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Effect of hypoosmotic stress by low salinity acclimation of Mediterranean mussels *Mytilus galloprovincialis* on biological parameters used for pollution assessment

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ABSTRACT

In the present study, we investigated the progressive acclimation of the mussel *Mytilus galloprovincialis* to different reduced seawater (SW) salinities and its effect on several biochemical markers and biotests. Mussels were purchased from a local mariculture facility during summer (SW temperature 27 °C, salinity 37.5 psu) and winter (13 °C, 37 psu) seasons, and transferred to the laboratory for acclimation to reduced SW salinities (37, 28, 18.5 and 11 psu). At the beginning and at the end of acclimation processes tests of mussel survival in air were provided. After 14 days of acclimation the DNA integrity, p38-MAPK activation, metallothionein induction, oxygen consumption rate, and condition index were measured. Survival in air (SOS test), as a physiological index of mussel's health and vitality, had significantly lower LT50 values (11 psu) in the summer than in the winter, and it seems to be negatively affected by acclimation in comparison to controls (37 psu and mariculture). Condition indexes (CIs) were not significantly different, but mussel's acclimation resulted in decline (i.e., a negative trend), especially of CI-2 and CI-3 calculated on the basis of mussel tissue weight and shell sizes. Oxygen consumption rate (V_{O_2}) of *M. galloprovincialis* acclimated to reduced salinities was a concentration-dependent process and increased considerably to about 51 and 65% in lower SW concentrations (28 and 18 psu) compared to control mussels (37 psu). DNA integrity, determined by Fast Micromethod®, was negatively impacted by salinity acclimation and corresponding physiological stress as well. Some differences in 1D protein expression patterns between control groups and mussels acclimated to 28, 18.5 and 11 psu (SW) were established. Reduced SW salinities (18.5 and 11 psu) resulted in significantly higher p38-MAPK phosphorylation, whereas the SW salinity of 28 psu decreased p-p38 significantly compared to control (37 psu). The concentration of metallothioneins in mussels' gills was reduced at 28 and 18.5 psu, while it was significantly higher at 11 psu. Results indicated that SW salinity variation (i.e., hypoosmotic stress) in the marine environment can affect all investigated parameters. This investigation expands our understanding of multifactorial effects of the physical marine environment on the specificity of investigated biomarkers and biotests, providing insight into the acclimation, adaptive and stress response processes of mussels. Effects of environmental factors have to be considered in sampling strategies for monitoring programmes to prevent false interpretation of results.

1. Introduction

The mussel *Mytilus galloprovincialis* has been widely used as a sentinel organism for pollution assessment in coastal areas and for biomonitoring of marine environments (Viarengo and Canesi, 1991; Pavičić et al., 1993; Cajaraville et al., 2000; Petrović et al., 2001, 2004; Jakšić et al., 2005). Systematic monitoring was launched with the "Mussel Watch" program in the US in the 1970s (Goldberg,

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1975). More recently, mussels have been the subject of several European research programmes, for example, MEDPOL (Gabrieldes, 1997) and BIOMAR (Narbonne et al., 1999).

Laboratory studies have shown the potential of using different biochemical responses/processes such as the induction of heat shock protein and metallothionein synthesis, and DNA integrity, as biomarkers and biotests for environmental studies (Pavičić et al., 1987; Bierkens et al., 1998; Viarengo et al., 1999a; Schröder et al., 2000; Bihari et al., 2002; Jakšić and Batel, 2003). However, field studies have shown a complexity of environmental impacts to marine organisms. In marine organisms some biomarkers have important cellular functions under "normal" and "stressful" states in the course of normal physiological adaptation to changing environmental conditions during daily and seasonal variations (Fader et al., 1994; Hamer et al., 2004; Luedeking and Koehler, 2004; Ivanković et al., 2005). Some disadvantages of using mussels, such as *Mytilus* spp., as indicators of pollution include our relative lack of knowledge of basic bivalve physiology. A better understanding of how mussels respond to complex mixtures of environmental parameters (Moore, 1985), with several of these parameters also exhibiting seasonal fluctuations (Viarengo et al., 1991; Vidal et al., 2002; Geffard et al., 2005), is required.

The main difficulty of using biomarkers in a monitoring programme is the interference of natural environmental factors, together with pollution, in the biological responses of monitored organisms. When external conditions are closely controlled, biological responses can be accurately assessed and related to exposure events (McCarthy and Shugart, 1990). In such cases, the use of biomarkers to monitor the health of the environment has been proposed.

At the Working Group on Biological Effects of Contaminants (WGBEC) ICES Meeting (1999), there was a discussion regarding the effects of changing salinity on biomarker responses used in monitoring programmes. Monitoring programmes often involve organisms from environments such as estuarine and intertidal zones, where salinity can greatly fluctuate or a natural salinity gradient is present (Monserrat et al., 2007; Prevodnik et al., 2007). Consequently, biomarkers that are used in biomonitoring programmes must be able to distinguish between the impact of environment factors and anthropogenic contaminants in marine organisms (Hylland, 2006). Therefore the WGBEC suggested the use of hydrographic data (temperature, salinity, etc.) for the interpretation of biological effects in an integrated monitoring programme to assess ecosystem health (water, sediment, biota). Unlike chemical monitoring, biomarker responses reflect the actual physiological status of the organism monitored at the molecular, cellular and individual levels of biological organisation.

Seawater salinity changes and thermal stress have been considered the most important determinants (stressors) of organisms distribution in rocky intertidal zones (Helmuth and Hofmann, 2001; Anestis et al., 2007). While salinity is relatively constant in the open Adriatic sea (36.96 ± 0.77 psu), in intertidal zones and estuaries, close to under-sea fresh water springs and during rainy days in closed lagoons, salinity can vary significantly from 4 to 38 psu (unpublished data of the monitoring programme "Project Adriatic"). *M. galloprovincialis* occurring in the intertidal zone of the Adriatic coast in Croatia are also exposed to a wide range of environmental temperatures, from approximately 10 °C water temperature (0 °C air temperature) in the winter to 27 °C (35 °C) during the summer. Due to the physical characteristics of the seawater, the marine environment can be extremely stressful to its inhabitants. Environmental stress, through extreme temperatures and salinities, including their variations, may affect abundance of species and their fitness, hence affecting biodiversity at all levels including the levels of genes, genomes, individuals, populations, species, communities and biota in general (Nevo, 2001; Parsons, 2005). Each organism and any species has the capacity of adaptation, based on regulating processes, and once stress and/or toxic exposure passes the threshold of toxicity, irreversible attacks can lead to a pathological state, which results in a significant deterioration of the individual's performance and later can lead to the organism's death (Manduzio et al., 2005).

Therefore, the purpose of this study was to test the impact of experimental salinity stress (i.e., acclimation to different salinities) on commonly used biomarkers for environmental pollution assessment. In the present work we investigated the acclimation of the mussel *M. galloprovincialis* to different SW salinities (37, 28, 18.5 and 11 psu), at high and low seawater temperatures and its effect on mussel survival in air (stress on stress), DNA integrity, changes in 1D profile (pattern) of total expressed proteins, metallothionein levels, mitogen-activated protein kinase (p38-MAPK) phosphorylation, oxygen consumption and condition index. Data obtained from this study will provide a reference for future biomonitoring studies and will contribute to studies of waste management and marine environmental protection, such as the "Project Adriatic".

2. Material and methods

2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma–Aldrich, if not otherwise stated.

2.2. Test organisms

The Mediterranean mussels *M. galloprovincialis* Lamarck, 1819 (Mollusca: Bivalvia) were obtained from a mariculture facility (Limski kanal, Northern Adriatic, Croatia) and transferred to the laboratory for the acclimation experiment. Mussels of the genus *Mytilus* are one of the most common marine molluscs, and thus, are significant components of coastal ecosystems (bioremediation) and are important for human food consumption.

2.3. Hypoosmotic stress—mussel acclimation to lowered SW salinity

The collected mussels (size 50–60 mm) were divided into 4 groups (40 specimens per group) and kept separately in well-aerated aquaria (40 L) during 14 days of progressive seawater (SW) salinity acclimation. The first group was kept in 100% SW (37 psu) and used as controls. The second group was finally subjected to 75% SW (28 psu), the third group to 50% SW (18.5 psu) and the last group to 30% SW (11 psu). SW was exchanged in aquaria once a day, and salinity was reduced each day as shown in Fig. 1. When SW in aquaria reached the final acclimation salinity for the group, salinity was maintained constant until the end of the acclimation period. During acclimation, salinity, pH, temperature, oxygen saturation and mortality were measured each day just before the seawater exchange using a WTW Multimeter P4. The pH, temperature and oxygen saturation remained constant during the acclimation treatment. At the beginning (field control) and at the end of the exposure period five mussels (or more depending on mortality) of each adaptation group, as well as from the control group (37 psu), were dissected and the gills removed, immediately frozen, and stored at −80 °C until analysed. Mussel acclimation experiments were performed as duplicates in both summer and winter periods.

For measurement of oxygen consumption during the acclimation study, mussels of 2.5 cm size were used because of limitations

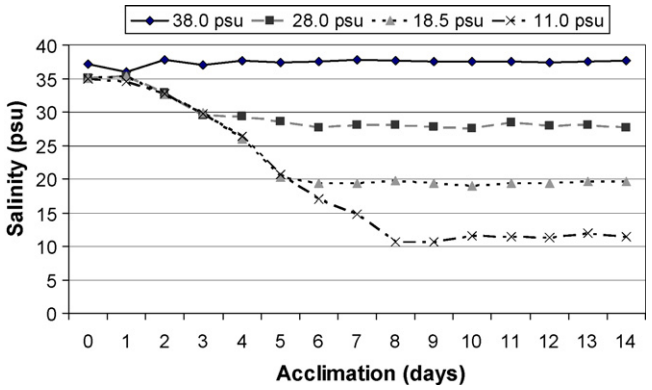

Fig. 1. Diagram of reduced salinity treatment of mussel during the summer (August) acclimation. Similar acclimation was also performed during the winter (March) period.

in chamber size, and acclimation was performed only during the winter period separately from the other experiments.

2.4. Stress on stress test (SOS test)

SOS test was performed on 30 mussels placed in a box with top cover (100% humidity) at a constant room temperature (air-conditioned) of 27 °C in the summer and 13 °C in the winter period. Boxes were checked daily for mortalities, and mussels were considered dead when the valves gaped and did not close when manipulated. The number of dead specimens was recorded in order to calculate the LT50 (median lethal time for 50% of sampled animals). LT50 was calculated using the Toxicologist regression program PROBIT ver. 1.0 according Buhagiar and Abel (1990). A confidence (fiducial) limit of 95% was used to test for significant differences between groups. Mussels were tested for LT50 values immediately after they had been obtained from the mariculture facility and after their acclimation to different salinities (37, 28, 18.5 and 11 psu).

2.5. Condition index

A variety of condition indices (CIs) have been used to measure the well-being/nutritional status of bivalves according to Lundebye et al. (1997). Three of them were used in the present study:

CI-1: Soft tissue wet weight (g) × 1000/shell weight (g).
CI-2: Soft tissue wet weight (g) × 1000/(length × width/height (mm)).
CI-3: Soft tissue wet weight (g) × 1000/(shell length (mm)).

2.6. Oxygen consumption

The oxygen consumption rates of mussels (2.5 cm size) were measured in a closed experimental system in the Perspex chamber according to Lucu and Pavičić (1995). The chamber contained 50 ml of filtered seawater and measurement was performed at a constant room temperature of 20 °C. Oxygen consumption rate (V_{O_2}) was measured after the medium was fully air saturated, and recorded by a digital PHM 72 Radiometer O_2 analyzer (Copenhagen). The top of the PO_2 electrode (E-5046, Radiometer, Copenhagen) extended through the cover into the chamber. The chamber was provided with a magnetic stir bar to maintain a uniform O_2 tension.

Individual mussels were placed in the chamber filled with the experimental solution until full saturation was attained. After closing the chamber, and after visual observation that mussels opened their shells, the decrease of the oxygen tension in the chamber was taken as a measurement of mussel's tissue respiration. Measurements stopped when oxygen saturation in the medium fell below 10% of the maximum saturation level. All corrections of oxygen solubility coefficients (actual barometric pressure and temperature) and calculations of V_{O_2} were done as previously described (Lucu and Pavičić, 1995). The V_{O_2} was expressed as μl O_2 h^{-1} per gram of mussel soft tissue (ww).

2.7. DNA integrity determination (Fast Micromethod®)

Mussel gills (100 mg) were homogenized with 2 ml of 10% DMSO solution in TE buffer, pH 7.4 (1 mM EDTA, 10 mM Tris–HCl) under liquid nitrogen in a mortar with a pre-cooled pestle. The pellets were collected in test tubes and stored at −80 °C. The amount of DNA in dissolved sample aliquots was determined using a YOYO fluorochrome dye (Molecular Probes). Sample homogenates were diluted with 10% DMSO in TE buffer, until desired concentrations (100 ng/ml DNA) were obtained.

DNA integrity was analysed within 3 min by the Fast Micromethod® applied according to Batel et al. (1999). The method is based on the ability of the specific fluorochrome dye PicoGreen (Molecular Probes) to make a very stable complex with dsDNA (Cosa et al., 2001) in highly alkaline conditions instead of ssDNA. The gill homogenates were lysed first, upon which the interaction between dye and DNA occurred. NaOH solution was added initiating the alkaline denaturing of dsDNA, while at the same time the reduction of the dsDNA-PicoGreen complex was followed by fluorometry. DNA denaturing was followed directly, for 20 min, in the microplate by measuring fluorescence (excitation 485 nm/emission 520 nm) using a Fluoroscan Ascent (Labsystems, Finland). Results were calculated after 5 min of denaturing and expressed as strand scission factors (SSFs) as a value for DNA integrity.

2.8. SDS-PAGE analysis of total mussel gill proteins

One-dimensional (1D) gill protein analysis was performed by discontinuous polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) with slight modifications (Hamer et al., 2004, 2005a). Mussel gills (100 mg wet weight) were homogenized in 0.1 ml buffer (0.5 M NaCl, 0.1 M Tris, 0.01 M EDTA, pH 7.5) containing 10 μl 40 mM phenylmethyl-sulphonylfluoride (PMSF) in acetone. Additional homogenization was done after dilution with 0.3 ml of the same buffer and after centrifugation (12,000 × g/4 °C/10 min) the supernatants were stored at −20 °C. Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard.

For analyses of protein expression patterns after acclimation, 20 μg of total mussel gill protein (n = 5) was applied together with a loading buffer to a 10% SDS-PAGE gel. Samples of all acclimation groups were run separately in the same 10% gel. Gels were washed in distilled water, and stained with GelCode® Blue Reagent (Roth, Karlsruhe, Germany) overnight. After washing (3 × 30 min), gels were scanned using an Odyssey laser scanner and software (LI-COR Corp., USA) at 700 and 800 nm (300 dpi). Obtained TIFF files were analysed with the Odyssey software (LI-COR Biosciences, USA) and TotalLab program TL120 (Nonlinear Dynamics, USA). By scanning the total band-lane areas, which minimizes errors in estimations, band quantifications were performed. Molecular weights were determined using Bio-Rad broad range standards. The results were normalized according to an applied MW marker (carbonic anhydrase, 29 kDa) as 100%. A similarity analysis from the acclimation expression protein profiles was performed using an UDGMA algorithm.

2.9. p38-MAPK phosphorylation determination

A total of 20 μg of mussel gill protein was used for the SDS-PAGE separation on a 12% gel. The number of mussels/samples per acclimation group was three ($n = 3$). Western blot analysis was performed as previously described (Böhm et al., 2000). After transfer, membranes were incubated with a rabbit anti-human phosphorylated p38 (p-p38 Thr180/Tyr182; Santa Cruz Biotechnology) as the primary antibody. Following incubation and washing steps, membranes were incubated with a goat anti-rabbit IgG Alkaline Phosphatase (Sigma). Detection of the immuno-complex was carried out using a solution of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). After visualization, membranes were scanned and band intensity, which is relative to the amount of protein expression, was quantified as relative optical density (ROD; pixels/mm^2) using the program Odyssey ver. 1.2. Results of p38-MAPK phosphorylation (whole bands) were normalized according to control mussels acclimated to 37 psu SW as 100%. The molecular weight of the detected p-p38 band was determined using a MW Sigma Marker.

2.10. Metallothionein quantification

Metallothioneins (MT) were determined in cytosolic fraction (50,000 × g supernatant) of gills homogenized in three volumes of 0.02 M Tris–HCl buffer, pH 8.6, containing sucrose (0.5 M), leupeptine (0.006 mM), PMSF (0.5 mM) and β-mercaptoethanol (0.01%), on an ice-bath with a Potter–Elverhjem homogenizer. The homogenate was further placed in organic solvent (ethanol/chloroform) for precipitation prior to the spectrophotometric quantification of SH groups against reduced glutathione standard (GSH), as previously described by Viarengo et al. (1997). For comparison, MT concentrations were expressed as wet mass tissue (μg MT g^{-1} ww) as well as per total cytosolic proteins (μg MT mg^{-1} proteins) determined using Commassie Brilliant Blue reagent (Bradford, 1976).

2.11. Statistical analyses

The results of the mussel acclimation experiments are given as mean ± S.D. of 5 gill samples, if not differently noted. The measured values of CIs, DNA integrity (SSF), p-p38-MAPK, metallothioneins and oxygen consumption were compared among different acclimated group using an Analysis of Variance (ANOVA) followed by a Tukey post hoc test. Statistical significance was accepted at a $P < 0.05$ throughout (*). A two-way ANOVA was applied in order to evaluate main and interactive effect of salinity and temperature on the modulation of MT levels. A principal component analysis based on a correlation matrix was applied to assess the association among salinities and the mussels' responses at subcellular (MT, total cytosolic proteins, p38-MAPK, DNA damage) and whole organism's levels (CI, SOS survival, cumulative mortality).

3. Results

In order to elucidate the effect of salinity changes (prolonged hyposaline stress) on different biomarkers, biotests and physiological indicators used for monitoring environmental pollution, the marine mussel *M. galloprovincialis* was used as an object of investigation and its gills as the target tissue.

3.1. Mortality during acclimation

During both acclimation experiments at high/low seawater temperature (27/13 °C) an increased mortality of mussels subjected to the lowest SW salinity (11 psu) was observed, especially during the summer period (Fig. 2). Acclimation of small (2.5 cm) mussels for oxygen consumption measurement during the winter period (15 °C SW temperature) showed a strong effect with a 95% mortality of mussels subjected to the lowest acclimation salinity (11 psu, 30% SW) (data not shown).

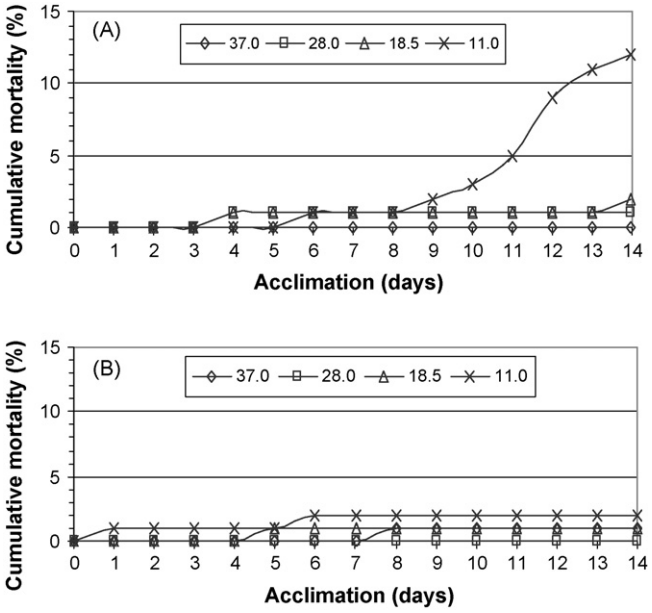

Fig. 2. Effect of hypoosmotic stress on the mortality of mussel *M. galloprovincialis* during the 14-day acclimation period to different seawater salinities (37–11 psu). (A) Summer (August) and (B) winter (March).

3.2. Stress on stress test

SOS test, i.e., survival in air as a physiological index of mussel's health and vitality, after acclimation to the first three SW concentrations did not change the LT50 values significantly: Lim 5.74 (5.45–6.02), SW 37 psu 5.32 (5.01–5.62), SW 28 psu 4.26 (fiducial limits too wide), SW 18.5 psu 5.37 (4.55–6.16). However, the 30% SW (11 psu) had significantly lower LT50 values: 2.76 (95% fiducial limits for LT50 from 2.42 to 3.08). There was also a strong effect of mussel acclimation to SW 11 and 28 psu compared to control (37 psu) during the summer period (Fig. 3A).

During the winter period, mussel acclimation did not significantly affect the SOS survival time (LT50) (Fig. 3B), but after an additional abrupt exposure to SW of 37 psu we observed a strong increase in the mortality of mussels acclimated to 18.5 and 11 psu SW (data not shown).

3.3. Condition index

The condition index did not significantly vary with salinity acclimation (37, 28, 18.5 and 11 psu), but almost all applied condition indexes declined with increased hypoosmotic stress (Fig. 4). CIs expressed as percentage of control mussel group (100% SW, 37 psu): CI-1 (100, 98.0, 97.1, 93.0%), CI-2 (100, 139.0, 65.3, 61.0%) and CI-3 (100.0, 113.1, 81.5, 76.4%) showed a strong effect of acclimation.

3.4. Oxygen consumptions

The V_{O_2} of mussels acclimated to reduced SW salinities was a concentration-dependent process and increased considerably in lower salinity concentrations (28 and 18 psu) compared to that

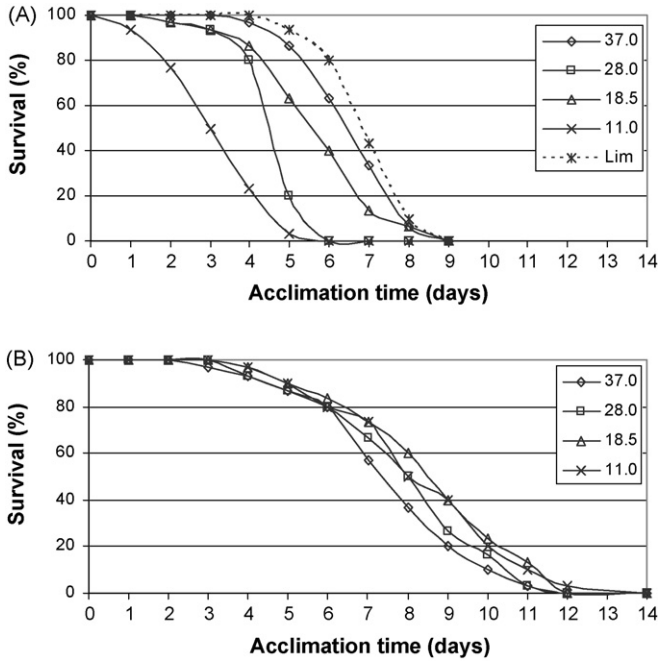

Fig. 3. The SOS test survival time of mussels after acclimation to different SW salinities: Lim mariculture, 37, 28, 18.5 and 11 psu. (A) Summer and (B) winter period.

measured in normal SW salinity for the Adriatic Sea (37 psu). The V_{O_2} of mussels was found to increase after acclimation to 28 and 18 psu SW by 51 and 65%, respectively, compared to V_{O_2} measured in control mussels (37 psu) (Fig. 5).

3.5. DNA integrity

Our results showed a significantly lower DNA integrity status in gill homogenates from mussels acclimated to salinities of 18.5 and 11 psu (50 and 30%, respectively) than control ones in both experimental campaigns (Fig. 6). Furthermore, the DNA integrity from mussels sampled in the winter period showed a significantly

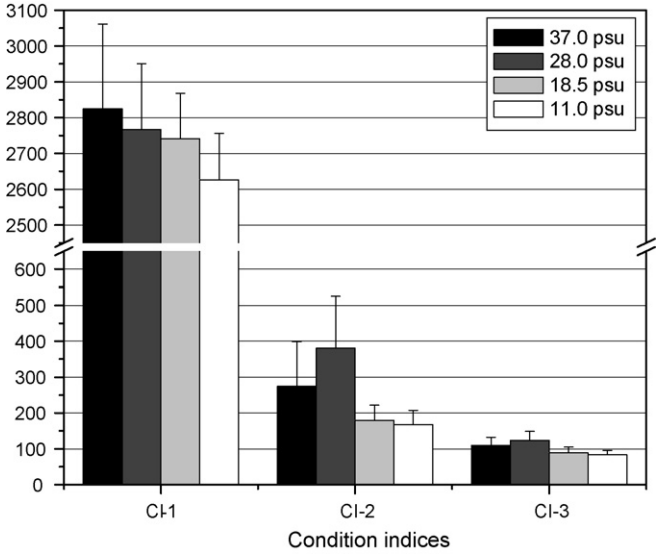

Fig. 4. Effect of salinity stress (37, 28, 18.5 and 11 psu) on condition indices of mussel *M. galloprovincialis* calculated on the basis soft tissue wet weight (g) × 1000 divided by: (CI-1) shell weight (g), (CI-2) length × width/height (mm) and (CI-3) shell length (mm). Data represent means ± S.D. obtained from five mussels.

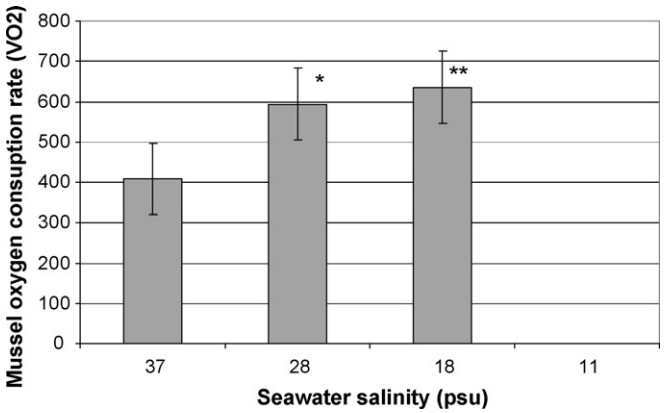

Fig. 5. Average oxygen consumption rate (V_{O_2}), which was measured during 60 min and expressed as $\mu l\, O_2\, h^{-1}$ per gram of mussel soft tissue (ww). Prior to measurements, mussels were acclimated to 37, 28, and 18 psu SW salinities for 2 weeks. We did not measured oxygen consumption rate of mussels acclimated to 11 psu salinity because of pronounced mortality. Data represent means ± S.D. obtained from five mussels. Statistically significant differences between respective acclimation groups and 37 psu SW are indicated by asterisks ($^{*}P < 0.05$; $^{**}P < 0.01$).

lower DNA integrity status than those sampled during the summer season (control 37 psu).

3.6. SDS PAGE analysis of total mussel gill proteins

Total gill protein analyses were performed only after summer acclimation to different salinities [37 psu (100% SW), 28.5 psu (75% SW), 18.5 (50% SW) and 11 psu (30% SW)]. The acclimation of mussels to different salinities affects 1D protein profile (pattern) of expressed proteins (bands and their intensities), as indicated by the similarity dendrogram and dashed lines in Fig. 7.

3.7. p38-MAPK activation

p38 analyses of mussels acclimated to different reduced SW salinities (18.5 and 11 psu) showed significantly increased p38-MAPK phosphorylation, whereas mussels acclimated to the SW salinity of 28 psu had a significantly lowered expression of the phosphorylated p38 (Fig. 8). The detected p-p38-MAPK band had the expected size of 42 kDa.

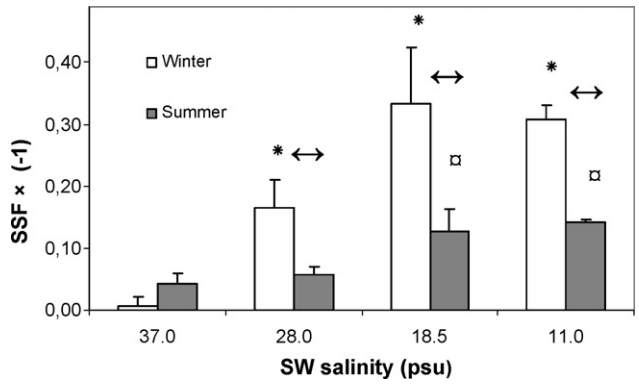

Fig. 6. The negative DNA integrity SSF × (−1) values measured in gill homogenates of mussels acclimated to different SW salinities during high (summer) and low (winter) seawater temperatures. The results are presented in mean values of 4 or 5 gills (each derived from a single mussel) samples with the corresponding standard deviations. Statistically significant differences ($P < 0.05$) between mussels subjected to SW of 37 psu and lower salinities in the winter (*) and summer (¤), and between mussels acclimated to same salinity in winter and summer (↔) season are indicated.

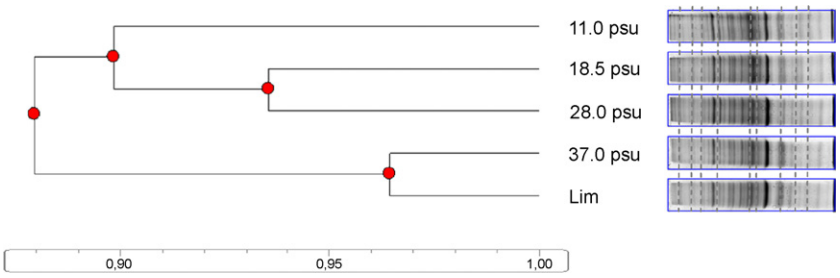

Fig. 7. Similarity analysis of total mussel gills proteins after 1D SDS-PAGE separation: Lim mariculture, mussels subjected finally to 37, 28, 18.5, and 11 psu SW salinity during a 14-day acclimation in summer. Differently expressed proteins are indicated by dashed lines.

3.8. Metallothionein induction

The variations in MT concentration in mussels that were experimentally subjected to natural (37 psu) and diluted seawater (28, 18.5 and 11 psu) were compared (Fig. 9), in order to study the relevance of MT in compensatory adjustments of mussels to hyposaline stress under two seasonal temperatures (13 and 27 °C). MT level was estimated on the basis of tissue wet weight (ww) (Fig. 9A) and total cytosolic proteins (Fig. 9B).

In general, MT (ww) levels in the summer tended to be consistently depressed in comparison with corresponding groups acclimated during the winter period, except for mussels subjected to the lowest salinity (11 psu), which displayed similar MT levels in both seasons. By comparing graphs presented in Fig. 9 the seasonal temperature effect was more pronounced if MT would be estimated on a tissue wet weight (Fig. 9A) than on a cytosolic protein basis (Fig. 9B) even though both generally displayed similar salinity-mediated MT fluctuation patterns. Hyposalinity-affected variation of MT (ww) levels was more pronounced in the summer than in the winter as indicated by the respective F values, 17.4 and 10.4 (one-way ANOVA, $P < 0.05$). Following a progressive MT decline in 37 and 18.5 psu, a significant elevation was recorded at 11 psu, the MT level being 1.3-fold that of control (37 psu; $P < 0.05$) after summer acclimation.

In spite of the fact that winter MT levels in gills of mussels acclimated to 11 psu were only slightly above those from controls (37 psu), the statistical significance of an overall salinity effect was assessed ($P < 0.05$) taking into account data from both seasons. Mussels acclimated in 18.5 psu were more significantly affected in an opposite direction showing a 1.5-fold decline of MT (ww) with respect to 37 psu ($P < 0.01$).

As presented in Table 1, a two-way ANOVA was performed separately for MT content normalized for, tissue wet weight and cytosolic protein basis in order to evaluate the importance of the main salinity and seasonal effect as well as their interac-

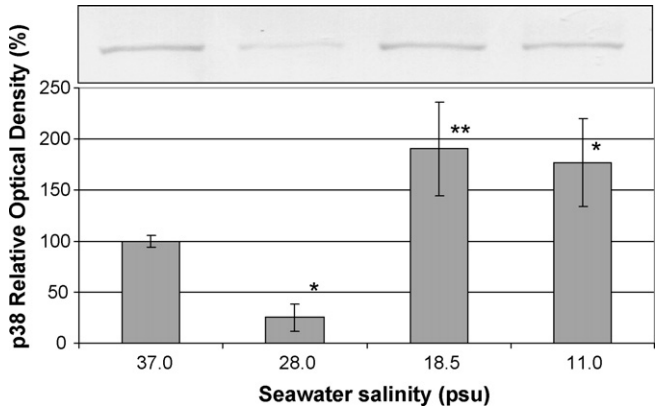

Fig. 8. Phosphorylation levels of p38-MAPK as a result of hypoosmotic stress of mussel acclimated to different lowered SW salinities. A representative immunoblot after SDS-PAGE separation is shown on the top of the graph. Relative optical densities (ROD) were measured using whole detected band area and expressed in percentage using ROD intensities of control mussel (37 psu) as 100%. Statistically significant differences between respective acclimation groups and 37 psu SW are indicated by asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$).

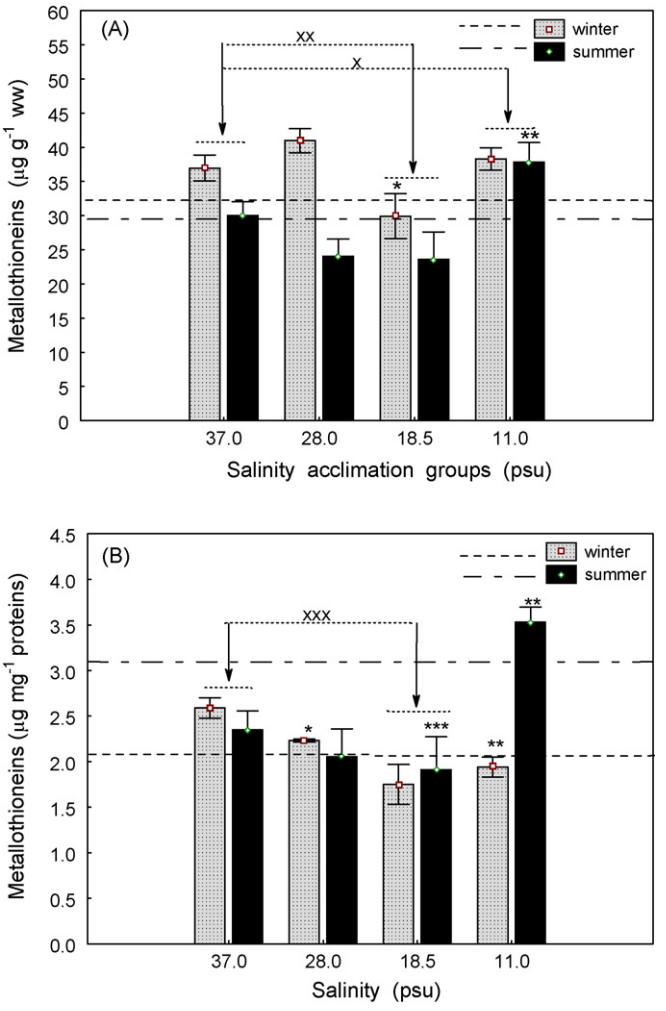

Fig. 9. Effect of lowered salinity (37, 28, 18.5 and 11 psu) on metallothionein levels (mean ± S.D.; $n = 6$) determined in gills of mussels after 14 days of acclimation at two seasonal temperatures (winter and summer). MT level was estimated (A) on a tissue wet weight and (B) on total cytosolic protein basis. Basal MT level recorded in mussels prior to laboratory acclimation for each season is indicated by dashed horizontal lines; winter (- - -) and summer (– – –). Statistically significant difference (Tukey HSD, post hoc comparison) for each season ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) as well as overall salinity effect ($^{X}P < 0.05$; $^{XX}P < 0.0$; $^{XXX}P < 0.001$) on MT between respective acclimation group and 37 psu salinity is indicated.

Table 1
Summarized results of two-factors analysis of variance (ANOVA/ANCOVA; crossed model) for metallothionein concentrations expressed on (A) tissue wet weight basis, (B) on cytosolic protein basis in gills of *M. galloprovincialis* after 14 days acclimation to natural (37 psu) and diluted seawater (28, 18.5 and 11 psu) during winter and summer season

	(A) MT ($\mu g\,g^{-1}$ tissue ww)[a]				(B) MT ($\mu g\,mg^{-1}$ proteins)[a]			
	d.f.	M.S.	*F*	*P*	d.f.	M.S.	*F*	*P*
ANOVA								
Salinity	4	202.0	14.4	<0.000	4	1.6	17.0	<0.000
Season	1	669.3	47.8	<0.000	1	3.3	35.1	<0.000
Season × salinity	4	119.6	8.5	<0.000	4	1.8	19.2	<0.000
ANCOVA								
Salinity	3	193.1	13.6	<0.000				
Season	1	27.9	2.08	0.155				
Season × salinity	3	119.7	8.4	<0.000				
Proteins (covariate)	1	1.7	0.12	0.729				

For evaluation of the effect of salinity, season and their interaction (salinity × season) data related to basal MT level recorded in mussels prior to acclimations were taken into account (d.f., degree of freedom; M.S., mean square; *F*, ratio; *P*, significance level).
[a] Main effect.

tions (season × salinity), taking into account data sets from both seasons.

The marked seasonality of MT observed in Fig. 9A is consistent with the prevalence of the main seasonal effect over salinity as demonstrated by the respective *F*-values 47.8 and 14.4 ($P < 0.0001$) with MT expressed on a tissue wet weight basis. The interaction effect (season × salinity) was less pronounced ($F = 8.5$) being most significant in the 28 psu salinity where the largest difference between winter and summer MT (ww) levels were observed.

When MT was expressed on a cytosolic protein basis (Fig. 9B) the main seasonal effect was considerably reduced ($F = 35.1$), although salinity ($F = 17.0$) and interaction effects ($F = 24.1$) were more pronounced in comparison with corresponding effects assessed in the MT (ww) data. Furthermore, post hoc comparisons of MT between single acclimation groups for both seasons showed that the statistical effect of the overall salinity was only significant between the MT levels at 18.5 and 37 psu ($P < 0.001$).

Contrary to estimations on a tissue wet weight basis, MT levels on a protein basis in mussels acclimated to 11 psu were not significantly different from 37 psu, reflecting, presumably, seasonal variations in the general protein metabolism modulated by hyposaline acclimation. Results presented in Fig. 10A displaying distinct seasonal salinity-related dependence of cytosolic proteins, including levels prior and after acclimation to low salinity, clearly indicated that cytosolic proteins were more strongly affected by low salinity in the winter (41.3) than in the summer (15.5), as assessed by the respective *F*-ratios attributable to fitted regression lines. Consequently, a 1.85-fold higher level of cytosolic proteins between winter and summer acclimations recorded in mussel gills subjected to 11 psu salinity would be the result of a marked underestimation of winter MT levels as shown in Fig. 9B. Therefore, in spite of the higher MT in the summer, the underestimated level in the winter necessarily contributes to the less pronounced difference in the overall salinity effect between the 11 and 37 psu salinity acclimation groups than if MT would be expressed on a protein basis.

The marked reduction of the MT (ww) seasonality ($F = 2.1$) has been obtained when using an Analysis of Covariance (ANCOVA), taking into account levels of total cytosolic proteins as a covariate. The overall salinity effect retained high significance ($F = 13.6$) and interaction effects ($F = 8.4$), which was also comparable with the MT data normalized on a tissue wet weight basis. By comparing salinity-mediated fluctuations of MT (ww) levels for each season, separately, with the overall salinity effect as shown by ANCOVA (Fig. 10B), it may be observed that by removing the part of MT seasonality evidently attributable to seasonal variations in cytosolic

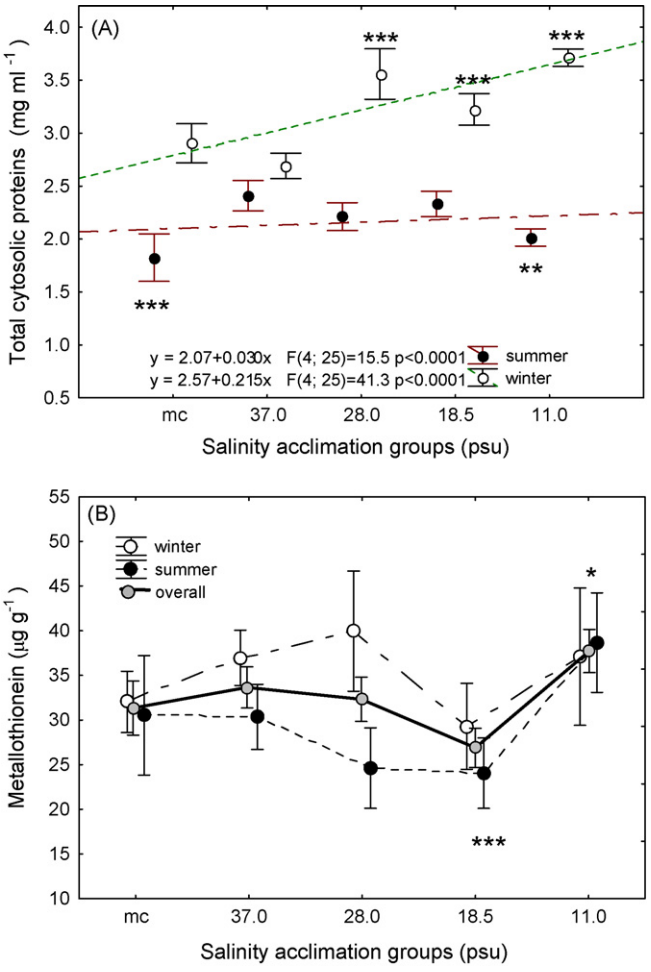

Fig. 10. (A) Seasonal dependence of total cytosolic proteins (means ± S.D., $n = 6$) in gills of mussels acclimated to progressive reduced salinities during 14 days in winter and summer period. Cytosolic proteins were determined prior to laboratory acclimation (denoted as "mc", mariculture) and in four groups of mussels ranged between 37 and 11 psu post-acclimation. Evaluation of salinity effects for winter and summer acclimations [one-way ANOVA, $F(4; 25)$, $P < 0.0001$]. (B) Evaluation of the overall salinity and interaction effects (season × salinity) on MT level (LS means ± 0.95 CI, $n = 6$) by ANCOVA taking into account total cytosolic proteins as a covariate.

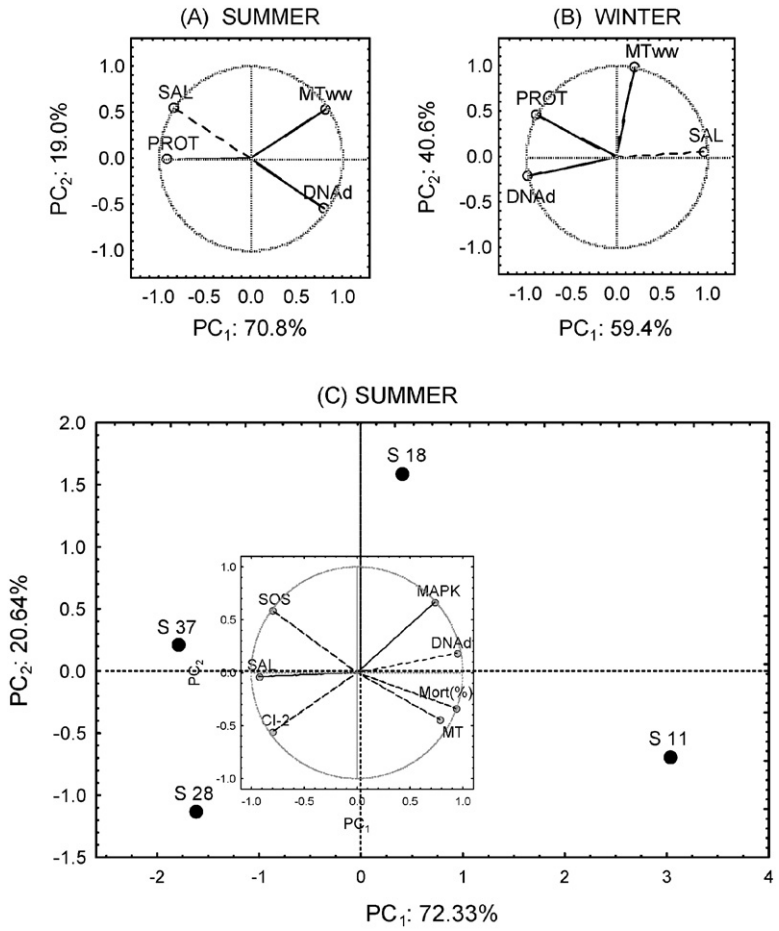

Fig. 11. Comparison of the seasonal PCA variable association patterns defined by four parameters (salinity, SAL; metallothionein, MTww; DNA damage, DNAd and PROT, total cytosolic proteins) for (A) summer and (B) winter acclimations. (C) PCA defined by seven parameters, including also p38-MAPK, condition index-CI-2, SOS survival time and cumulative mortality (Mort%) determined only for the summer acclimation. The variable coordination circle is presented jointly with the complementary cases analysis showing distribution of salinity acclimation groups (S 37, S 28, S 18, S 11) in the ($PC_1 \times PC_2$) coordination plane.

proteins in the winter, the lowest salinity-mediated increase of MT level was attained. The resulting overall salinity effect on MT levels assessed in gills of mussels acclimated to 11 psu retained a similar level of statistical significance as MT recorded at 37 psu salinity ($P < 0.05$), assessed by ANOVA.

3.9. PCA

The PC analysis was performed for each season separately taking into account salinity (as a supplementary variable) and data of three biochemical/molecular parameters (MT, total cytosolic proteins and DNA integrity status) as explanatory variables determined using the gills of mussels subjected to hyposaline acclimation during summer (Fig. 11A) and winter (Fig. 11B) seasons.

The prevailing PC_1 component, the most indicative of salinity-induced biochemical responses in mussel gills, was more pronounced in the summer (70.8%) than in the winter (59.4%) season, with respect to the proportion of the total variance. For both seasons PC_1 was mostly characterized by a close inverse relationship between salinity and DNA integrity (all positive correlations >0.8–0.9) indicating that the magnitude of hyposaline stress may be strongly linked to enhanced DNA susceptibility to oxidative damage. The strong association of MT with PC_1 in the summer (0.8), in contrast with markedly reduced MT loading in the winter (0.2) may suggest that hyposaline stress-mediated MT induction tended to be more closely associated to prooxidative conditions in the warmer period of the year.

The association of total cytosolic proteins with PC_1 also displayed differential seasonal patterns. Following the summer acclimation to low salinity (−0.8) the level of cytosolic proteins (−0.9) was inversely related to DNA integrity (0.8) and MT levels (0.8), suggesting the prevalence of lysosomal degradation of non-essential, partially oxidized proteins contrary to the MT induction. In the winter, an inverse relationship between salinity (0.96) and cytosolic proteins (−0.87) could observed, also closely associated to DNA integrity status (−0.98) indicating the progressive increase of cytosolic protein levels with the magnitude of hyposaline/oxidative stress.

The minor PC_2 component, which was evidently was more strongly expressed in the winter (40.6%) than in the summer (19.0%), may be attributed to responses recorded in mussels acclimated to moderate hyposalinity stress ranging from 28 to 18.5 psu. In the winter, due to a marked positive association of PC_2 with MT (0.98) and cytosolic proteins (0.46) but an insignificant relationship to salinity (0.05) and DNA integrity (−0.2), MT levels seem to be better linked to seasonal changes in the general protein metabolism than to cellular prooxidant/antioxidant imbalance. In the summer, PC_2 was mostly linked to salinity (0.5) and MT (0.5), both of them relating negatively to oxidative DNA susceptibility (−0.6), which may be ascribed to the decline of MT level within salinity ranges

from 28 to 18 psu, and presumably facilitated by prevailing prooxidant processes under extremely high summer temperatures.

The additional PC analysis presented in Fig. 11C was performed, including data from the four additional parameters recorded only for the summer acclimation, such as p38-MAPK, the condition index of mussels, cumulative mortality (%) following hyposaline acclimation and survival time assessed by SOS test. It should also be mentioned that MT data were normalized on a protein basis and the concentration of total cytosolic proteins was omitted from this model.

The specific variable association patterns of the major PC_1 (72.3%) and minor PC_2 (20.6%) components, as presented jointly with a complementary case analysis, evidently reflected the partitioning of four salinity acclimation groups in the $PC_1 \times PC_2$ coordinate plane. The largely prevailing component, PC_1, may be attributed to compensatory responses induced by hyposaline stress of the largest magnitude (11 psu) as opposed to groups of mussels acclimated to normal seawater (37 psu) and moderate hyposalinity (28 psu). Interrelations among variables were, in general, in agreement with the PC_1 pattern presented on Fig. 11A, indicating that mussels subjected to extreme hyposalinity (−0.93) in the summer displayed an enhanced susceptibility to DNA damage (0.94) and higher proportion of cumulative mortality (0.94) than mussels acclimated to 37 and 28 psu in the same period. Additionally, the marked association of PC_1 with cellular responses implicated in stress signalling and antioxidant defence such as p38-MAPK (0.74) and MT induction (0.78), was observed. The condition index CI-2 (−0.80) and SOS (−0.80) were inversely related to cellular responses attributable to prooxidant imbalance (DNAd, p38-MAPK and MT) indicating that the higher nutrition/energy status was linked to the prolonged survival time of mussels subjected to natural seawater (37 psu) as well as to those experiencing the less intensity stress (28 psu), as opposed to mussels acclimated to extreme hyposalinity (11 psu).

The minor PC_2 component related to the salinity acclimation groups 28 and 18.5 psu was mainly associated to p38-MAPK phosphorylation (0.67) and SOS (0.59), but negatively correlated with condition index CI-2 (−0.56) and MT (−0.44). Accordingly, mussels acclimated to 18.5 psu, in spite of the lower nutritional status, unexpectedly exhibited better survival capability in comparison to those acclimated to 28 psu, which presumably might be attributable to physiological modulation of reversible p38-MAPK signalling and MT antioxidant activities.

4. Discussion

The main objective of this experimental laboratory study was to gain a better understanding of the multifactorial effects of the physical environment on the commonly used bioindicator species *M. galloprovincialis*. Additionally, this study assessed the advantages and restrictions associated with the implementation and specificity of commonly investigated biomarkers/biotests as indicators of environmental pollution in biomonitoring studies. Several recent studies have demonstrated that mussels exposed to different pollutants or stressors, are less tolerant to additional stress exposure and *vice versa* (Thomas et al., 1999). Furthermore, fluctuations in environmental conditions can cause a broad range of cellular responses and organismic adaptations, including behavioural avoidance and alterations in reproduction (Schroth et al., 2005).

Our results showed that there are significant effects/differences of all measured parameters between control mussels (37 psu, 100% SW) and mussel acclimated to 11 psu (30% SW). This result indicates that salinity variation (hypoosmotic stress) in the marine environment can affect all measured parameters. The present findings highlight that salinity and temperature effects need to be taken into account when interpreting the results of biomarkers and bioassays used as indicators of water quality and organisms' health in biomonitoring studies.

4.1. Stress on stress

Our results support Bayne's (1986) suggestion that acclimation stress or tissue pollutant accumulation may reduce the ability of these mussels to tolerate additional environmental changes, in this case stress due to air exposure, especially during the summer period. Mussel health, indicated by the survival time (LT50) of animals acclimated to SW of 11 psu, when maintained in air at sea temperature shows several doubts on whether stress on stress response provides a simple and sensitive indicator of environmental pollution, which could be integrated with mussel watch studies (Hellou and Law, 2003).

As determined by the present study, survival time of aerial exposure was not totally salinity acclimation dependent, since mussels exposed to lowered salinity (18.5 psu) did not have significantly reduced survival times compared to the other acclimation groups. This finding is similar to that of Eertman et al. (1993) investigating the possible effect of lowered salinity on the SOS survival. They studied mussels' survival under laboratory and semi-field conditions with mussels acclimated for 15 days to seawater with lowered salinity (35, 28 and 23 psu), keeping food availability and concentration of suspended particles identical in all experiments. They demonstrated that after 1 and 4 days of acclimation to 28 and 23 psu SW salinities, mussel survival time in air was significantly reduced in comparison to control mussels (35 psu). However, in their study a similar reduction was not observed after 7 and 15 days of adaptation time. Furthermore, mussels adapted for 15 days to 23 psu had a significant increase in survival time in air compared to the other groups. Eertman et al. (1993) suggested that reduced salinity in the field may affect the SOS values negatively in mussels by a shortening in survival time, which supports our conclusions.

Nevertheless, laboratory studies are difficult to compare to field exposures where uncontrolled ecological factors may influence the response. Therefore, we suggest that, at least, continuous measurements of temperature and SW salinity should be performed when using mussels as biomonitors of pollution response. Subtle differences in environmental factors, such as temperature, salinity, pH, bacterial proliferation, water currents, and food availability, could obscure physiological differences and energetic impacts on individual mussels.

Babarro and de Zwaan (2002) showed that environmental factors play a significant role in determining the course of bacterial infection and death of bivalves exposed to anoxia. Recent studies have applied this concept to the survival/stress on stress responses of *Mytilus* sp., and suggested a positive correlation between pollutant accumulation and reduced capacity of mussel to survive in air (Hellou and Law, 2003; Bihari et al., 2007). Anoxic survival response of mussels is a simple and low-budget method for the investigation of whether environmental variables might have perturbing effects, which may lead to the individual's reduced fitness (Widdows et al., 1979; Viarengo et al., 1995; de Zwaan and Eertman, 1996). It has been shown, especially during prolonged anoxia and high seawater temperatures, that anoxic survival time may be strongly underestimated due to bacterial proliferation (Babarro and de Zwaan, 2002).

Intertidal organisms are distributed within their range of tolerance to changes in environmental conditions associated with tidal changes (Tsuchiya, 1983). Even though intertidal animals are highly tolerant to tidal emersion, abnormal oceanographic and weather conditions sometimes exceed their physiological tolerance result-

ing in mass mortality (Joergensen, 1990; Gosling, 1992). During field application of the SOS test the tidal height of collected mussels is important, because of emergence time at this particular height. High seawater and air temperatures as well as high and low tidal regimes can affect SOS results. Our preliminary field study showed better SOS results of mussels from mariculture (continuously submerged) in comparison to field "wild" intertidal mussels that were constantly emerged due to tidal fluctuations (data not shown).

4.2. Mussel growth, respiration, energy, fitness

Condition indexes did not give significant differences between mussel acclimation groups, but acclimation resulted in decline (i.e., a negative trend; Fig. 4), especially of CI-2 and CI-3 calculated on the basis of mussel tissue weight and shell sizes.

4.2.1. Scope of growth

In long term acclimation experiments (weeks), mussels can be acclimated to lower salinities, but the reported growth rates of individuals subjected to only 13 psu was reduced to almost zero (Bøhle, 1972; Almada-Villela, 1984). However there are several environmental factor (e.g. temperature) breakdown points in mechanisms of organisms' adaptation, resulting in an increase in the metabolic rate and a decline in filtration rates (Widdows, 1978; Widdows et al., 2002). These results are in accordance with our results of the decrease of CIs and soft tissue wet weight.

4.2.2. Energy

Rainer et al. (1979) showed the effect of reduced salinity treatments on adenylate energy charge (ATP, ADP, AMP). In three estuarine molluscs the mean energy charge for each species decreased by 17% or more when salinity was reduced from 35 to 10 psu. Changes with reduced salinity were also found in the concentration of individual adenylates and several adenylate ratios other than energy charge (Atkinson, 1971).

Environmental stresses, especially climatic are an underestimated feature of organisms' habitats in the wild. Resource depletion and, hence, inadequate nutrition becomes the norm during those circumstances, so that organisms often struggle to survive. Stress is an environmental probe that targets the predominant carrier of energy, the adenosine triphosphate (ATP). Therefore, stress reduces energetic efficiency, that is, the organism's fitness, but increased energetic efficiency should evolve during adaptation of organisms to their habitats (Parsons, 2007).

4.2.3. Fitness

The calculated CI values of mussels subjected, under laboratory conditions, to prolonged hyposaline acclimation may indicate and can be explained by sub-lethal stress and reduced food availability. This may represent a stress on the mussels that may then be reflected in an altered maintenance of energy requirement for the animal and consequent physiological responses. *M. galloprovincialis* is an osmoconformer and maintains its tissue fluids isoosmotic with the surrounding media by mobilization and adjustment of the tissue fluid concentration of free amino acids (Bayne, 1986). However, mobilizing amino acids may result in protein loss, increased nitrogen excretion and reduced growth. *Mytilus* species are known to exhibit a defined behaviour to reduced salinity, initially by closing its siphons to maintain the salinity of the water in its mantle cavity, which allows some gaseous exchange and therefore maintenance of a longer aerobic metabolism. If salinity continues to fall the valves close tightly (Davenport, 1979; Rankin and Davenport, 1981), and in extreme low salinities a large proportion of mussels may die.

4.2.4. Oxygen consumption

Our results confirmed the significant increase in oxygen consumption rates of mussels acclimated to different lower salinities (Fig. 5). The V_{O_2} of *M. galloprovincialis* depends on their previous acclimation history and includes *de novo* synthesis and/or induction of adaptation enzymes and processes (Neufeld et al., 1980). The oxidative metabolism is enhanced by mussel exposure to dilute seawater, as similarly found for crabs (Lucu and Pavičić, 1995), and is provided mostly through phosphorylation pathways, oxidation by glycolysis and Krebs cycle.

M. galloprovincialis is regarded as euryoxic, and tolerant of a wide range of oxygen concentrations, including zero (Gosling, 1992). Adult mussels are capable of anaerobic metabolism, and during aerial exposure (emersion) mussels close their valves, resulting in a low rate of oxygen exchange and consumption (Luedeking and Koehler, 2004). Therefore, mussels conserve energy and utilize anaerobic metabolism. This anaerobic metabolism also increases at low temperatures and some of the end products may be cryoprotectant (de Vooys and de Zwaan, 1978; de Vooys, 1991). Although *M. galloprovincialis* is highly tolerant to hypoxia, this incurs a metabolic cost and, hence, a reduced growth, which has already been recorded (Almada-Villela, 1984).

4.3. Non-genotoxic causes of DNA damage?

It is interesting to understand the correlation between DNA integrity (damage) and salinity changes (hypoosmotic stress) in marine euryhaline organisms, since their metabolism, energy consumption and fitness components can be brought into focus by performing such investigations. Many authors have described and applied DNA integrity determination techniques for DNA damage estimations (Zahn et al., 2000; Dolcetti and Venier, 2002; Pérez-Cadahia et al., 2004; Cornet, 2006). However, these techniques do not satisfy the required efficiency and rapid need of biomonitoring studies. Our method of choice, the Fast Micromethod®, allowed a fast and simple determination of DNA integrity and estimation of organisms' physiological stress after lower salinity acclimation (Batel et al., 1999; Jakšić and Batel, 2003).

As with other organisms, DNA damage may arise as a result of processes unrelated to mutagen exposure and castogenicity. For example, both physical and chemical stressors (e.g. high temperature and increased salinity) are known to cause DNA damage through processes linked to lysosomal enzyme destabilization as part of a general stress response. In such cases, evidence of damage is usually reversible and is lost once the organism is returned to its normal conditions (Dixon et al., 2002).

DNA integrity as a biomarker of exposure to genotoxins is highly specific for various stressor types and the mechanistic relationship between stressors and ecologically relevant factors could help us to understand and establish causal relationships. DNA integrity as indicator includes genotoxic exposure events and DNA repair rates, and especially that repair depends on the organism's fitness status (general stress response), in our case mussels.

4.4. Protein expression pattern—synthesis, degradation and cell signalling

The detected proteins represent the equilibrium between synthesis and degradation rates, and the observed pattern changes can be used as an indicator of the organism's metabolism and gene expression state (Fig. 7). This approach can be used, with little information about the identities of the proteins investigated. Many investigators have examined protein expression patterns (protein synthesis) after exposure of the organism to different stresses using

1D and 2D gel electrophoresis (Apraiz et al., 2006; Supek et al., 2008).

Previously, we have detected two bands of stress-70 proteins (70 and 72 kDa) by Western blot analysis after one-dimensional electrophoresis of gill tissue homogenates of the mussel *M. galloprovincialis* (Hamer et al., 2004). Experimental exposure of mussels to different lower salinities reduced HSP72 and induced HSP70, and a positive correlation between field seawater temperatures and HSP70 content was found at investigated sites of Rovinj coastal area, Croatia (Hamer et al., 2004). Furthermore, sites ranged according to total annual HSP70 protein contents in mussel gills and total PAHs content in the sediment showed the same increased order (Bihari et al., 2006; Pavičić-Hamer and Hamer, 2007). Indeed, it is known that natural fluctuations in environmental temperatures and several other physical and chemical parameters can result in the induction of some proteins (e.g. HSPs) and cellular adaptive stress responses (Conway de Macario and Macario, 2000; Minier et al., 2000; Hofmann et al., 2002; Hamer et al., 2004).

Our results suggest that variation of seawater salinity can alter 1D protein expression patterns of the mussel *M. galloprovincialis*. Further investigation using "fingerprinting" by running conventional 1D SDS-PAGE in bulk and analyzing gel banding patterns using machine learning methods (Bierkens, 2000; Supek et al., 2008) and proteomic approach (2D protein analysis) will be undertaken to search for specific protein expression signatures (PES) as indicator of exposure to different environmental factors and/or specific pollutants (Shepard et al., 2000; Hamer et al., 2005b; Apraiz et al., 2006; Pytharopoulou et al., 2006).

Gaitanaki et al. (2004) have examined the effects of decreasing and increasing SW salinities (50–120%) in *M. galloprovincialis* during a short exposure period (30 min) on the expression of p38-MAPK phosphorylation. They showed that hyposmotic solutions with salinity 60% of normal SW did not induced a significant phosphorylation, but a 50% salinity induced a considerable kinase phosphorylation similar to our results (Fig. 8). The mitogen-activated protein kinase cascade regulates changes in gene transcription by transmitting extracellular stimuli from the plasma membrane to the cell nucleus and plays an important role in organisms' responses to environmental stresses (Böhm et al., 2001). The p38-MAPK responds to low oxygen, oxidative stress, thermal stress, and hypo- and hypertonicity in *Mytilus* sp. (Canesi et al., 2002; Gaitanaki et al., 2004; MacDonald and Storey, 2006). Anestis et al. (2007) studied the behavioural, metabolic, and molecular stress responses of *M. galloprovincialis* during long-term acclimation to increased ambient temperature. In their study, acclimation to temperatures higher than 24 °C caused an increase in mortality and induced the expression of HSP72, increased phosphorylation of p38-MAPK and JNKs, both indicating the activation of MAPK signalling cascades.

4.5. Metallothioneins – oxidative stress – seasonality

Results obtained in the present study are in general agreement with the growing body of evidence reported in the literature that strong variations of various environmental stimuli may induce prooxidative processes in tissues of bivalve molluscs. These processes are mostly associated with alterations on structure/functionality of phospholipid membranes, proteins and nucleic acids (Nicholson, 2001; Chelomin et al., 2005; Manduzio et al., 2005).

We found in the present study a close inverse relationship between salinity and susceptibility of DNA to oxidative damage in both summer and winter, presumably indicating an imbalance of prooxidant/antioxidant status in mussel gills depending on the magnitude of hyposaline stress. Concentrations of metallothioneins and of total cytosolic proteins were both also markedly affected by extreme hyposalinity (18.5 and 11 psu), although they displayed distinct seasonal patterns of PCA for winter and summer acclimations. The observed fluctuations may be related to differential, seasonally dependent antioxidant defence capacity of bivalve molluscs extensively studied by several authors (Viarengo et al., 1991; Vidal et al., 2002; Manduzio et al., 2004; Borković et al., 2005).

Our results are also in agreement with the concept that metallothioneins, sulphydryl-rich, mainly cytosolic metal-binding proteins are particularly susceptible to oxidative damage due to the specific kinetically reactive zinc-thiolate bonds (Maret, 2004) therefore capable of transducing cellular redox into zinc signal by MT-mediated modulation of key antioxidant defence responses (Chung et al., 2004). Therefore, due to the well known capacity of MT for direct oxyradical scavenging and inhibition of lipid peroxidation associated with MT expression in mussels through both trace metals and oxidative pathways (Viarengo et al., 1999b; Dondero et al., 2005), this multifunctional protein may be considered as an integral non-enzymatic part of cellular antioxidant defence.

Assuming that both MT neosynthesis and degradation in gills are closely related to prooxidant processes promoted by strong variations in ambient parameters (including food availability), they indispensably affect physiological requirements of bivalve molluscs for essential trace metals (i.e., Zn and Cu) during their annual reproductive cycle (Viarengo et al., 1988; Serra et al., 1999; Dragun et al., 2004; Geffard et al., 2005). The observed fluctuations of MT levels in our study, particularly pronounced below 28 psu salinity, may be explained by interactive effects of salinity and temperature, presumably modulated by seasonally dependent internal stimuli. Therefore, the inverse related responses, showing the significant MT decrease from 28 to 18.5 psu, followed by a marked increase at 11 psu may suggest interactive functionalities depending on the magnitude of hyposaline stress. Our findings are in agreement with previously reported results of Viarengo et al. (1988) indicating interactive effects of temperature and salinity on subcellular copper partitioning, therefore affecting MT induction in gills of copper-exposed and control mussels. Among non-metal exposed mussels the low salinity effect (22 psu versus 36 psu) on the partial redistribution of tissue copper toward cytosolic fraction was noticeable, resulting by similar MT temperature dependency as found in our study regarding the markedly higher MT level at 23 °C compared to 13 °C.

The observed fluctuations of MT levels tentatively linked to the changes in subcellular metal distribution may be also supported by the fact that the prevailing part of intracellular trace metals in gills of *M. galloprovincialis* is bound to a particulate fraction (Serra et al., 1999), which may be partially remobilized in the cytoplasm. Under the strong prooxidative conditions redox active metals, such as Cu and Fe, concentrated in secondary lysosomes of marine invertebrates (Viarengo and Nott, 1993) may be relocated to the cytoplasm and nucleus due to lysosomal membrane permeabilization (Kurz et al., 2004), presumably facilitating oxidative DNA damage. Therefore, MT levels in gills of bivalve molluscs, more likely, reflect oxidative stress-induced changes in intracellular trace metal availability rather than an environmental or tissue metal concentrations (Bonneris et al., 2005; Domouhtsidou et al., 2004). Consequently, physiologically modulated variations in the subcellular partitioning of trace metals between soluble and insoluble fractions may be related to variation of basal MT levels irrespective to the external metal bioavailability.

Considering the salinity induced MT variations in gills of mussels in the present study, it should be emphasized that the organisms were not exposed to experimentally elevated ambient metal concentrations. Consequently, the observed MT fluctuations may be, more likely, ascribed to the dynamics of the

neosynthesis/degradation rates presumably modulated through prooxidant/antioxidant imbalance depending on the amplitude of hyposaline stress rather than the enhanced ambiental trace metal bioavailability within 14 days of hyposaline acclimation.

The results obtained in the present study also support the arguments that salinity-mediated changes of metallothioneins and total cytosolic proteins determined in gills of mussels were significantly affected by seasonal temperature in close association with internal factors. The observed seasonality was more pronounced if MT would be expressed on a tissue mass rather than on a protein basis, showing significantly higher levels of both MT and total cytosolic proteins in the winter than in the summer. The similar seasonal pattern was recorded in mussels employed in several field studies (Bodin et al., 2004; Ivanković et al., 2005; Gorbi et al., 2008), where MT was used as a biomarker of trace metals exposure. Seasonality tended to be less pronounced if MT was normalized on a protein basis (Bocchetti and Regoli, 2006). According to our results the most reliable approach for evaluation of salinity/interaction effects on MT variation might be provided by the ANCOVA results that included total cytosolic proteins as a covariate, which resulted in minimizing the relative importance of seasonality attributable to variation of cytosolic proteins without affecting MT-salinity dependency.

The results obtained in this study reveal that mussels acclimated to hyposaline stress exhibit significant seasonal dependence between MT and total cytosolic proteins, being mostly pronounced under the lower salinities tested. In the winter, an enhanced DNA damage susceptibility in mussels was observed, presumably attributable to less pronounced antioxidant enzyme activities than in the summer (Sheehan and Power, 1999; Vidal et al., 2002; Manduzio et al., 2004; Borković et al., 2005). Generally, in the winter, lower antioxidant defence capacity of mussel gills, compared to the digestive gland, may be compensated by the higher basal MT and glutathione-*S*-transferase (GST) activity (Manduzio et al., 2004), both sharing similar transcriptional regulatory pathway via zinc-mediated gene activation (Chung et al., 2004). We also found that hyposalinity mediated variation of MT levels in the winter, evidently less pronounced than in the summer, tended to be more closely linked to the general protein metabolism than to alteration in the oxidative status of the tissue, as indicated by the higher DNA integrity status. Our findings are consistent with fluctuations of MT concentrations in gills of estuarine crabs in response to salinity changes, being more closely related to variations in the general protein metabolism than to accumulated metal concentrations (Legras et al., 2000; Mouneyrac et al., 2001). The observed higher basal level of total cytosolic proteins in the winter than in the summer, including that of MT, may be related to the less developed lysosomal system and lower intensity of phagocytotic activity (Marigomez et al., 1996), thus slowing the rate of macromolecular turnover accompanied to oxyradical generation.

During summer acclimations, the observed decrease of cytosolic proteins at 11 psu salinity may indicate a faster ROS generation linked to the more rapid turnover of non-essential proteins by lysosomal proteases in providing free amino acids pool for cellular volume regulation (Gosling, 1992; Neufeld and Wright, 1996). The same could also be true for the neosynthesis of many classes of intracellular protective proteins, including MT and stress-70 proteins (Hamer et al., 2004). It should be mentioned that the physiological importance of macromolecular recycling via controlled autophagy has been mostly reported in the digestive gland lysosomes of several bivalve genuses, including *Mytilus* (Tremblay and Pellerin-Massicotte, 1997; Guerlet et al., 2007). Due to the elevated membrane permeability and presence of lipofuscin granules, indicative of autophagic reactions, the similar adaptive mechanism may also occur in lysosomes of specific gill cells, including haemocytes (Nicholson, 2001; Gomez-Mendikute et al., 2005). It may be presumed that autophagy in gills should probably be more associated with the production of nutrients required for relocation of cell energy towards compensatory defence processes in response to diverse environmental stressors rather than to seasonal changes in tissue reorganization during the reproductive cycle.

The synthetic multivariate approach provided by the PC analysis by integrating biochemical/cellular responses with the physiological parameters after summer acclimation, may be indicative of coordinated functioning of two distinct systems of adaptations. This should be attributed to substantially different strategies (tolerance versus resistance), depending on the amplitude of salinity changes as previously proposed by Berger and Kharazova (1997). Accordingly, mussels subjected to natural seawater and to hyposaline stress of lower intensity (37 and 28 psu) were presumably fully acclimated within 14 days, contrary to those acclimated to extreme hyposalinity (11 psu), whose compensatory adjustment to stressful conditions had not been completed. Mussels acclimated to 11 psu salinity displayed a lower degree of DNA integrity, evidently exceeding their adaptive capacity, indicated by the higher percentage of cumulative mortality compared to mussels acclimated to the moderate hyposaline stress. In gills of mussels acclimated to extreme hyposalinity, the relatively higher level of biomarkers attributable to cellular antioxidant defence and stress signalling, such as MT and p38-MAPK phosphorylation, was evident as opposed to mussels acclimated to moderate stress.

Responses to moderate salinity changes, considered as tolerance since a higher condition index was recorded, compared to mussels acclimated to hyposaline stress of the largest magnitude may be linked to the lower energy demanding processes. These responses presumably imply a degradation of tissue proteins by phagocytotic processes underlying volume regulation by utilizing organic osmolytes. On the contrary, resistance to extreme hyposalinity tentatively implies a higher energy cost, mostly determined by changes in protein regulation of ionic content and cell volume, including ATP-driven sodium translocating pumps (Pierce, 1982; Pagliarani et al., 2006).

Transition from the tolerance toward a resistance level, according to interrelations among variables correlating with the minor PC_2 component, may take place at salinities ranging between 28 and 18.5 psu. Therefore, mussels developing resistance at 18.5 psu salinity, despite of the lower condition index, but exhibiting prolonged anoxic survival ability, seem to be provided with a more efficient compensatory mechanism than those acclimated to 28 psu, which presumably involves lower energy cost activities mostly related to antioxidant defence at the constitutive level. The intermediate salinity range with tolerance towards resistance is largely characterized by the inverse relationship between MT and p38-MAPK phosphorylation. This result suggests a physiological adjustment through reversible cellular switching based on the modulation of intracellular free zinc by MT chelating/delivery activities depending on the cellular redox and energetic status (Maret, 1994, 2004).

Referring to the marked activation of p38-MAPK phosphorylation observed in the gills of mussels acclimated to 18.5 psu salinity, as previously reported in mantle tissues of *M. galloprovincialis* (Gaitanaki et al., 2004), the observed lowest MT level at 18.5 psu, in the present study, may indicate a prevailing oxidative degradation of the presynthesized basal MT level followed by oxidative zinc release required for Zn-mediated p38-MAPK activation (Kefaloyianni et al., 2005). The same result was also found in gills of mussels subjected to acute thermal stress (Kefaloyianni et al., 2005).

The similar oxidative/nitrosative stress-dependent mechanism dealing with MT-modulated Zn-activation of p38-MAPK and subsequent K^+ channels phosphorylation was recently proposed in rodent neuron cells (Bossy-Wetzel et al., 2004). In connection with that our study provides additional evidence that mussels subjected to extreme hyposalinity may be supplied by a compensatory mechanism implying Zn-MT modulation in activation of p38-MAPK, which may be linked to ion transport processes. This concept is also in agreement with our recently published results on the progressive increase of both cytosolic zinc and MT induction in the posterior gills of the shore crab *Carcinus aestuarii* during acclimation to hyposaline stress and concomitant activation of Na^+/K^+ ATP-ase (Lucu et al., 2008).

4.6. Proposal for the future implementations of biomarkers in biomonitoring studies

Our findings should be of particular ecotoxicological relevance for pollution assessment using biomarkers in areas under strong estuarine influences, which are impacted, evidently, by significant confounding effects of environmental stressors, such as salinity and temperature. Mussels in nature seldom undergo only one stress at a time as in most laboratory experiments. For biomonitoring purposes (field study) it is necessary to collect mussels continuously submerged, to avoid stresses due to desiccation and anaerobiosis. It may be unrealistic to assume that temperature, salinity and pollution are the only stresses that influence measured biomarkers and biotests. A combination of anthropogenic pollution and natural stressors results in several adverse effects occurring under *in situ* conditions, and their interpretation requires further research.

Survival and growth of mussel populations is largely dependent on the ability of mussels to tolerate strong fluctuations of environmental conditions and can be affected by additional pollution stress. It is essential to characterize the recent physical and chemical state of sampling sites and to study natural variations in the biomarker responses of molluscs in order to differentiate the pollution-induced effects from the effects of natural/environmental stresses.

The present study expands our understanding of multifactorial effects of the physical environment on the specificity of investigated biomarkers as indicators of environmental pollution. The search for biomarkers as measures of the biological effects of pollution and environment factors is far from finished, with new biomarkers being developed, while old are tested, and re-evaluated.

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