,3	Arginine/serine-rich splicing factor 2 like	1,32			gi|115843186	*Strongylocentrotus purpuratus*	52	2	17	15.62	9.63
89	21	6.1	Peptidyl-prolyl cis-trans isomerase B		1,58		gi|241171270	*Ixodes scapularis*	59	1	4	21.51	7.01
90	14	5,6		**1,52**									
91	16	5,1		**1,68**									
92	15	6.55	Destrin	1,363	1,18	1,25	Q3YAN6_MACMU	*Macaca mulatta*	60	2	7	12.27	6.55
93	14	8.5	Peptidyl-prolyl cis-trans isomerase A	−3,33	1,51		gi|1706258	*Blattela germanica*	102	7	13	18.10	9.05
94	14	6.2	Cu/Zn superoxide dismutase		1,28		gi|34481600	*Mytilus edulis*	168	9	31	16.05	5.84
95	14	5.2	Histone H4	1,53		2,225	H4_SOLST	*Solaster stimpsoni*	82	2	21	11.3	11.2
96	13	6.1	Histone H2	−5,67		−12,4	Q6WV83_MYTED	*Mytilus edulis*	83	4	20	13.78	10.69
97	13	8,3			dis.	dis.							
98	12	7,1				5,32							
99	11	6,5		1,61									
100	11	4,2				1,94							
101	18	9,2		−11,1									
102	10	5,3			2,76								

after 10 h of emersion at normal temperature (Anestis et al., 2010). Authors proposed that progressive decline of oxygen tension in body fluid could contribute to trigger HSP70 expression since they observed simultaneous activation of p38MAPK, a potential upstream regulator of HSP70 expression. Gills are more exposed but also more responsive to environmental stresses than mantle, which may explain why the induction of stress protein occurs earlier than in mantle (Letendre et al., 2011).

4.1.5. Energetic metabolism

Variations in the expression level of spots corresponding to fructose bisphosphate aldolase and phosphoglycerate kinase are observed in emersed mussels, which may be due to adjustments of energetic metabolism to face reduced oxygen tension in tissues and fluids. Two spots corresponding to fructose bisphosphate aldolase (maybe isoforms) showed clear increase of expression in intertidal mussels whatever the contamination state of animals. Fructose bisphosphate aldolase catalyzes the fourth step of glycolysis, which consists in the cleavage of fructose 1,6-bisphosphate in glyceraldehyde 3-phosphate. As regards phosphoglycerate kinase, this enzyme intervenes in the seventh step of glycolysis where it catalyzes the transfer of a phosphate group from 1,3-bisphosphoglycerate to ADP and is therefore responsible for the formation of ATP. Its expression level rose during air-exposure with high statistical significance ($p = 0.0095$) but only in clean mussels. Over-expression of these proteins in emersed mussels probably reflects the onset of fermentative metabolism, which allows to provide sufficient ATP level for the weak energy consumption occuring then.

Table 4
Eigenanalysis of the correlation matrix showing the five principal components. Data are expressed as % of the total variability.

Principal component	PC1	PC2	PC3	PC4	PC5
Eigenvalue	17,939	11,261	10,048	9029	7359
Cumulative	17,939	292	39,248	48,277	55,636

4.1.6. Gene expression

An increase in histone H4 expression level is detected simultaneously to a strong decrease in histone H2 level in emersed intertidal mussels, as well as an increase in arginine/serine rich splicing factor and pur-alpha protein, a regulator of DNA expression and replication. These variations may reflect the modulation of gene transcription in the response to air-exposure. Indeed, transcriptomic response to emersion and consequent hypometabolism consists in down-regulating the expression of a majority of genes but activating specific ones mainly involved in energetic metabolism and antioxidant defences (Larade and Storey, 2007; Letendre et al., 2011). Histones are likely to undergo methylation, acetylation and phosphorylation among other PTM, that alter their interaction with DNA and proteins, regulate factor access to genes and consequently transcriptional activity. Changes in the amount of covalently modified histones have been demonstrated in resting *C. elegans*, as well as increased histone deacetylase activity in hibernating hypometabolic squirrels (Storey and Storey, 2007). Apparent changes in expression level of histones H4 and H2 on gels may possibly be due to such PTM rather than real expression regulation. This would explain why observed molecular weight were concordant with calculated ones whereas observed isoelectric points were more acidic than theoritical ones. Basic proteins are actually difficult to detect with our method so that native forms of histones could be invisible here contrary to modified ones.

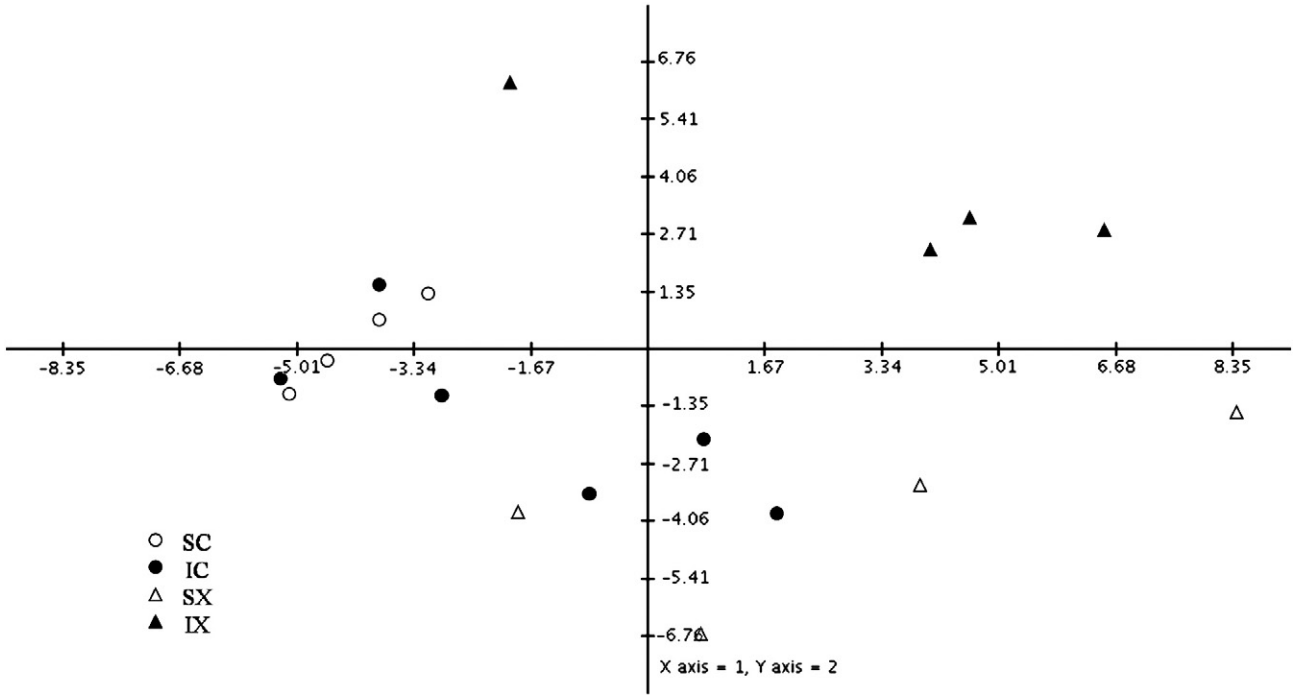


Fig. 5. PCA score plots for the two first components obtained by analysing 172 variables (spots) and 18 samples (gels) after 14 days of exposure to B[a]P and phenanthrene and tidal cycles. SC: subtidal control; IC: intertidal control; SX: subtidal exposed; IX: intertidal exposed.

4.2. Effets of PAHs exposure

4.2.1. B[a]P and phenantrene accumulation in tissues

Mussels came from a site used as a reference but exhibited all the same background PAH body burdens before the experiment. In consequence, background intoxication of animals cannot be excluded *a priori*. However, at the beginning of the experiment after 1-week acclimatisation, near-zero values were observed concerning B[a]P and phenanthrene body burdens, which enable us to assume that the observed differences in antioxidant activities and proteomic profiles were mainly due to the microcosm exposure.

Levels of PAHs contaminants in whole body of mussels were equivalent after 14 days of continuous exposure between subtidal and intertidal conditions. A slower kinetic of B[a]P accumulation is noticed in intertidal mussels in comparison to subtidal counterparts. The difference in the accumulation behaviour of B[a]P between intertidal and subtidal mussels may be due to reduced contact between intertidal mussels and seawater due to the 6 h-emersion periods. Tissues of subtidal mussels were exposed to a greater quantity of B[a]P than their intertidal counterparts during the first days of treatment and this difference could explain *a priori* some differences between their proteomic profiles. Nonetheless, it must be noticed that continuous submersion also allows to depurate more xenobiotic compounds than if emerged half of the time (Durand et al., 2002), and therefore probably to compensate in part the greater uptake.

4.2.2. Effects on cellular defences and protein expression

PAHs exert their toxic effects mainly through oxidative stress, even in bivalves which capacities of metabolisation are limited (Livingstone et al., 1990). 7 day-exposure to B[a]P generated an increase in antioxidant activity and lipid peroxiation of mussel gills (Maria and Bebianno, 2011). Besides, blue mussels exposed to a single dose of B[a]P showed in mantle higher level of lipid peroxidation in intertidal condition than in subtidal condition in spite of equivalent B[a]P burdens (Durand et al., 2002), which indicated an interactive effect of emersion–submersion cycles and of contaminant exposure on the levels of oxidative damages. In the present experiment, we observed a significant increase in GST activity exclusively in intertidal mussels exposed to the mixture of B[a]P and phenanthrene, confirming the highest degree of stress these animals underwent. More precisely, this reflected the induction of metabolising processes initiated by PAHs exposure. It has been shown that GST activity is correlated to the presence of aromatic compounds with 5 to 6 rings such as B[a]P (Gowland et al., 2002). Besides, GST possesses also a peroxidase activity so that observed induction of activity could be indirectly linked to an alteration of the redox status. In a different manner, Cu/Zn SOD activity was inhibited in exposed animals in comparison to controls when in subtidal context. Proteomic analysis showed however that Cu/Zn SOD expression level rose in subtidal contaminated mussels in comparison to controls, which contrasts with the change of activity. Such discrepancy was also observed by Hansen et al., who studied the effect of copper exposure on metalloproteins in brown trout and found a stagnation of SOD mRNA levels but a decrease of activity in the course of the treatment (Hansen et al., 2006). They invoque post-translational modifications as important regulators of SOD activity in addition to modulation of transcription. The disparity between regulation of protein expression and activity in the present work may be explained by an overexpression of Cu/Zn SOD in PAHs exposed animals but not in the active form of the enzyme. Indeed, it is synthesised as an apo-SOD that has to be activated through the addition of metallic cofactors, a process that can be dependant from a specific chaperone and from oxygen according to the organism (Galiazzo et al., 1991; Rae et al., 2001; Leitch et al., 2009). Further investigations have to be performed to elucidate this discordance, which underlines that regulation of enzymes expression is not necessarily accompanied with parallel variation of activity.

PAHs exposure also impacted expression levels of chaperone proteins since increased expression of HSC70 and peptidyl prolyl isomerases were observed in PAHs-exposed subtidal mussels. These indicate an enhancement of cytoprotective mechanisms most probably due to oxidative damages ensuing from PAHs presence in the organisms. However, a strong decrease of a HSP70 spot was noticed at the same time in a similar manner to that observed in the specific response to air exposure. Besides this stress response, expression modifications of proteins involved in energetic metabolism, signal

transduction, cellular defences, protein folding and cytoskeleton were registered in response to PAHs contamination. Effect of PAHs on bivalve proteome have been investigated in several studies dealing with sediment or water extracts (Knigge et al., 2004; Olsson et al., 2004; Manduzio et al., 2005) or individual exposure (Riva et al., 2011) that revealed alteration of such important cellular functions. However, specific actors varied according to contaminant, dose and model used. The present work did not aim at revealing markers of B[a]P and phenanthrene contamination so that apart from stress response, individual changes of protein expression induced by PAHs contamination are not discussed here. Number of regulations were noticeable though: in comparison to the effects of exposure to tidal cycles, proteomic profile was slightly affected by exposure to B[a]P and phenanthrene; moreover, modifications consisted in as many over-expressions as under-expressions. It has to be pointed out that PAHs were solubilised in water with DMSO solvent, which may have specific effects on protein expression. Authors are aware that comparison to DMSO control would enable the substraction of these effects. However, the main objective, *i.e.* understanding molecular effects of regular emersion and potential disruption of these processes by contaminants, was not compatible with such a design. We believe that this experimental feature should not prevent from bringing out interactive effects of contamination on intertidal physiology.

4.3. Interference of PAHs contamination on proteomic response to air exposure

Studying the interactive effects of PAHs exposure and intertidal condition allow to understand how chemical contamination of environment may affect physiological adjustments linked to intertidal way of life. Similarly to emersion effects in clean context, the proteomic response to air exposure in PAHs-contaminated mussels was composed of several changes of which a majority was characterized by over-expressions.

4.3.1. Maintenance of energetic adjustments and cytoskeletal rearrangement

When mussels were exposed simultaneously to intertidal condition and PAHs mixture, a weak part of the regulations observed in clean intertidal mussels in response to air exposure were conserved. For instance, over-expression of cytoskeletal proteins and regulators were observed in emersed mussels whatever the contamination state of animals, although some regulations disappeared. These suggest that cytoskeleton rearrangement as a putative functional response to emersion-induced stress was maintained in spite of chemical stress.

Likewise, fructose bisphosphate aldolase expression was also increased during emersion in both control and PAHs-exposed mussels. Hence, metabolic adjustments due to emersion seem to be conserved even if mussels undergo chemical insult. These processes are indeed crucial for energy production and maintenance of sufficient ATP levels despite hypoxia. Disruption of such process would reflect a high level of stress and probably some morbidity. In subtidal mussels, PAHs generated a decrease of arginine kinase expression that is not found in intertidal exposed ones. Arginine kinase is the equivalent of mammalian creatine kinase; both phosphorylate or dephosphorylate a phosphagen molecule allowing the formation or consumption of ATP. Hence, they regulate ATP and proton buffering and consequently metabolism and energetic transport (Ellington, 2001). These phosphagen kinases probably play important role in cells displaying high and variable rates of aerobic energy production. Besides, increased reaction velocity of arginine kinase has been demonstrated in abalone subjected to hypoxia (Shofer and Tjeerdema, 1998). The usefulness of this enzyme when oxygen is lacking during emersion may explain why its over-expression is observed in spite of PAHs toxicity. The apparent variations of expression of two histones, which are putatively linked to the regulation of gene expression, still appeared in the proteomic response to air-exposure in context of PAHs contamination. Energetic adjustments due to emersion indeed consist in the regulation of expensive mechanisms like gene expression, in addition to metabolism modulation. Maintenance of histones regulation in the face of water deprivation even when mussels were exposed to pollutant underlines the importance of the regulation of transcriptional activity during hypometabolic state. Nonetheless, changes of expression of proteins involved in gene expression (pur-alpha protein and splicing factor) occuring during emersion in clean context were not found in contaminated context.

4.3.2. Alteration of stress response

Contrary to the energetic and structural adjustments that were mostly conserved even in presence of contaminants, the pattern of stress protein expression found in intertidal mussels is dramatically altered when the latter are subjected to PAHs exposure at the same time. In particular, the stress protein induction that was clearly observed in response to air exposure in clean context disappeared in contaminated context at least among identified proteins. Yet, a strong increase in HSP90 was recorded at the same time. These are very abundant proteins that play important roles in protein conformation and activation and cell signaling, downstream to HSP70 action (Fabbri et al., 2008). Present results suggest that combination of natural and anthropic stresses lead to interactive effects on stress response that are different from the addition of those observed in response to single stress (Bierkens, 2000; Schulte, 2007). Addition of stresses could have interferent effects that nullify each other but stress response seems to be proportional to the degree of insult until a threshold above which it stagnates or even decreases, probably because of the energetic cost (Olsson, 2005). Thus, the dramatic loss of HSP70 induction suggests that the insult due to multiple stresses may represent a too high challenge for mussels.

4.3.3. Interactive effects of tidal regime and chemical stress on proteomic profiles

The Principal Components Analysis of the data brought supplementary insights on the global impact of the different conditions tested (Fig. 5), although the total variance that principal components explained was low (Table 2). Plots representing subtidal control samples are remarkably close to each others in comparison to other plot groups, which probably reflects a homogeneity of proteomic profiles when no adverse conditions are encountered. This does not occur anymore when mussels are subjected to stress. The second component clearly separates intertidal exposed (SX) from subtidal exposed (IX), which suggests that PC2 would be correlated to tidal conditions. However, intertidal and subtidal controls are not discriminated at all by this component. The separation of intertidal condition from subtidal condition only in contaminated context could indicate that intertidal condition generated changes that were more pronounced when mussels were exposed to pollutants at the same time. These observations are similar to those made by Masson, who studied the effects of intertidal condition and organic contamination on *Mytilus edulis* transcriptome (Masson, 2008). IX plots, which represent animals subjected to both chemical and tidal stresses, are the most negatively correlated to second principal component and relatively isolated from other plot groups on graph, suggesting a synergistic effect of these two kinds of stresses.

5. Conclusion

Present results showed clearly the interference of the cellular response to pollutants and the mechanisms linked to intertidal physiology. Emersion triggered adjustments of cellular skeleton, metabolism and chaperoning processes mainly; those were mostly maintained when mussels underwent environmental contamination except for stress response which was dramatically altered. These findings reflect the impact of chemical insult in a context that is

already stressful and energy-consuming. Ecophysiological conditions of organisms is a priority aspect to take into account in the assessment of environmental stresses. It allows not only to avoid confounding factors, but also to better understand the impact of contaminants on physiological parameters and to consider the multiplicity of stresses that wild organisms are likely to undergo.

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