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Effects of increasing temperatures on biomarker responses and accumulation of hazardous substances in rope mussels (*Mytilus galloprovincialis*) from Bizerte lagoon

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Abstract:

This study examined the influence of increasing temperatures in spring and summer on biochemical biomarkers in *Mytilus galloprovincialis* mussels sampled from Bizerte lagoon (northern Tunisia). Spatial and seasonal variations in a battery of seven biomarkers were analyzed in relation to environmental parameters (temperature, salinity, and pH), physiological status (condition and gonad indexes), stress on stress (SoS), and chemical contaminant levels (heavy metals, polycyclic aromatic hydrocarbons (PAHs), and PCBs) in digestive glands. Integrated biological response (IBR) was calculated using seven biomarkers (acetylcholinesterase (AChE), benzo[a]pyrene hydroxylase (BPH), multixenobiotic resistance (MXR), glutathione S-transferase (GST), catalase (CAT), malondialdehyde (MDA), and metallothioneins (MT). Seasonal variations in biological response were determined during a critical period between spring and summer at two sites, where chemical contamination varies by a factor of 2 for heavy metals and a factor 2.5 for PAHs. The analysis of a battery of biomarkers was combined with the measurement of physiological parameters at both sites, in order to quantify a maximum range of metabolic regulation with a temperature increase of 11 °C between May and August. According to our results, the MT, MDA, CAT, and AChE biomarkers showed the highest amplitude during the 11 °C rise, while the BPH, GST, and MXR biomarkers showed the lowest amplitude. Metabolic amplitude measured with the IBR at Menzel Abdelrahmen—the most severely contaminated station—revealed the highest metabolic stress in Bizerte lagoon in August, when temperatures were highest 29.1 °C. This high metabolic rate was quantified for each biomarker in the North African lagoon area and confirmed in August, when the highest IBR index values were obtained at the least contaminated site 2 (IBR = 9.6) and the most contaminated site 1 (IBR = 19.6). The combined effects of chemical contamination and increased salinity and temperatures in summer

appear to induce a highest metabolic adaptation response and can therefore be used to determine thresholds of effectiveness and facilitate the interpretation of monitoring biomarkers. This approach, applied during substantial temperature increases at two sites with differing chemical contamination, is a first step toward determining an environmental assessment criteria (EAC) threshold in a North African lagoon.

Keywords: Biomarkers ; Mussels ; Lagoon biomonitoring ; Integrated biomarker response

Introduction

In Mediterranean lagoon ecosystems, exchanges with the open sea and water circulation are mainly governed by winds and atmospheric pressure (Rougier et al. 2000). These systems are characterized by shallow waters (Armi et al. 2010), which allow the wind to mix the entire water column, thus enhancing diatom proliferation (Jarry et al. 1990). Bizerte lagoon, located in northern Tunisia, is linked to the Mediterranean Sea by a single entrance channel. It is situated near various industrial units and agricultural areas and has been used for shellfish farming since 1964 (Beji 2000). It is the recipient of a variety of industrial waste, pesticides and chemical fertilizers through soil erosion and runoffs, which has led to a decrease in bivalve and fish production (ANPE 1990). Indeed, direct and indirect discharges of urban and industrial wastes and runoffs have resulted in chemical contamination of the lagoon by various toxic compounds such as organochlorine pesticide OCPs (Cheikh et al. 2002), halogenated aromatics compounds such as PCBs (Derouiche et al. 2004), polycyclic aromatic hydrocarbons (PAHs) (Trabelsi and Driss 2005), polybrominated diphenyl ethers (PBDEs) and their methoxylated analogs (Ben Ameur et al. 2011) and heavy metals (Ben Garali et al. 2010).

Biomarkers are increasingly used to assess environmental quality and, in particular, the chemical quality of lagoons, in the aim of assessing the biological effects of environmental contaminants on aquatic organisms living in them (Ben Ameur et al. 2012). They are among the emerging tools used in monitoring programs to assess the biological effects of chemical contaminants (Allan et al. 2006; Depledge 2009) and can be used to reveal environmental stresses caused by contaminants and other environmental variables. The integration of biomarkers and chemical analysis is therefore an essential factor of success for establishing links between stress and pollution (Galloway et al. 2004a; Thain et al. 2008).

In poikilothermic organisms such as bivalves, ambient temperature is one of the major factors driving physiological and biochemical processes (Pfeifer et al. 2005). Mussels may be exposed to extreme temperature fluctuations and major changes in body temperature within a short period of time during the hot season (Sokolova 2004) and are capable of challenging sustained seasonal variations in environmental temperatures (Banni et al. 2011).

In a previous study, we investigated oxidative stress and detoxification response in *Mytilus galloprovincialis* after exposure to thermal stress (18-20-22-24-26°C) and a sublethal dose of benzo [a] pyrene (Kamel et al. 2012). Our results demonstrated the negative effects of acute heat stress on mussel response, manifested by cellular genotoxicity and cytotoxicity.

In order to study the *in situ* influence of elevated temperatures on mussels, we used rope mussels (*M.galloprovincialis*) as sentinel organisms to assess (i) variations in a suite of biomarkers, (ii)

the capacity of mussels to accumulate hazardous substances and (iii) related effects of increasing summer temperatures in Bizerte lagoon. For this purpose, biomarkers of oxidative stress were measured in the gills and digestive glands of mussels in late spring and summer 2011. We particulary took the following criteria into consideration, in line with the results of large-scale biomonitoring programs such as MED POL and OSPAR (UNEP-RAMOGE. 1999; ICES 2010): survival in air (SoS), condition index, gonadic condition index, neutral red retention, benzo [a]pyrene hydroxylase (BPH) activity, glutathione S-transferase (GST) activity, catalase (CAT) activity, acetylcholinesterase (AChE) activity, multixenobiotic resistance (MXR), metallothioneins (MT) and lipid peroxidation.

Survival time in air can indicate the general health of organisms (Viarengo et al. 2007). Mussel survival time can be considered as a "Stress on Stress" (SoS) response. Similarly, the Condition Index (CI) can provide general information on the more general energy budget allocation of mussels (Gomiero et al. 2011). The CI summarizes the physiological activity of organisms (growth, reproduction, secretion, etc.) in given environmental conditions (Lucas and Beninger, 1985) and is mainly used for two purposes: first, to guarantee the quality of meat for the marketplace (Orban et al. 2002) and second, as an ecophysiological measurement of animal health status.

Activity of the biotransformation enzyme benzo[a]pyrene hydroxylase (BPH) can be used indirectly to measure CYP450 1A activity, which is involved in the biotransformation of xenobiotics (Snyder 2000). This biotransformation enzyme has previously been used as a biomarker of exposure to organic pollutants and, in particular, PAHs (Akcha et al. 2000; Burgeot et al. 2006; Banni et al. 2010). Glutathion S-transferase (GST) is involved in Phase II of biotransformation and thus in the detoxification of numerous environmental chemicals, as it catalyzes the conjugation of glutathione to electrophilic compounds (e.g. epoxides of PAHs), hence rendering them less reactive and more water-soluble (Cheung et al. 2001; Pan et al. 2009).

P-glycoprotein (P-gp), multidrug resistance (MDR), two ATP-driven membrane pumps and the lung resistance protein (LRP), which is the main vault protein (MVP), are part of the Phase III system and involved in the excretion of conjugated metabolites. MXR is an MDR-like system that has been identified in marine invertebrates (McFadzen et al. 2000; Smital et al. 2000). The relevance of MXR has been demonstrated through its potential ability to protect aquatic organisms from DNA damage (Waldmann et al. 1995). MXR protein expression is inducible by exposure to toxic compounds (Minier and Moore. 1996). Quantities of these proteins vary significantly across differentially polluted sites (Minier et al. 2000).

Catalase (CAT) is an antioxidant enzyme used as a biomarker of the oxidative stresses induced by a wide range of contaminants, including organic xenobiotics, heavy metals and PAHs (Livingstone 2001; Sureda et al. 2011). Malondialdehyde (MDA) is used as marker of membrane phospholipid oxidation through lipid peroxidation. Increased MDA levels in organisms may be related to the degradation of an environmental site due to decreased water quality (Charissou et al. 2004). Studies carried out on marine species have shown lipid peroxidation to be a relevant index of toxin-induced chemical injury (Avery et al. 1996).

Acetylcholinesterase (AChE) is an enzymatic biomarker of neurotoxicity and responsible for acetylcholine degradation. AChE activity is inhibited by the presence of pesticides such as organophosphorous compounds, carbamates and various heavy metals or PAHs in mussels (Bocquené et al., 1993; Mora et al. 1999). Tissue levels of metallothionein proteins (MT) were estimated in mussel digestive glands in order to evaluate their biological effects on sentinel organisms and assess trace metal pollution in the aquatic environment. MTs are involved in both homeostasis and detoxification (Viarengo and Nott 1993) and their accumulation is more obvious in gills, digestive glands and kidneys, hence reflecting the significant role of these tissues in the uptake, storage and excretion of metals (Bebianno and Langston. 1992).

Biomarker evaluation in mussels is a major tool used in aquatic environment biomonitoring to assess the causal relationship between exposure to environmental pollutants and long-term effects on individuals and populations (Bolognesi et al. 2004; Bodin et al. 2004). However, the exploration of large datasets of chemical and biological measurements requires a coherent suite of assessment criteria and tool indexes in order to provide standardized interpretations for monitoring purposes. In this aim, we attempted to determine assessment criteria for measuring biological effects by applying the OSPAR Strategy; we also applied the integrated biomarker response index (IBR) in order to interpret a global response from the various measured biomarkers. Background assessment criteria (BAC) and environmental assessment criteria (EAC) have already been developed for mussels and fish in the framework of the integrated marine environmental monitoring of chemicals and their effects in the Northeast Atlantic (Davies and Vethaak. 2012). The IBR index was computed using the biomarker measured in *Mytilus galloprovincialis* in order to assess ecological risks associated with pollution at Bizerte lagoon study sites.

The aim of this study was to examine the influence of increasing temperatures in spring and summer on the biomarker responses of mussels inhabiting a North African lagoon, recognized as a sensitive area in terms of temperature changes and pollution. Biomarker variations were initially compared during the highest temperature elevation, between spring and summer. The

amplitude response of the biomarkers was then studied across two sites characterized by different levels of chemical contamination.

Materials and Methods

Study area

This study was performed at two sites in Bizerte lagoon (Fig.1). The Menzel Abdelrahmen site (S1) (37°13′ N, 9°51′ E), located between the port and the former wastewater (ONAS) discharge, is characterized by urban effluents (Mahmoud et al. 2010), while the Baie des Carrières site (S2) (37°13'N, 9°49' E), located in the channel between the lagoon and Mediterranean Sea, is subjected to intensive maritime traffic and various impacts from the channel area characterized by a strong hydrological dynamics (Khessiba et al. 2001; Cheikh et al. 2002).

Sampling strategy

Mussels of similar sizes (5-7 cm shell length) were sampled monthly from both sites in late spring and summer 2011. The same cohort of juvenile mussels from the same native area (Bizerte lagoon) was selected for this study. Two sites with contrasting levels of chemical contamination, S1 Menzel Abdelrahmen and S2 Baie des Carrières, were selected in the lagoon. These sites, which respectively show high and low chemical contamination levels, are suitable for comparison thanks to their homogeneous physicochemical parameters within the lagoon area.

The mussels were immediately transported to the laboratory. In the laboratory, two samples of 30 individuals were used for biometric analyses (shell length and soft body wet weight) and for the stress on stress test. 50-60 mussels were then scarified for excision of the digestive gland (DG) and gills (G), for the purpose of chemical and biomarker analyses. The sampled tissues were stored in liquid nitrogen at -80°C prior to the assays. All biomarkers were analyzed in the same mussel, whereas stress on stress and chemistry were analyzed on other individuals from the same cohort.

Water quality was assessed at each sampling time. The physicochemical quality of Bizerte lagoon waters was monitored in situ. Salinity ($g.l^{-1}$), temperature (°C), dissolved oxygen ($mg.l^{-1}$) and pH were measured at the sampling sites using a Multi 350i Multimeter.

Chemical analyses

Determination of PAH content

Three distinct pools were prepared (1 pool = 3 digestives glands). PAH content in the digestive gland fractions was determined by gas chromatography (GC) coupled with mass spectrometry (MS), based on a protocol described by Baumard and Budzinski (1997). An HP GC (Hewlett-Packard, Palo Alto, California, USA), equipped with a split/splitless injector, was used. PAHs were quantified relative to perdeuterated PAHs (Baumard and Budzinski 1997). The response factors of the various compounds were measured by injecting a standard reference material solution (SRM 2260 (24 aromatic hydrocarbons in toluene) (NIST, Gaithersburg, Maryland, USA), spiked with the same solution containing perdeuterated PAHs as that used for spiking mussel digestive glands. The detection limits for PAH congeners was approximately 50 pg/g. Results were expressed in µg/g dry weight.

Determination of PCB content

A standard mixture of twelve PCB congeners (PCB-18, 28, 31, 52, 44, 101, 149, 118, 153, 138, 180 and 194) at 10 µg mL^{-1} in heptane was purchased from Supelco (CIL, USA). These standard solutions were further diluted by *n*-hexane to obtain mixed fortifying and GC calibration standard solutions for all compounds.

PCB compounds were analyzed using the method described by Guo et al., 2008. Freeze-dried digestive gland tissue (10 g) was Soxhlet-extracted with *n*-hexane:acetone (4:1; v/v) for 16 h at a rate of five cycles per hour. The extract was concentrated using a rotary evaporator. An aliquot of 1 mL was used for gravimetric determination of the extractable lipid content. The remaining lipids were removed by treatment with concentrated sulfuric acid (4×10 mL), after adding BDE-77 as internal standard. In addition, a clean-up was performed on a column (40×0.5 cm ID) packed with 5 g of activated Florisil and topped with 1 g of anhydrous sodium sulfate. The extract was eluted with 50 mL of dichloromethane and *n*-hexane (1:9; v/v). The eluate was finally concentrated in a Kuderna-Danish to 0.5 mL and was then ready for instrumental analysis. The limit of detection, calculated as three times the signal to noise ratio, ranged from 500 to 1,000 pg g^{-1} lipid weight for PCBs. Results were expressed in ng/g dry weight.

Determination of heavy metal content

Digestive gland tissue (five distinct pools prepared as described below) was thawed and dried at 508°C to a constant weight. Digestion of the samples was performed in a microwave oven (CEM-MDS 81D), in high-pressure vessels with concentrated nitric acid (Amiard et al. 1987). Cd, Cu, Zn and Ni concentrations were determined by atomic absorption spectrophotography with an acetylene flame for Cu and Zn, and a graphite furnace for Cd and Ni (Amiard et al.

1987). Internal controls, based on standard reference materials with certified metal content, together with international intercalibration exercises, were carried out to validate this procedure. The limit of detection of Cd, Cu, Zn and Ni was 0.05 $mg.g^{-1}$ wet weight. Results were expressed in µg/g dry weight.

Physiological indexes

The Condition Index (CI) was determined as an indicator of mussel physiological status. CI is an ecophysiological measurement of animal health that summarizes physiological activity (growth, reproduction, secretion etc.) in given environmental conditions.

Shell thickness, length and width were measured using a 0.05 mm precision caliper as described by Fisher, Schneider, and Bauchot (1987). Once the biometric measurements had been completed, the valves of each individual (30 specimens) were opened carefully and all tissue was removed from the shells. Before weighing, excess moisture was removed from all parts of the animals using absorbent paper. After recording total weight and visceral mass wet weight, the tissue was then dried at 70°C for 48 h.

The Condition Index (CI) was calculated as follows:

CI = MDW/ (TW-SDW) * 100 (Lucas and Beninger, 1985)

Whereby MDW is meat dry weight (g), TW is total weight and SDW is shell dry weight (g).

Stress on stress

The survival in air (SoS) test was performed on 30 animals from each site on their arrival at the laboratory, using the method described by Viarengo et al. (1995). The mussels were placed in a plastic box at a constant room temperature of 18 ±1 °C with less than 100% humidity. Mortality was checked daily. Mussels were considered dead when they did not produce any response to an external stimulus, after their valves gaped, or they did not react when placed in sea water. Results were expressed in number of survival days.

Biochemical Analysis

Ten mussels (n = 10) from each site were analyzed individually for each biomarker measurement.

Prior to biochemical analysis, the digestive glands were homogenized in a phosphate buffer (0.1 M [pH7.5]). The resulting homogenate was centrifugated at 100,000 *g* for microsomal fractions and 9,000 *g* for cytosolic fractions (S9). The quantities of proteins present in the microsomal

fractions and S9 fraction were determined according to the Bradford (1976) method, using Coomassie Blue reagent (BioRad).

Benzo[a]pyrene hydroxylase (BPH)*:* each pool of microsomal fractions of digestive glands was prepared at 4°C using differential centrifugation, as described by Michel et al. (1994). The quantity of S9 proteins was determined on the basis of the obtained supernatant (S9), according to the Bradford method (1976), using bovine serum albumin as standard. BPH activity was then assayed in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), using the fluorimetric assay of Michel et al. (1994), as adapted to the microplate reader by Akcha et al. (2000). Results were expressed in nmol min^{-1} $.mg^{-1}$ proteins.

Glutathione S-transferase (GST) activity was measured in digestive gland cytosol according to the method of Habig et al. (1974), using 10 lg cytosolic protein CDNB (Sigma-Aldrich Chemical, St. Louis, MO) as a substrate and glutathione reduced-form GSH (1 and 4 mM final concentrations, respectively) in a 100 mM sodium phosphate buffer (pH 7.5). GST activity was determined by kinetic measurement at 20°C using a Jenway 6105 spectrophotometer (k = 340 nm). Results were expressed as µmol GSH-CDNB produced/min/mg protein.

Catalase (CAT) was determined using the Clairbone method (Clairbone 1985). The reaction mixture (final volume 1 mL) contained 0.78 mL 0.1 M phosphate buffer (pH 7.5) and 0.2 mL 0.5 mM H_2O_2. After 30 s of preincubation, a reaction was triggered by adding 0.02 mL of the (S9) solution containing CAT fractions. CAT activity was assessed by kinetic measurement at 20°C using a Jenway 6105 spectrophotometer (k = 240 nm). Results were expressed as µmol hydrogen peroxide transformed/min/mg protein.

Lipid peroxidation was estimated in terms of thiobarbituric acid reactive species (TBARS), with the use of 1, 1, 3, 3-treaethyloxypropane as standard. The reaction was assessed at 532 nm using TBA reagent as described by Buege and Aust (1978). MDA content was expressed as nmol equivalent MDA/mg protein.

Acetylcholinesterase (AChE): gill tissues were homogenized in a phosphate buffer (0.02 M, pH 7, Triton X 100, 1/2 w/v), then centrifugated at 9,000 *g* for 20 min at 4 °C. The supernatant (S9) was used to determine AChE activity using the modified method of Ellman et al. (1961) by Bocquené et al. (1993). Protein concentration was determined with the Bradford (1976) method, using bovine serum albumin as standard. Results were expressed in µmol.min^{-1} $.mg^{-1}$ protein.

Multixenobiotic resistance (MXR) proteins was determined with the Western blot method (Minier et al. 2000), using an anti-hamster Pgp C219 monoclonal antibody (Centocor

Diagnostics, Malvern, PA, USA) and an alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma).

Metallothionein content (MT) was evaluated using the spectrophotometric method described by Viarengo et al. (1997), based on cysteine residue titration of a partially-purified MT extract. MT protein levels were determined with a spectrophotometric assay for MTs, using Ellman's reagent (0.4 mmol/L DTNB in 100 mmol/L KH_2PO_4) at pH 8.5 in a solution containing 2 mol/L NaCl and 1 mmol/L EDTA. Reduced GSH standard solutions were used for calibration (2–100 mmol/L) and data was expressed in micrograms MT per milligram protein, taking into consideration mussel MT molecular weight and number of cysteine residues (21 residues) (Viarengo et al. 1997).

Integrated biomarker response determination

The biomarkers were then considered simultaneously by calculating the renewed version of the Integrated Biomarker Response (Beliaeff and Burgeot. 2002) by Devin et al. 2013. Once the standardized value of each biomarker had been calculated, all possible circular permutations of the k biomarkers were computed. This resulted in a matrix of IBR values, enabling calculation of the median IBR for a site and cross-site comparison of IBR values.

BAC and EAC determination

Background assessment criteria (BAC) and environmental assessment criteria (EAC) were drawn up using the 10th and 90th percentiles of data. BAC was estimated using data from Site 2 (least-contaminated site and lowest temperature) and described background level threshold values. EACs are recently derived from toxicological data or expert knowledge and indicate a significant risk to the organism (Davies and Vethaak. 2012). In our study, EAC were calculated during the highest temperatures and at the most-contaminated site S1.

Statistical Analysis

The experimental data was initially tested for normality and homogeneity of variance, in order to meet statistical demands. Data was expressed as mean±standard deviation (SD). Data statistical analysis was performed using one-way analysis of variance (ANOVA) and Duncan's test for multiple range comparison ($\rho<0.05$) was considered as significant. Different letters (a, b) indicated significant differences between groups. The Pearson correlation coefficient was also calculated in order to study the relationships between the various biochemical and chemical

analyses. Differences in mussel size between the two sites were evaluated using Student test ($\rho < 0.05$) for each sampling date. All biological, chemical and physico-chemical data were further subjected to principal component analysis (PCA), this analysis was used to discriminate the abiotic parameters effects on biological response in mussels from two sites in the lagoon. eighteen variables were taken into consideration: concentrations of for traces metals, Σ PAHs and Σ PCB concentrations, physico-chemical parameters (Oxygen dissolved, salinity, and seawater temperature), seven biomarkers (BPH, CAT, GST and AChE activities, MXR, TBARS and MT levels), as well as the condition and the gonadic indices of mussels. All statistical tests were performed using the Statistica 6 software package (StatSoft, USA).

Results

Monitoring of environmental parameters

Environmental parameters revealed changes in water quality according to sampling time (Table 1). Water temperature increased in Bizerte lagoon during the study period and a marked increased in temperature (i.e. + 11°C) was observed between May and August 2011. In a corresponding way, a significant increase in lagoon salinity was recorded in August (37.8 psu) versus May (32 psu). The lowest concentration of dissolved oxygen was recorded in August (6.2 $mg.L^{-1}$). This decrease in dissolved oxygen concentration was the result of an increased Chlorophyll a concentration. Indeed, Chlorophyll a concentrations in lagoon waters were found to be higher in August (3.642 $\mu g.L^{-1}$) than in May (2.185 $\mu g.L^{-1}$). The increases in temperatures and salinity, associated with a relative decrease in dissolved oxygen we observed during the summer months, is favourable to the eutrophication of Bizerte lagoon (Khessiba et al. 2005). There were no variations in pH, turbidity, nitrite, nitrate, ammonia and silicate concentrations throughout the study period exept pH with decrease of 0.5.

Chemical analyses

Trace metal concentrations in mussel digestive glands showed both temporal and spatial variations. Generally speaking, metal concentrations (Cd, Cu, Zn and Ni) as a whole were significantly higher in the digestive glands of mussels sampled from Site 1 versus Site 2 ($\rho<0.05$) and contamination levels at the respective sites differed by a factor of 1.5. Metal trends were similar at both study sites (Figure 5), with the lowest values in May and the highest values in August. Moreover, concentrations of all metals were significantly correlated with water temperature ($\rho<0.05$).

All measured trace metals showed a significant correlation with MT accumulation at both sites. Cd was significantly correlated to MT ($r = 0.59$; $\rho < 0.05$) and ($r = 0.50$; $\rho < 0.05$), respectively at Sites 1 and 2. (Table 2). PCA analyses were performed to obtain a global vision of the results according to the mean values of pollutant levels in digestive gland, biomarker responses in mussel's tissues and physic- chemical parameters of water (Figure 5). Both sites showing that the accumulations of Cd, Cu, Zn and Ni were grouped and related to the water temperature. Almost all polycyclic aromatic hydrocarbon (PAH) compounds measured in digestive glands were three times higher in mussels from the Menzel Abdelrahmen site (Site 1) versus the Baie des Carrières site (Site 2). PAHs content was significantly higher in the digestive glands of mussels collected from Site 1 versus Site 2 ($\rho < 0.05$) (Table 2). The highest values of ΣPAHs concentrations were found in August at Site 1 (ΣPAHs=2.03 $\mu g.g^{-1}$dry weight) and in July at Site 2 (ΣPAHs=0.92 $\mu g.g^{-1}$dry weight) (Table2.). ΣPAHs concentrations were significantly correlated with water temperature at Site 1 only ($r = 0.66$; $\rho < 0.05$). Total PAHs at Site 1 showed a significant correlation with the detoxification enzymes oh phase I BPH activity ($r = 0.58$; $\rho < 0.05$) and phase II GST activity ($r = -0.84$; $\rho < 0.05$).

Concentrations of polychlorinated biphenyls (PCBs) in digestive glands were significantly higher in mussels from Site 1 versus Site 2 ($\rho < 0.05$). The monthly trends of ΣPCB at both sites were similar, with increased levels between May and August. The trends of each PCB compound were similar at both sites, with generally higher levels in May for PCB-18, PCB-52, PCB-44 and PCB-194 and in August for the remaining compounds. A significant correlation was found at both sites between PCB concentrations and CAT activity, with ($r = 0.80$; $\rho < 0.05$) and ($r = 0.48$; $\rho < 0.05$) respectively at Sites 1 and 2.

Physiological parameters

During the sampling period the size of the mussels showed no difference between the two study sites ($\rho < 0.05$) with ($\rho = 0{,}594$; $\rho = 0{,}786$; $\rho = 0{,}879$; $\rho = 0{,}724$) respectively in May, June, July and August. Both stations appear homogeneous with a seamless growth factor inside the lagoon.

Physiological response to temperature changes was determined by examining condition and gonadic indexes (CI and GI). CI calculated during the study period remained constant in May and June, then showed a slight decrease in July, offset by an increase in August. However, these variations were not significant ($\rho < 0.05$) (Figure 2). In mussels sampled at Site 1, CI ranged from 35.80± 5.64 to 37.27± 4.58 in May 2011 and August 2011 respectively. Mussels collected from Site 2 showed condition indexes ranging from 38.33± 7.16 to 42.9± 6.8 during the study period.

Similarly, there were no significant differences in GI throughout the sampling period at the two sites. Physiological parameters seems to be related to abiotic factors such as pH and dissolved oxygen in seawater (Figure 5).

Stress on stress curves and LT_{50} values for all sampling months at both study sites are reported in Figure 3. LT_{50} values ranged from 5 to 9. No differences were observed across the study sites. Survival time was longer at both sites in May than in August. There were no significant physiological differences related to chemical contamination at the two stations. However, mussel metabolic adaptation response was naturally more intensive when the temperature was at its highest in August, hence apparently affecting the resilience of immersed mussels.

Biotransformation enzymes

The markedly high water temperature at our study sites appeared to influence the level of expression of all analyzed biomarkers. Indeed, the three studied biomarkers involved in biotransformation mechanisms (BPH, GST and MXR) revealed a very noticeable monthly variation starting in July, when the water temperature rose sharply from 19°C to 26°C (Table 1). In fact, BPH activity measured in the digestive glands of mussels sampled from the Menzel Abdelrahmen site showed a very significant ($\rho<0.05$) induction in August (p=0,00005), when the water temperature reached 29°C (Figure 4A). BPH activity was significantly higher ($\rho<0.05$) in animals sampled from the Menzel Abdelrahmen site (Site 1) versus Site 2 (F=23, 1747; p=0,0001). The highest values (0.31 and 0.27 $nmole.mn^{-1}.mg^{-1}$ proteins) were recorded at Site 1 in June and August respectively, but did not correspond to a progressive temperature elevation. No significant monthly variations were recorded at Site 2. BPH activity appears to be the most biomarker related to PAH accumulation (Figure 5), thus indicating the induction effect of PAH contamination on enzyme of phase I.

Variations in GST activity in mussel digestive glands were also observed during the study period. GST activity ranged from 91.8 to 176.8 $\mu mole.mn^{-1}.mg^{-1}$.proteins at Site 1 and 129.8 to 157.9 $\mu mole.mn^{-1}.mg^{-1}$ proteins at Site 2 (Figure 4B). GST decreased progressively as temperature increased. A significant difference in GST activity at both sites was also observed in June ($\rho_1=0.000021$; $\rho_2=0.0236$) and August ($\rho_1=0.000025$; $\rho_2=0.0010$) respectively for site 1 and site 2.

MXR protein expression in the gills of mussels collected monthly in late spring and summer appeared to be more affected by water temperature elevation in August (Figure 4C). MXR protein levels were significantly lower at Site 2 than at the more contaminated Site 1 ($\rho<0.05$) (F=50.930 ; p=0.000001). The highest levels were observed in August at both sites, with 218.4 OD/µg proteins and 125.9 OD/µg proteins at Sites 1 and 2 respectively.

Oxidative stress

Oxidative stress biomarkers were highest when water temperature was at its highest in July and August. Moreover, CAT activity was significantly higher in the digestive glands of *M.galloprovincilais* collected from both sites in July and August (Figure 4F). CAT activity ranged from 159.8 to 375.6 µmole.mn^{-1}.mg^{-1}proteins at Site 1 and from 153.6 to 340.4 µmole.mn^{-1}.mg^{-1}proteins at Site 2. The amplitude of response was similar from May to June and no clear inter-site trends were observed during the study period.

MDA accumulation (Figure 4E), evaluated as TBARS in mussel digestive glands, differed significantly at the two sites ($\rho<0.05$) (F= 15.285 ; p= 0.001). At Site 1, MDA accumulation ranged from 2.6 to 4.9 nmole.mg^{-1} proteins and the highest value was recorded in August. At Baie des Carrières (Site 2), MDA content was homogenous and increased progressively according to temperature. No significant temporal variations were observed.

Neurotoxicity

AChE activity measured in *M.galloprovincialis* gills (Figure 4G) showed this biomarker to be significantly inhibited in mussels sampled in July and August versus those sampled in late spring (May and June). AChE activity ranged from 21.5 to 60.3 µmole.mn^{-1} .mg^{-1} proteins in the gills of mussels from Site 1 and from 40.1 to 61.2 µmole.mn^{-1} .mg^{-1} proteins in the gills of mussels from Site 2 (F=18.893 ; p= 0.00038). The amplitude of response was higher at the more contaminated Site 1. A significant difference was observed in AChE activity in mussels sampled from Site 1 versus Site 2 during the summer months (June, July and August 2011) ($\rho<0.05$). Lower levels of activity were detected in mussels sampled from Site 1 and significant differences were also recorded during the biomonitoring survey conducted at the same site.

Metal-related stress

MT accumulation (Figure 4D) evaluated in mussel digestive glands revealed significant temporal variations. The temperature-dependent accumulation of MT was shown to be more pronounced in animals sampled from both sites in August. MT concentrations in the digestive glands of mussels from Site 1 were significantly higher than in those from Site 2 throughout the study period ($\rho<0.05$) (F=46.526 ; p=0,000002). MT content ranged from 126.54 µg.mg^{-1} proteins in May to 203.95 in August and from 64.77 µg.mg^{-1} proteins in May to 150.29 in August in the digestive glands of mussels from Site 1 and Site 2 respectively. MT response appeared to be month-related at both sites, with lower values observed in May versus August.

IBR index

The IBR index demonstrates an increase of the metabolic response in relation with the temperature (Fig 6) and secondly with the salinity in the two sites 1 and 2. The lowest IBR values (from 2 to 6) were calculated in the two sites when the temperature is less than 18°C , in May and June. The IBR index increase greatly from June to August when the temperature raise from 19°C to 29°C. The greatest increase of IBR (from 5 to 19.6) is obtained in the most contaminated site 1 when the highest temperature and the highest chemical contamination are cumulated.

Discussion

The purpose of this study was to quantify the biological effects of the highest summer water temperatures on biomarker responses analyzed in mussels from Bizerte lagoon at two sites with differing degrees of chemical contamination (PAHs, PCBs, trace metals). Various works have reported a close relationship between thermal stress and biomarker response in marine molluscs (Regoli 1992; Domouhtsidou and Dimitriadis. 2001; Moore et al. 2006a, 2007; Zhang et al. 2006). In our study, significant alterations to biochemical markers were observed in mussels sampled from the most-contaminated Site 1 versus Site 2. Indeed, biomarker responses in *M.galloprovincialis* showed clear temporal and spatial variations. Temperature has been considered as the primary factor affecting natural enzyme activity (Leiniö and Lehtonen. 2005); and salinity can be considered as a second factor in the Bizerte lagoon. These variations therefore probably reflect environmental parameter changes associated with contaminant bioavailability. The biological availability and toxicity of trace metals are determined by their chemical speciation which is dominated by organic complexation along a gradient of salinity in estuary (Money et al., 2011)

Temporal variations in the Condition Index of mussels from both sites showed very similar patterns during the study period, with an increase reported in August. The Condition Index of mussels is affected by a variety of extrinsinc and intrinsic factors, such as water temperature and salinity, food availability and animal gametogenic cycle (Okumus and Stirling. 1998). Mussel survival (SoS) during prolonged aerial exposure can be used as a simple physiological index of pollution (Viarengo et al. 1995). This study showed that mussels collected from both sites in May had a greater capacity for survival in air than those sampled in August. The stress on stress (SoS) test is a physiological biomarker used to evaluate mussel resistance to air exposure

(Eertman et al. 1993). Various studies have demonstrated that bivalves exposed to contaminants have reduced tolerance to anoxia (Viarengo et al. 1995). We found a similar level of resistance and a clear-cut monthly pattern at both sites. However, decreased resistance was revealed in August, in particular in mussels from Site 1, confirming that this site is more extensively-affected by chemical contaminants such as metals, PAHs and PCBs, although reproductive cycles and variations in body reserves should also be taken into account. Lagoon mussels undergo a period of sexual inactivity in July and August (Banni et al. 2011), so that body reserves and, in particular, glycogen derived from food easily available in lagoon waters in this season (Bressan and Marin. 1985) can be used to better withstand stresses caused by air exposure. These factors may significantly reduce the responsiveness of the survival-in-air parameter.

The cytochrome P450 family, belonging to Phase I (functional reactions) and GST, involved in Phase II (conjugative reactions) of the biotransformation process, are the main enzymes used as biomarkers of organic pollutant detoxification (Regoli et al. 2002). Phase I and Phase II detoxification mechanisms were therefore investigated in our study by evaluating BPH and GST activity (Figure 5). We reported a significant increase in BPH activity in animals sampled from Site 1 in June and August versus animals from Site 2. This increase in the Phase I biotransformation process hence correlated with increased Σ PAH bioaccumulation in June and August. Inhibited GST activity at both sites was higher in June and August in the site 1 comparatively to the BPH induction. In this case the phases I and II seems indicates the same metabolic effort of detoxification (Figure 4).

MXR protein levels were significantly higher at Site 1 than Site 2 throughout the study period. This could be explained by the contamination levels found in mussels from Site 1 (Menzel Abdelrahmen) (fig 1 C). MXR prevents the bioaccumulation of toxic xenobiotics or endogenous metabolites by transporting them out of the cell (Minier and Moore. 1996). The increase in MXR protein levels in August can be explained by the increased water temperature. Indeed, abiotic factors such as temperature (Minier et al. 2000) and organic pollutants have been shown to affect MXR protein levels, as we observed in *M.galloprovincialis*. In this case, the phase III of detoxification is not correlated with the phases I and II.

Oxidative stress biomarkers in *M.galloprovincialis* showed spatial variability, with significant MDA accumulation in mussels from Site 1 versus Site 2 throughout the study period, whereas CAT activity was significantly higher in August only. The activity of CAT - a primary enzyme in the antioxidant defence system and often one the earliest antioxidant enzymes to be induced

(Capello et al. 2013) - was also investigated in this study. CAT values showed temporal fluctuations at both study sites. These biological responses may also be modulated by seasonal changes in both environmental and biological factors, potentially influencing responsiveness and sensitivity to pollutants (Dellali et al. 2001). Our results were in total agreement with the fluctuations in CAT activity according to temperature found in *M.galloprovincialis* mussels and *Ruditapes decussatus* clams in Bizerte lagoon (Dellali et al. 2001). Pellerin-Massicotte (1994, 1997) reported increased CAT antioxidant activity as being associated with increased lipid peroxidation at high temperatures in *Mytilus edulis* blue mussels. MDA levels are proportional to the extent of lipid peroxidation (Aust. 1985) and act as a marker for membrane lipid oxidation. Numerous studies have underlined MDA concentration as a major parameter for assessing oxidative stress in organisms (Roméo and Gnassia-Barelli. 1997). MDA levels in *M.galloprovincialis* sampled from Site 1 were highest in August. This increase appears to be due to an increase in heavy metals, inducing lipid peroxidation (Avery et al. 1996). Wastewater from numerous sewers is discharged directly into the eastern sector of the lagoon; the city of Menzel Abdelrahmen may also be responsible for enriching Site 1 with various pollutants (Mahmoud et al. 2010). In general, organisms with a reduced antioxidant status may be more prone to lipid peroxidation and therefore have higher MDA levels (Cossu et al. 2000).

From June onwards, AChE activity measured in the gills of mussels sampled at Baie des Carrières (Site 2) was slightly higher than at the Menzel Abdelrahmen (Site 1). Neurotoxic pesticides such as carbamates, organophosphates and pyrethrins are known to exert an inhibitory effect on AChE activity (Bocquené et al. 1993; Binelli et al. 2005). Other classes of compounds, such as heavy metals (Lionetto et al. 2003), may influence the AChE activity of an organism by altering the pathway of enzyme synthesis, or simply by affecting the general health of the organism by reducing enzyme production. This suggests an extensive domestic use of potential pollutants such as pesticides in summer. Indeed, AChE activity is commonly used to diagnose pesticide exposure in environmental monitoring studies (Mora et al. 1999; Davies et al. 2001), but can also be indirectly inhibited by other organic compounds.

Metallothionein (MT) measurements have been widely used to assess the effects of metal pollution in both laboratory (Viarengo et al. 1997) and field studies (Serafim and Bebianno. 2001). In this study, we reported a significant increase in MT levels in the digestive glands of mussels sampled from Site 1 versus Site 2. Temporal variations in MT levels at both sites were significantly correlated with water temperature, as previously shown by Sefarim et al. (2002).

The salinity could also influence the MT levels because the biological availability and toxicity of trace metals are determined by their chemical speciation which is dominated by organic

complexation along a gradient of salinity in estuary (Money et al., 2011). The salinity variation (hypoosmotic stress) in the marine environment can affect lot of biomarkers as MT, oxygen consumption rate and survival in air (SoS test) in *M. Gallorpovincialis* (Hamer et al., 2008) (Figure 5)

We observed a clearly significant spatial difference in the accumulation rate of MDA, MT and MXR for all measured biomarkers during the study period, although the highest values were measured in mussels from the Menzel Abdelrahmen site. Moreover, mussels from this site appeared to be more vulnerable to and affected by the higher water temperatures in July and August. This could be explained by the combined effect of chemical contamination at this site and higher temperatures in summer.

The IBR constitutes a practical and robust tool for assessing sensitivity to contaminants using combined biomarker responses. The IBR can be used to reflect the biological effects of contamination measured at various sites, regardless of variations in the biomarker sets used for index calculations (Beliaef and Burgeot. 2000, Sefarim et al. 2012). In our study, application of the IBR allowed us to identify the most highly-impacted site. The two sites studied in Bizerte lagoon (Sites 1 and 2) showed marked temporal and spatial variations (Figure 5). The IBR index showed biomarker responses to be higher in July and August at both sites. At Site 1, all measured biomarkers were shown to be significant with regards to overall biochemical response. IBR elevation was particularly significant between June and July. When the temperature rose from 19°C to 26°C, the IBR increased from 5 to 18 at Site 1. These results highlighted Menzel Abdelrahmen (Site 1) as being the most highly-impacted site. Furthermore, correlation analysis showed a highly significant correlation between the IBR index and water temperature (r=0.661; r=0.519), respectively at Sites 1 and 2. The strong fluctuation of the biological response observed with the IBR index allowed us to characterize a maximal metabolic effort in August in the site 1 and a minimal metabolic effort in May in the site 2 (Figure 6). In this study, both BAC and EAC thresholds were calculated according to the OSPAR approach (Davis and Vethaak. 2012) for caged mussels collected from a lagoon (Table 3). We calculated BAC based on the two study sites in Bizerte lagoon and our chemical data and biomarker results, considering Baie des Carrières (Site 2) as the least-contaminated site. EACs were calculated during the highest temperatures and highest metabolic response in August and at the most-contaminated Site 1 (Menzhel A.). Only two thresholds (AChE and SoS) determined in Bizerte lagoon could be compared with the OSPAR thresholds for *M.galloprovicialis* mussels. The background response of acetylcholinesterase in *M.galloprovincialis* mussels in Bizerte lagoon derived from OSPAR

2012 data (study performed on *M.galloprovincialis* wild mussels in the Mediterranean Sea (Spain) in the same season (Campillo-Gonzalez, pers. comm in 2012 ICES report)). This difference (EAC OSPAR = 10 nmol.min^{-1}.mg^{-1}proteins versus EAC Bizerte = 30 nnmol.min^{-1}.mg^{-1}proteins), seen between two Mediterranean sites, highlights the need to adapt thresholds to different area typologies (i.e lagoon in North Africa and open sea in Spain). The determination of EAC and BAC in Bizerte lagoon is a first step towards defining thresholds at two sites with different chemical contamination during sharp temperature rises in North Africa. A more exhaustive definition is needed to express metabolic response magnitude for a full physiological cycle.

Conclusion

This study revealed that increased water temperatures in summer, combined with the presence of organic and inorganic contaminants, can directly influence the biological response of mussels. The combined increases of temperature and salinity exceptionally caused major variations in biomarkers at the most highly-contaminated site (Menzel Abdelrahmen). The biomarkers found to be the most sensitive to the combined effects of chemical contamination and high temperatures were AChE inhibition, the induction of CAT activities and a large accumulation of MT, MDA and MXR proteins. The highest metabolic response was confirmed in August, when differences in the IBR values of the least-contaminated Site 2 and the most-contaminated Site 1 were also highest. This preliminary study, conducted at a lagoon in northern Tunisia, allowed us to propose preliminary EAC and BAC over a specific period of high temperatures and high salinity, when metabolic response was at its most intense. Another more accurate and designated study to determine response thresholds in Bizerte lagoon mussels should be performed over the course of one or two complete physiological cycles. This approach demonstrated the value of choosing a sensitive period in the mussel life cycle to facilitate biomarker interpretation and biomonitoring in a North African lagoon.

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Descriptions of Figures

Figure 1: Map of the study area and location of sampling sites at the Bizerta Lagoon. Site 1: S1 Menzel Abdelrahmen, site 2: S2 Baie des Carrières.

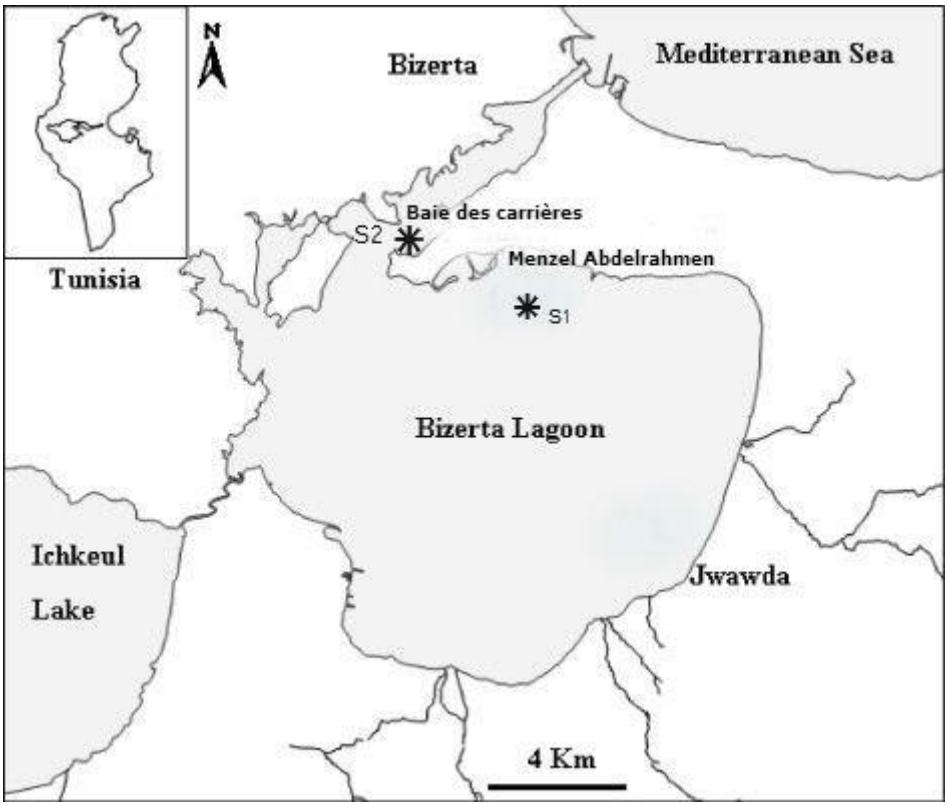
Figure 2: Temporal and spatial variations in the condition index (A) and gonadic index (B) of *M.galloprovincialis* collected from the Menzel Abderahmen (Site 1) and Baie des Carrières (Site 2) in late spring and summer. Superscript "a" indicates $\rho < 0.05$ (significantly different by ANOVA, multiple comparison and Duncan's test across sites (n=30)). Superscript "b" indicates $\rho < 0.05$ (significantly different by ANOVA, multiple comparison and Duncan's test among month (n=30)).

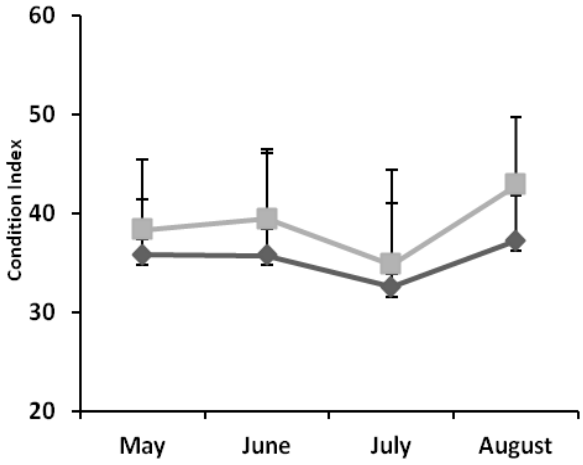
Figure 3: The Stress on stress response of mussels collected during summer 2011 from A. Menzel Abdelrahmen (Site 1) and B. Baie des Carrières (Site 2), both located in Bizerta Lagoon. . Superscript "a" indicates $\rho < 0.05$ (significantly different by ANOVA, multiple comparison and Duncan's test across sites (n=30)). Superscript "b" indicates $\rho < 0.05$ (significantly different by ANOVA, multiple comparison and Duncan's test among month (n=30)).

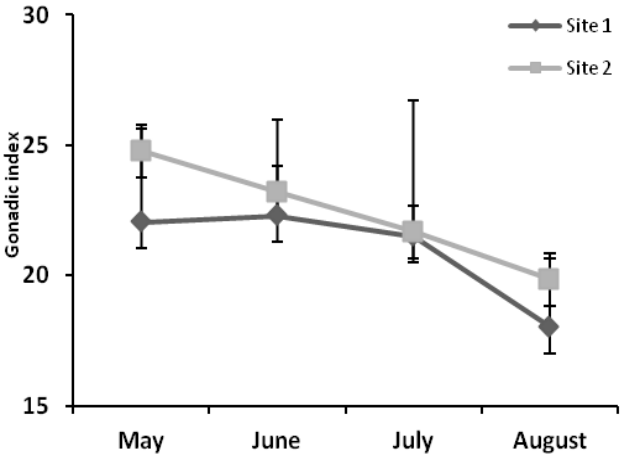
Figure 4: Spatial and temporal variations in biomarker response of *Mytilus galloprovincialis* collected from the Bizerta Lagoon, Menzel Abdelrahmen (Site 1) and Baie des Carrières (Site 2). A. Digestive gland BPH activity. B. Digestive gland GST activity. C. Densitometric analysis of MXR proteins as detected by Dot blot. D. Digestive gland metallothionein accumulation. E. Digestive gland MDA content. F. Digestive gland CAT activity. G. Gill AChE activity. Data represents means ±SD. Superscript "a" indicates $\rho < 0.05$ (significantly different by ANOVA, multiple comparison and Duncan's test across sites (n=10)). Superscript "b" indicates $\rho < 0.05$ (significantly different by ANOVA, multiple comparison and Duncan's test among month (n=10)).

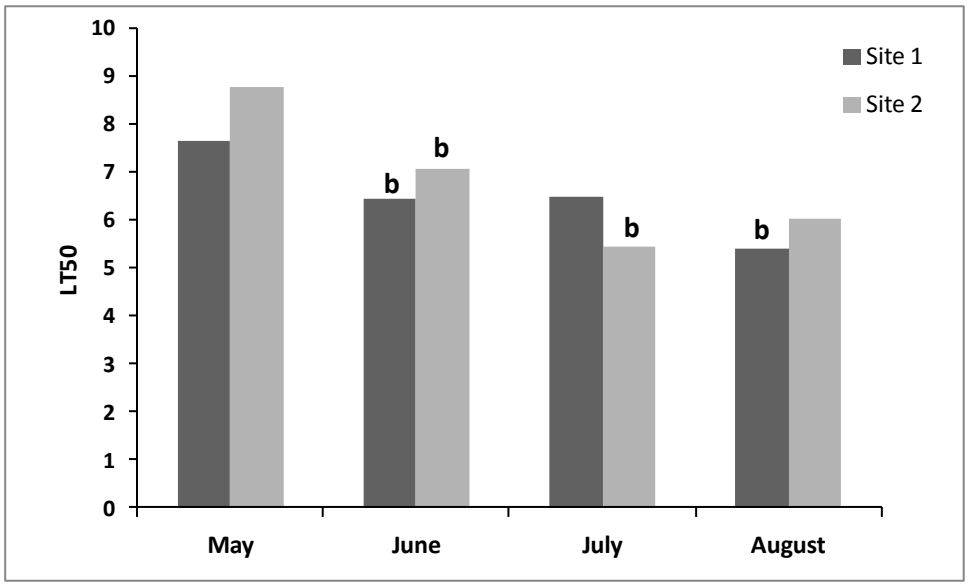
Figure 5: Principal component analysis (PCA) based on mean values: chemical, biolochemical, biological and physic chemical water variables (PC 1 vs PC 2) in the mussel collected from the Bizerta Lagoon, a) Menzel Abderahmen (Site 1) and b) Baie des Carrières (Site 2)

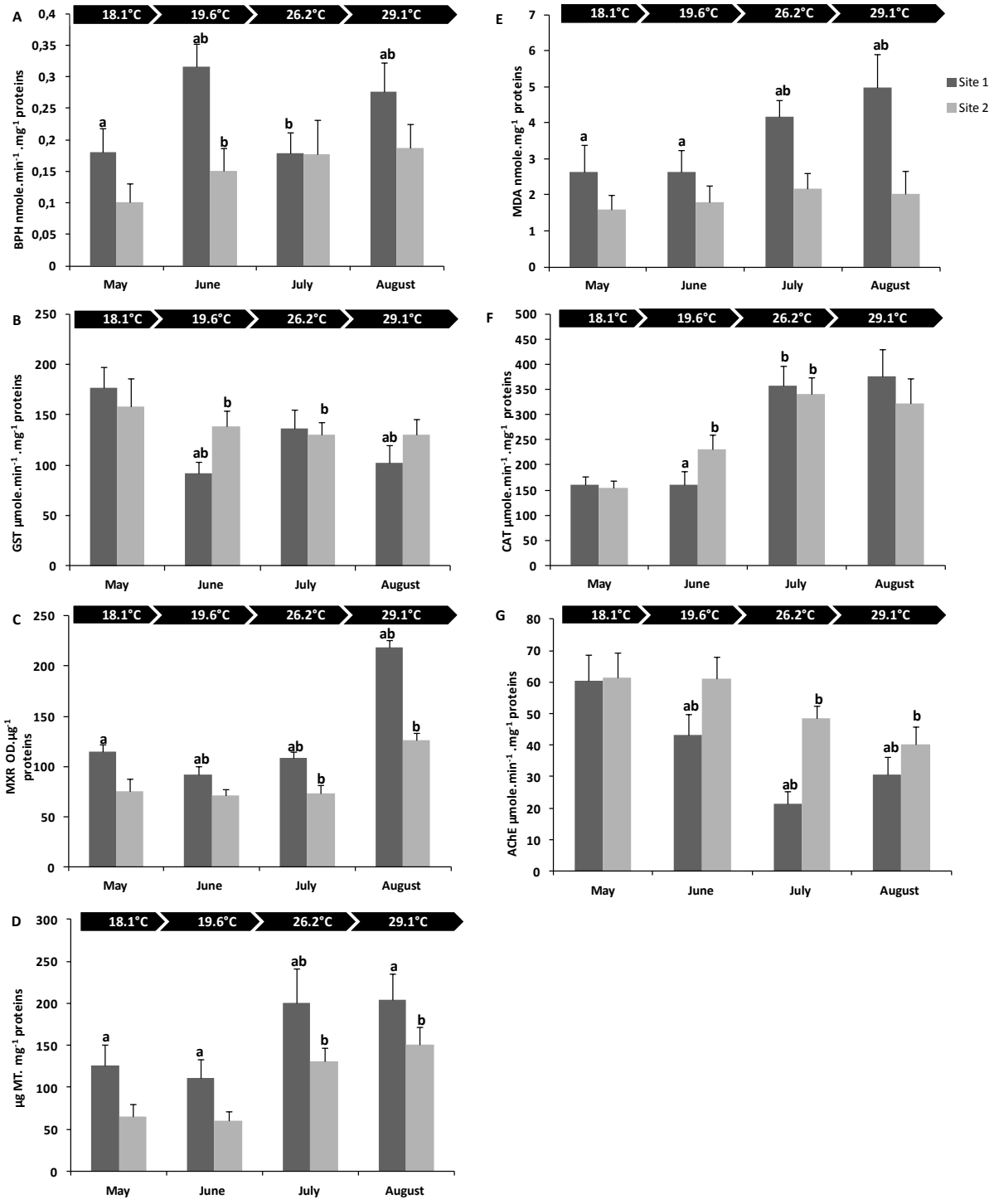
Figure 6: Variations in water temperature and Integrated Biomarker Response calculated for a set of biomarkers measured in caged mussels sampled from two sites (Site 1 Menzel Abdelrahmen and Site 2 Baie des Carrières) at the Bizerta lagoon during the hot months.

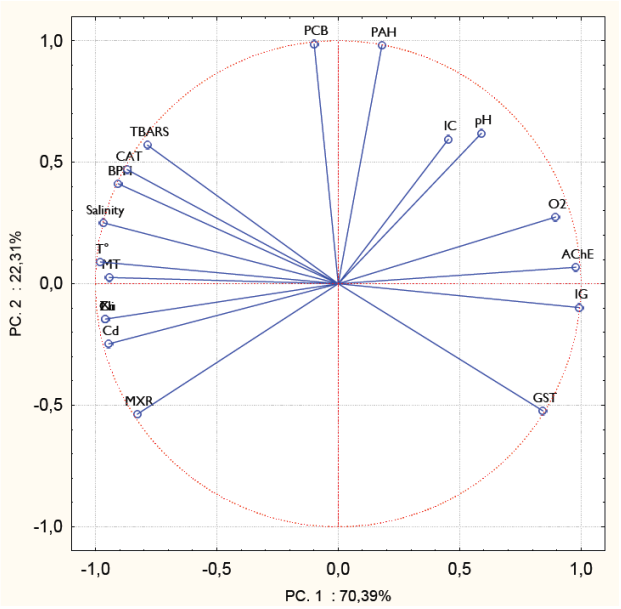

N
Bizerta
Mediterranean Sea
Baie des carrières
S2
Menzel Abdelrahmen
S1
Tunisia
Bizerta Lagoon
Ichkeul
Lake
Jwawda
4 Km


60
50
40
30
20
Condition Index
May
June
July
August

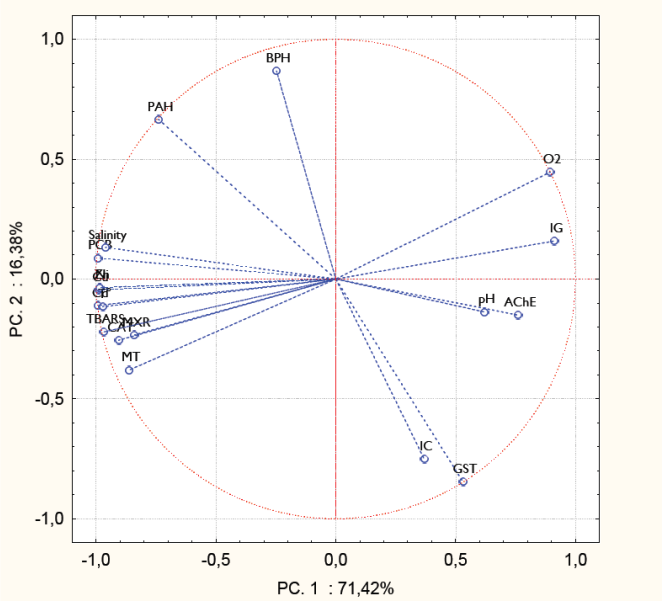

30
25
20
15
Gonadic index
Site 1
Site 2
May
June
July
August


10
9
8
7
6
5
4
3
2
1
0
LT50
Site 1
Site 2
b
b
b
b
May
June
July
August

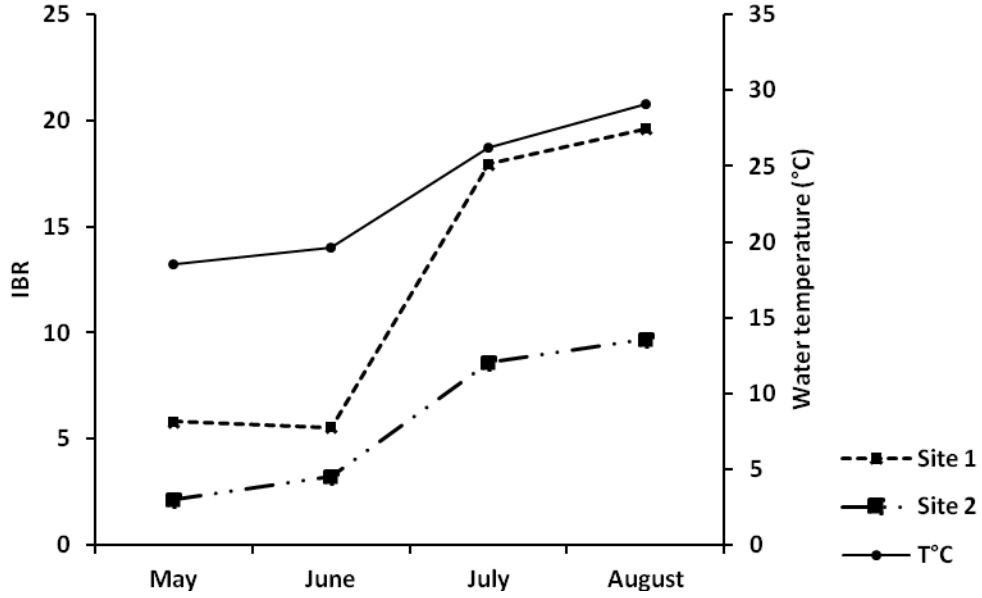

A
18.1°C
19.6°C
26.2°C
29.1°C
BPH nmole.min-1.mg-1 proteins
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
a
ab
b
ab
May
June
July
August
E
MDA nmole.mg-1 proteins
7
6
5
4
3
2
1
0
Site 1
Site 2
B
GST µmole.min-1.mg-1 proteins
250
200
150
100
50
0
F
CAT µmole.min-1.mg-1 proteins
500
450
400
350
300
250
200
150
100
50
0
C
MXR OD.µg-1 proteins
G
AChE µmole.min-1.mg-1 proteins
80
70
60
50
40
30
20
10
0
D
µg MT. mg-1 proteins
300
250
200
150
100
50
0


PCB
PAH
IC
pH
TBARS
CAT
BPH
Salinity
O2
T°
AChE
MT
IG
Cd
MXR
GST
1,0
0,5
0,0
-0,5
-1,0
PC. 2 : 22,31%
PC. 1 : 70,39%

a


BPH
PAH
O2
IG
Salinity
pH
AChE
MXR
MT
IC
GST
1,0
0,5
0,0
-0,5
-1,0
PC. 2 : 16,38%
PC. 1 : 71,42%

b


25
20
15
10
5
0
IBR
35
30
25
20
15
10
5
0
Water temperature (°C)
May
June
July
August
Site 1
Site 2
T°C

Descriptions of Tables

Table 1. Physical and Chemical parameters in Bizerta lagoon. Values represents the mean and the standard deviation of temperature (T), salinity (sal), pH, dissolved oxygen (DO), turbidity (Tur), nitrite (NO_2^-), nitrate (NO_3^-), ammonia (NH_4^+), silicate (SiO_3^{3-}) and phosphate (PO_4^{3-}).

Table 2. PAH, PCB and trace metal concentrations in the digestive glands of *M.galloprovincialis.* The above values represent mean and standard deviation in pooled samples of digestive glands. Concentrations are expressed as (µg/g dry weight), (ng/g dry weight) and (µg/g dry weight) respectively for PAHs, PCBs compounds and trace metals. <DL: below detection limit. Superscript "a" indicates $\rho < 0.05$ (significantly different by ANOVA, multiple comparison and Duncan's test across sites). Superscript "b" indicates $\rho < 0.05$ (significantly different by ANOVA, multiple comparison and Duncan's test among month).

Table 3. Assessment of a set of biomarkers in *M.galloprovincialis* caged mussels at the Bizerta Lagoon. BAC= Background assessment criteria and EAC= Environmental assessment criteria.
a We cannot compare the EAC and BAC calculated in this study with those calculated for MT by Davies &Vetaack 2012 because of the two different methods of analysis.

Table 4. Values of Integrated Biomarker Response for a set of biomarkers measured in caged mussels from two sites (Site 1 Menzel Abdelrahmen and Site 2 Baie des Carrières) at the Bizerta lagoon during the summer.

	May	June	July	August
T (°C)	18.1± 2.2	19.6±2.1	26.2±2.8	29.1±3.1
Sal (psu)	32±2.9	32.7±3.4	33.4±3.4	37.8±2.9
pH	8.35±1.2	8.3±0.92	8.5±0.81	8±0.95
DO ($mg.L^{-1}$)	7.5±0.6	8±0.75	6.9±0.58	6.2±0.57
Tur (NTU)	1.6±0.18	1.8±0.95	2.1±0.24	2.5±0.18
Chl- a ($\mu g.L^{-1}$)	2.185±0.23	2.012±0.21	2.326±0.21	3.642±0.29
NO_2^- ($\mu mol.L^{-1}$)	0.57±0.05	0.615±0.01	0.535±0.06	0.595±0.03
NO_3^- ($\mu mol.L^{-1}$)	4.1±0.45	4.3±0.44	4.9±0.45	5.6±0.6
NH_4^+ ($\mu mol.L^{-1}$)	23.65±2.42	24±2.6	21.7±2.19	23.2±2.5
SiO_3^{3-}($\mu mol.L^{-1}$)	3.385±0.37	2.645±0.19	3.125±0.33	2.95±0.31
PO_4^{3-}($\mu mol.L^{-1}$)	0.5±0.07	0.715±0.06	1.09±0.13	1.3±0.17

Data represent the mean of five values measured in several sites on the lagoon at each sampling time.

Biomarkers	Tissue	Suggested BAC for the Bizerta lagoon	Suggested EAC for the Bizerta lagoon	BAC OSPAR (Davies et Vetaack, 2012)	EAC OSPAR (Davies et Vetaack, 2012)
AChE	Gills	43 (nmol.mn^{-1}.mg^{-1} P)	30 (nmol.mn^{-1}.mg^{-1} P)	15 (nmol.mn^{-1}.mg^{-1} P)	10 (nmol.mn^{-1}.mg^{-1} P)
CAT (µmol.mn^{-1}.mg^{-1} P)	Digestive gland	335	370	_	_
MDA (nmol.mg^{-1} P)	Digestive gland	2	5	_	_
MT[a] (µg.mg^{-1} P)	Digestive gland	144	203		
BPH (nmol.mn^{-1}.mg^{-1} P)	Digestive gland	0.2	0.3	_	_
GST (µmol.mn^{-1}.mg P)	Digestive gland	152	165	_	_
MXR (OD.µg^{-1} P)	Gills	111	187	_	_

Compound	Site 1 (Menzhel Abderahmen)				Site 2 (Baie des Carrières)			
	May	June	July	August	May	June	July	August
Acenaphtylene	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Acenaphtene	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Naphthalene	0,1±0.01	0,15±0.04	0,15±0.02	0,16±0.03	<DL	0,06±0.01	0,07±0.01[a]	0,08±0.01[a]
Phenanthrene	0,11±0.01	0,12±0.01	0,17±0.04	0,14±0.03	<DL	0,08±0.01	0,07±0.01[a]	0,06±0.01[a]
Anthracene	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Fluoranthene	0,19±0.03	0,22±0.04	0,25±0.04	0,2±0.04	0,18±0.05	0,17±0.02	0,16±0.02[a]	0,11±0.02[a]
Pyrene	0,22±0.04	0,21±0.05	0,2±0.04	0,21±0.04	0,15±0.04	0,15±0.03	0,15±0.02	0,1±0.01[a]
Benzo(a)anthracene	0,25±0.04	0,24±0.04	0,19±0.04	0,28±0.06	0,19±0.04	0,14±0.03[a]	0,15±0.03	0,12±0.02[a]
Chrysene	0,11±0.02	0,15±0.02	0,14±0.02	0,16±0.02	<DL	<DL	<DL	<DL
Benzo(b)fluoranthene	0,14±0.01	0,19±0.03	0,17±0.03	0,19±0.04	<DL	<DL	<DL	<DL
Benzo(e)Pyrene	0,09±0.01	0,12±0.01	0,12±0.02	0,13±0.01	0,06±0.01	0,06±0.01[a]	0,08±0.01	0,09±0.01
Benzo(a)Pyrene	0,22±0.03	0,3±0.07	0,28±0.05	0,31±0.05	0,08±0.01[a]	0,1±0.01[a]	0,09±0.01[a]	0,09±0.01[a]
Benzo(g,h,i)Perylene	0,2±0.04	0,28±0.07	0,25±0.04	0,25±0.03	<DL	<DL	<DL	<DL
Dibenzo[a,h]anthracene	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
PCB-18	0.44±0.04	0.48±0.06	0.53±0.04	0.28±0.03	0.23±0.04[a]	0.27±0.04**a**	0.26±0.04	0.20±0.02
PCB-28+31	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
PCB-52	1.44±0.14	1.60±0.20	1.79±0.19	0.93±0.11	0.78±0.12[a]	0.90±0.15[a]	0.88±0.12[a]	0.66±0.06[a]
PCB-44	1.68±0.16	1.11±0.14	1.24±0.13	0.64±0.07	0.54±0.08[a]	0.62±0.10[a]	0.61±0.08[a]	0.47±0.05[a]
PCB-101	9.54±0.92	10.57±1.33	11.80±1.25	9.94±1.15	5.15±0.80[a]	5.92±0.98[a]	5.84±0.79[a]	4.51±0.43[a]
PCB-118	8.72±0.84	9.71±1.22	8.52±0.90	9.44±1.10	4.73±0.74[a]	5.44±0.90[a]	5.36±0.73[a]	4.14±0.40[a]
PCB-149	3.71±0.36	4.13±0.52	4.61±0.49	8.52±0.99	2.01±0.31[a]	2.31±0.38[a]	2.28±0.31[a]	1.76±0.17[a]
PCB-138	9.80±0.94	10.91±1.37	12.17±1.29	13.97±1.62	5.31±0.83[a]	6.11±1.01[a]	6.02±0.82[a]	4.65±0.45[a]
PCB-153	16.98±1.63	18.12±2.28	16.39±1.74	20.09±2.33	14.01±2.19	19.38±3.22	18.30±2.49	13.63±1.31[a]
PCB-180	21.59±2.07	18.92±2.38	17.26±1.83	16.30±1.89	9.22±1.44[a]	10.60±1.76[a]	10.45±1.42[a]	10.24±0.98[a]
PCB-194	2.56±0.25	2.23±0.28	2.48±0.26	2.05±0.24	1.08±0.17[a]	1.25±0.21	1.23±0.17[a]	0.95±0.09[a]
Cd	0.34±0.06	0.36±0.07	0.43±0.06	0.49±0.09	0.18±0.02[a]	0.19±0.03[a]	0.19±0.04[a]	0.24±0.04[a]
Cu	2.53±0.11	2.68±0.11	3.01±0.19	3.70±0.15	1.72±0.10[a]	1.91±0.11[a]	1.96±0.12[a]	2.39±0.14[a]
Zn	36.90±1.95	39.11±2.07	43.17±2.28	53.96±2.85	22.84±1.40[a]	25.35±1.55[a]	26.04±1.60[a]	31.77±1.95[a]
Ni	2.84±0.23	3.01±0.25	3.33±0.27	4.16±0.34	1.68±0.16[a]	1.87±0.18[a]	1.92±0.19[a]	2.34±0.23[a]

	IBR Site 1	IBR Site 2
May	5,80	2,13
June	5,54	3,22
July	17,94	8,57
August	19,63	9,67

Dear Editor,

Thank you very much for you message concerning the review of our manuscript titled "Effects of increasing temperatures on biomarker responses and accumulation of hazardous substances in rope mussels (*Mytilus galloprovincialis*) from the Bizerta Lagoon". ESPR-D-13-01857. Authors also want to thank the advisors for their instructive comments and for the interest showed regarding our manuscript.

Answer to the Reviewer comments:

Reviewer #1:

Specific comments:

1. Page 3, line 82: please indicate the full name before the abreviation PBDEs. Done
2. Line 84: this is a continuation of the previous text, so paragraph should not be used here.

Response: this sentence is a connection between two paragraphs. We have reformulated this passage.

3. Line 97: "oxidative stress" and not "oxidative stresses; and delete "the" before Mytilus. Done
4. Line 98: delete "mussel" Done
5. Line 99: "benzo..." and not "Benzo..."; please notice that the name of the chemicals should not be started by caps logs unless it starts a sentence; please review all the text regarding this, there are similar mistakes in other points of the text. Done
6. Page 4, lines 114-115: What kind of index are you referring too? This should be clearly indicated.

Response

The condition index is mainly used for two purposes: first, as a gauge of the quality of meat for the market place (Orban et al., 2002), and second, an ecophysiological measure of the health status of animals. Indeed, it summarizes the physiological activity of the organisms (growth, reproduction, secretion, etc.) under given environmental conditions (Lucas and Beninger, 1985).

The reference used for calculating CI was added in materials and methods section.

7. Line 143: what kind of trace elements? Metals? This should be indicated.

Response: The sentence was reformulated as:

Tissue levels of metallothionein proteins (MT) were estimated in mussel digestive glands in order to assess the trace metals pollution in aquatic environment end evaluate their biological effects on sentinel organisms.

8. Lines 161-162: the sentence should be supported by a reference.

Response: The following reference was added in line 162 and to the final reference list.

Mediterranean Action Plan (2011) Development of assessment criteria for hazardous substances in the Mediterranean. UNEP (DEPI)/MED WG. 365/Inf.8

9. The objectives of the work and the rationale should be clearly indicated at the end of the discussion. Please notice that applying an index is not by itself a scientific objective and that no ecological risk assessment was performed. So, the central and specific objectives are not indicated in a suitable way.

Response

The objectives of the work were restate at the end of the introduction, as requested by the reviewer: L165 "The aim of this study was to examine the influence of increasing temperatures in spring and summer on the biomarker responses of mussels inhabiting a North African lagoon, recognized as a sensitive area in terms of temperature changes and pollution. Biomarker variations were initially compared during the highest temperature elevation, between spring and summer. The amplitude response of the biomarkers was then studied across two sites characterized by different levels of chemical contamination"

Application of composite indices is not a goal in itself, but this is the first work that applies these methods in this lagoon.

10. page 5, Material and Methods: why was this particular lagoon selected?

Response

This environment, in light of its location between Lake Ichkeul and the Mediterranean, is characterized, as are most Mediterranean lagoons, by a great variability in its ambient conditions, in particular insofar as the hydrological parameters, such as salinity, temperature and dissolved oxygen levels, are concerned.

11. Why only 2 sampling sites and why these particular ones?

Response

The aim of studding only 2 sampling sites was not to compare the contamination levels but to evaluate the physiological responses of *M.galloprovincialis* in the lagoon.

12. What about reference sites in another system that could be used as reference? As far as I could understand both sampling sites are contaminated, so how can the effects on mussels can be correctly evaluated?

Response

Selecting a reference site outside the lagoon does not appear relevant because we wanted to meet the homogeneity Exposure of the molds within the lagoon. This is a constraint of the study we had to integrate. A site at sea open would not have to compare homogeneous exposure areas between lagoon and open sea views. We have selected two sites different contamination with uncontaminated site and a site more contaminated. The difference in response biomarkers shows that the choice of the two sites is sufficient contrast in contamination chemical.

13. Why the sampling was made only in spring and summer? Why not also in the Autumn and Winter to also investigate seasonal variability? Notice that in summer mussels may be affected by the bivalve summer syndrome, especially in very warm summers, while in the spring they are actively reproducing what may introduce bias on the biomarkers results.

Response

The present work constitutes a continuation of previous investigation conducted by our group using the same organism (Banni et al.2011 and Kamel et al. 2012) in these works we establish the physiological response of the mussel during an annual cycle in the Bizerta lagoon, these results showed a very important variation in physiological response due to thermal variation in summer, we have also study the effects of increasing temperature in experimental condition (in laboratory).

14. The size of the mussels should be indicated and the size of mussels from the two sampling sites should be compared with an adequate statistical analysis (e.g. Students t-test). Done

Response:

Line 180 : Mussels of similar sizes (5-7 cm shell length) were sampled monthly from both sites in late spring and summer 2011.

Line 332; Differences in mussel size between the two sites were evaluated using Student test ($\rho < 0.05$) for each sampling date

15. Where standards used in chemical analysis determinations? This should be cleraly indicate, as well their type and the values obtained for validatio purposes.Line 238: what do you mean exactly by "when did not recover...". How was recover!y assessed?

Response

Standards were added in Line 206.

L 238: here recover it mean to go back. The term was replaced with “react”

16. Line 45: check the date of the reference Bradford, it appears here as 1979, in other sites as 1976 that I believe is the date you would like to indicate. Check also the name of the reagent in line 246. Done

Response:

it was a not careful mistake, the correct reference is Bradford 1976, this has been corrected in the text and in the reference list

16. All the description of biomarkers determination detechniques lack important details, please complete it carefully, indicate the equipment (model company) used, the temperature, some references are missing, etc. Done

Response:

All the section M & M has been verified, and the authors have added more details and reviewer recommendations were taken into account.

16. How many replicates where done? Individual animals or pooled samples? this should be clearly indicated for both chemical and biological analysis.

Response

Biological analysis : Ten mussels (n = 10) from each site were analyzed individually for each biomarker measurements.

Chemical analysis : PAH content in the digestive gland fractions (three distinct pools prepared (pool =3 digestives glands))

Determination of PCB content Freeze-dried digestive gland tissue (10 g)

Determination of heavy metal content: (five distinct pools prepared as described above)

17. The use of the Analysis of variance (ANOVA) is only adequate when ANOVA assumptions are fulfield. How were they tested (for each parameter) and if data transformations were made they should be indicated.

Response

The experimental data were first tested for normality and homogeneity of variance to meet statistical demands. Data was expressed as mean±standard deviation (SD).

18. What kind of ANOVA was done with what data precisely? this should be clear.

Response: Data statistical analysis was performed using using one-way analysis of variance (ANOVA) and Duncan's test for multiple range comparison ($\rho<0.05$) was considered as significant. This was added in the Ms, statistics sub-section (line: 323 -328)

19. Why was the Duncan test selected and not the Tukey (or other multicomparison test?)

Response

We applied the Duncan test because it's a multiple comparison procedure for comparing all means. This is a step by step procedure, based on a Studentized distribution ranks. This procedure indicates which means are significantly different from each other.

20. What do you mean by "Pearson correlation matrix"? Are you referring to the Pearson correlation coefficient? If so, are the data appropriate for its use? This should be checked. Anyway I found difficult to consider correlation because at least some of the biomarkers are expected to have a kind of bell shape distribution.

Response

The term "Pearson correlation matrix" was adjusted as suggested by reviewer. Authors totally agree with the reviewer comment regarding the rational in using the Pearson correlation coefficient. However, only significant correlations were reported in the text.

RESULTS

21. Table 1, correspond text in page 10 and legend of the table: What are exactly the values shown in the table? Pontual measurements? Means? If they are punctual measurements/determinations with replicates, they have no meaning. If they are means, the number of true replicates and how and when the measurements/determinations were made should be indicated. Where they done at low time or high tide? at What time of the day, etc? Variability (e.g. SD) should be given. This should be indicated in the material and methods section and in the table legend.

Response

Water quality was assessed at each sampling time. The physico-chemical quality of the Bizerta Lagoon waters was monitored in situ. Salinity (g/L), temperature (°C), dissolved oxygen (mg/L), and pH were measured at the sampling sites with a Multi 350i Multimeter.

Values represents the mean and the standard deviation of temperature (T), salinity (sal), pH, dissolved oxygen (DO), turbidity (Tur), nitrite (NO_2^-), nitrate (NO_3^-), ammonia (NH_4^+), silicate (SiO_3^{3-}) and phosphate (PO_4^{3-}).

Data represent the mean of five values measured in several sites on the lagoon at each sampling time.

22. The corresponding text in page 10 should be discussed after statistical analysis.

Response

We took into account the reviewer comments

Trace metal concentrations in the digestive glands of mussel showed both temporal and spatial variations. In general, the levels of all metals (Cd, Cu, Zn and Ni) were significantly higher in the digestive glands of mussels sampled from Site 1 versus Site 2 ($\rho<0.05$).

Almost all polycyclic aromatic hydrocarbon (PAH) compounds measured in mussel digestive glands were three times higher in those of mussels from the Menzel Abderahmen site (Site 1) versus the Baie des Carrières site (Site 2), the PAH content were significantly higher in digestive gland of mussel collected from Site 1 then Site 2 ($\rho<0.05$)...

Concentrations of polychlorinated biphenyls (PCBs) in digestive glands were significantly higher in mussels from Site 1 versus Site 2 ($\rho<0.05$). The monthly trends of ΣPCB at both sites were similar, with an increase in levels from May to August.

23. Chemical analysis Where there statistical significant differences between the sites for each determination?

Response

The statistical test was specified in the materials and methods section and the following was added to the table 2 description

a: Statistically significant differences ($P<0.05$) in comparison with site 1 at the same sampling period

24. The corresponding text in page 10 should be written according the results of statistical analysis.

This was adjusted in the point 21.

25. A table with the full results of statistical analysis of biological parameters should be given. The F values, degrees of fredoom should be given in addition to P values. The results section (page 11 and 12) should be written based on the results of statistical analysis.

Response

P and F values were added in results section, and a file containing manufacturing all statistical analyzes was joined.

26. I suggest to use a multivariate analysis approach integrating biological responses, physico-chemical parameters and chemical analysis (e.g. redundancy analysis) in order to relate biological responses and factors contributing to them.

Response

Reviewer recommendations were taken into account, ACP integrating biological responses, physico-chemical parameters and chemical analysis was performed with Statistica 6 and included in the manuscript at Figure 5

27. IBR values should be indicated in a table.

Table 4: Values of Integrated Biomarker Response for a set of biomarkers measured in caged mussels from two sites (Site 1 Menzel Abderahmen and Site 2 Baie des Carrières) at the Bizerta lagoon during the summer.

	IBR Site 1	**IBR Site 2**
May	5,80	2,13
June	5,54	3,22
July	17,94	8,57
August	19,63	9,67

Response

28. The figure 3 has no variability, this should be shown. Againg the sites should be compared by statistical analysis, as for the other biomarkers (for example first with a two way ANOVA with interactions with sites and time as main factors, and then relevant parameters should be integrated using a RDA analyis.

Response

This figure was adjusted and we applied ANOVA and Duncan's test.

29. The legend of the figures should be improved as indicated for tables, among other corrections. Done
30.
31. Correlations based on the Pearson correlation coeficient are not adequate because some of the parameters may have a bell shap or sigmoidal distribution.

Response

Authors totally agree with the reviewer comment regarding the rational in using the Pearson correlation coefficient. However, only significant correlations were reported in the text. Authors want also to highlight the fact that other statics analysis were tested "PCA" and thus authors are in agreement with the observation of reviewer and added an

ACP in addition coeficient pearson.

32. The discussion, conclusions and the abstract should be reformulated after the adequate analysis of data.

Response

The manuscript was reviewed by the authors, it has added more details in the materials and methods section, the results were discussed according to the statistical analysis.

Supplementary Material

Click here to download Supplementary Material: statistical analysis.docx