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Sampling Site Specific Biomarker Responses in Mediterranean Mussels from the Adriatic Sea

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Abstract
This study aims to explore the spatial and temporal patterns in biomarker responses during early spring and late summer in *Mytilus galloprovincialis* using samples from two Adriatic Sea ecosystems between 2009 and 2012. The condition index was higher in September at all sampling sites and suggests that mussels can store energy during summer for wintertime spawning and survival through the winter. Over the entire study period, higher values of metallothioneins indicated sites with higher levels of heavy metals (Boka Kotorska Bay), while acetylcholine esterase activity was inhibited at the Gulf of Trieste. Genotoxicity was similar among sampling sites. We summarized biomarker responses in a stress index, IBRv2, and found that sampling sites in the Gulf of Trieste had lower stress levels while the highest stress levels were detected in the Boka Kotorska Bay.

Keywords Pollution · Biomarkers · Health status · *Mytilus galloprovincialis* · Adriatic Sea

Anthropogenic pressures on marine ecosystems caused by urban development, industrialization and intensive agriculture are particularly pronounced in enclosed seas and bays due to their long water retention time, which increases the accumulation of pollutants. Pollutants have been a concern due to their potentially harmful effects on marine biota, with detrimental consequences for food production and ecological status of the marine environment (Hylland et al. 2017). Pollutants in the Adriatic basin have been studied in detail since the 1970s and certain hot spots along the eastern Adriatic Sea have been identified (Horvat et al. 1999; Ščančar et al. 2007; Kljaković-Gašpić et al. 2010; Joksimović et al. 2016; Bajt et al. 2019).

Exposure to pollutants can trigger biological effects in the cells, bodily fluids, tissues and organs of mussels. Such responses can be quantified using biomarkers (Beyer et al. 2017 and references therein). Several mussel biomarkers are used as an early warning tool to assess the biological effects of pollutants, some of which are used in various regional monitoring programmes including the ICES Working Group for the Biological Effects of Contaminants in the Atlantic Ocean, the OSPAR programme in the Northeast Atlantic, HELCOM at the Baltic Sea, and MEDPOL in the Mediterranean Sea (Lyons et al. 2017; Martínez-Gómez et al. 2017). The Marine Strategy Framework Directive (MSFD, Directive 2008/56/EC) considers biomarkers in sentinel species to be an important tool (Descriptor 8) for improving the ecological status of European waters. However, the use of biomarkers in monitoring programmes in the Mediterranean region has not yet been widely implemented (Hylland et al. 2017).

We measured responses in the Mediterranean mussel (*M. galloprovincialis*), a common sentinel species intensively used in ecotoxicological studies in the Mediterranean region (Beyer et al. 2017). The concentration of metallothioneins was measured as a specific biomarker of exposure to heavy metals while acetylcholine esterase inhibition was characterised as a neurotoxic effect of various pollutants. We also assessed the effects of genotoxic agents (by examining the formation of micronuclei) and general health status (through a condition index) as well as measuring environmental parameters (temperature, salinity, oxygen). These measurements were carried out between 2009 and 2012 in the northern and southern Adriatic Sea. Biomarker responses are a helpful tool for assessing the biological effects of pollutants.

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Biomarker responses can be summarized in a simplified index called IBRv2, which is based on biomarker deviation from a reference site (Sanchez et al. 2013).

Materials and Methods

The sampling sites were located in the Gulf of Trieste (Bay of Koper (ST1: 45° 32.890′ N, 13° 43.371′E), the Bay of Strunjan (ST2: 45° 31.695′N, 13° 36.098′E), the Bay of Piran (ST3: 45° 29.698′N, 13° 34.886′E)) and the Boka Kotorska Bay (Bay of Kotor (ST4: 42° 26.23′N, 18° 45.821′E); Bay of Herceg Novi (ST5: 42° 27.43′N, 18° 40.211′E)). Sites ST2 and ST4 are not known hot spots and have the lowest measured concentrations of pollutants (metals, PCB, PAHs) compared with other sampling sites according to 2008 measurements (Bajt et al. 2019). Sampling was carried out between 2009 and 2012 in early spring (March) and late summer (September) before spawning in order to evaluate the biomarker response during colder and warmer seasons. The mussels collected ranged between 40 and 60 mm in size. During mussel sampling, we measured abiotic seawater parameters (temperature, salinity, dissolved oxygen content) at a depth of 2 m for each sampling site. We used an MSS 90 multiparameter probe (Sea & Sun Technology) in the Gulf of Trieste and a Multi 350i handheld multi-parameter instrument (WTW, Xylem Brand) in the Boka Kotorska Bay.

The condition index (CI) was measured in twenty mussels at each sampling site following Lobel et al. (1991). The whole soft body was freeze dried and the shell length, height and width were measured using Vernier calipers. The dry tissue weight was measured to the nearest 0.001 g in tightly capped pre-weighed pots using an analytical balance and expressed in mg/cm^3. CI was expressed as the mean (± standard deviation) of twenty mussels per sampling site.

The spectrophotometric method described in Viarengo et al. (1997) was used to analyse metallothionein (MT) content in the hepatopancreas. We measured MT concentrations using five samples from each sampling site; each sample consisted of ten hepatopancreata combined into one sample. Concentrations were expressed as micrograms of MT per gram of tissue wet weight (μg/g w.w.) and reduced glutathione was used as a standard.

To measure acetylcholine esterase (AChE) activity in the mussel hepatopancreas or gills, we excised the tissue immediately after collection and snap froze it until analysis. The protocol is detailed in Tsangaris et al. (2016). Twenty mussels were analysed per sampling site. The activity was expressed in nmol of degraded acetylcholine (nmol/minute/mg proteins). AChE activity was expressed as the mean (± standard deviation) of twenty mussels per sampling site.

Micronuclei (MN) were evaluated in gills excised from ten mussels per sampling site, strictly following the protocol of Bolognesi and Fenech (2012). We inspected the slides under a light microscope at 1000-fold magnification using criteria for scoring MNs (Fenech 2007).

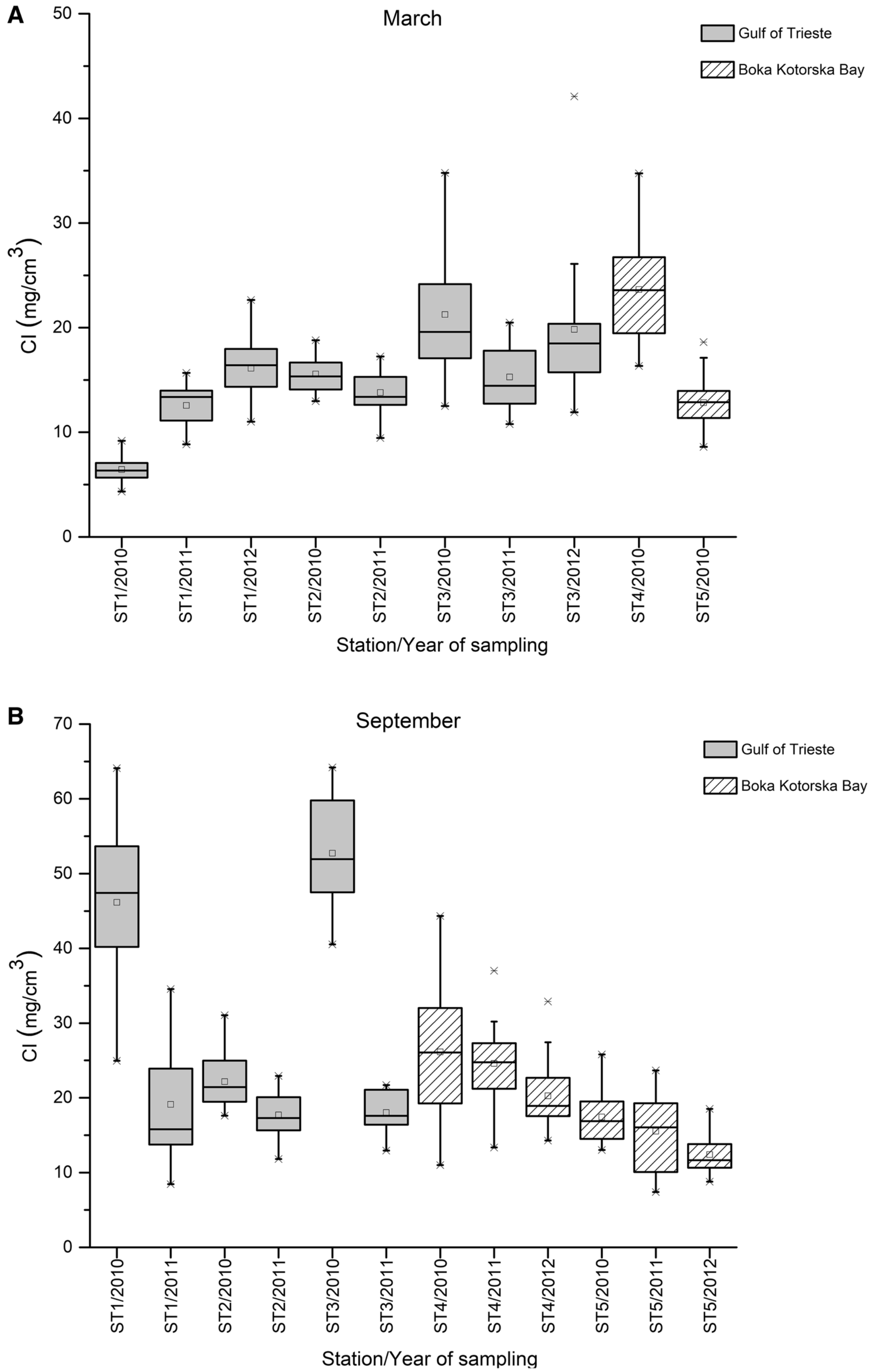
We assessed statistical differences in CI, MT concentration and AChE activity between seasons, years and sampling sites using a two-way analysis of variance (ANOVA). Differences in mean values were considered to be statistically significant at $p<0.05$ with a Tukey's post-hoc HSD test. The normality of the data was tested using the Shapiro–Francia test (Shapiro and Francia 1972), and the homogeneity of variance was tested using Levene's test. The results of CI and MT concentration in the digestive glands and AChE activity are presented as the means of the analysed replicates (± standard deviation) per sampling site and visualised in Figs. 1, 2 and 3.

Biomarker responses were evaluated using IBRv2, which is in principle based on the deviation between a disturbed and undisturbed state (reference site). Individual biomarker data were compared to biomarker data from a reference site. The data were log transformed to reduce variability and a biomarker deviation index (A) was then calculated for each biomarker at each site. IBRv2 is calculated from the absolute value of A as $\mathrm{IBRv2} = \Sigma \left| A \right|$ (Sanchez et al. 2013).

Results and Discussion

This study explores spatial and temporal patterns in biomarker responses measured during early spring and late summer from 2009 to 2012 in two locations on the Adriatic Sea, the Gulf of Trieste and the Boka Kotorska Bay. Environmental parameters, which are important to interpret biomarker responses, were measured at the time of mussel sampling. Seawater temperature in March ranged from 8.5 to 11.7°C in the Gulf of Trieste and between 9 and 15.1°C in the Boka Kotorska Bay. September temperatures reflect summer characteristics, ranging from 20.1 to 26.7°C in the Gulf of Trieste and 20.1°C to 27.9°C in the Boka Kotorska Bay. Summer seawater temperatures often exceed the thermal limits of *M. galloprovincialis*, the optimum temperature of which ranges from 20 to 25°C (Anestis et al. 2010). In this study, the greatest variability in salinity throughout the year was in the Boka Kotorska Bay, where it ranged from 2.50 to 35.8 PSU (at ST4), and from 12.5 PSU to 37.2 PSU (at ST5) due to underwater springs along the Karstic terrain of the coastline during heavy precipitation events (Magaš 2002). The Gulf of Trieste had a more uniform salinity throughout the year, ranging from 33 PSU to 37 PSU, mainly driven by river inflows (Malačič et al. 2012). The oxygen saturation of seawater is always lower in the Gulf of Trieste (5.40–8.82 mg/L) than in the Boka Kotorska Bay (7.4–11.81 mg/L) due to the inflow of freshwater. Organisms are often exposed to sub-optimal environmental conditions,

Fig. 1 Condition index (CI) for *Mytilus galloprovincialis* during March (**a**) and September sampling (**b**) between 2009 and 2012 at sampling sites in the Gulf of Trieste (ST1, ST2 and ST3) and the Boka Kotorska Bay (ST4 and ST5, Adriatic Sea). Boxes represent the inter-quartile range from the 25th to 75th percentile; line inside boxes: median; square: mean; cross: minimum and maximum values. Twenty mussels were analysed at each site during each collection



which are natural stressors that affect organisms' physiology and uptake of pollutants and, consequently, biomarker responses. The combined effects of various natural stressors and pollutants are common phenomena (e.g. heat stress can lead to higher uptake of certain metals). Investigations have revealed that such effects can often be synergistic (e.g. Holmstrup et al. 2010). Recent studies have shown that increased winter temperatures have a stronger impact on mussels due to an increased metal uptake than higher summer temperatures. Biomarker responses show similar

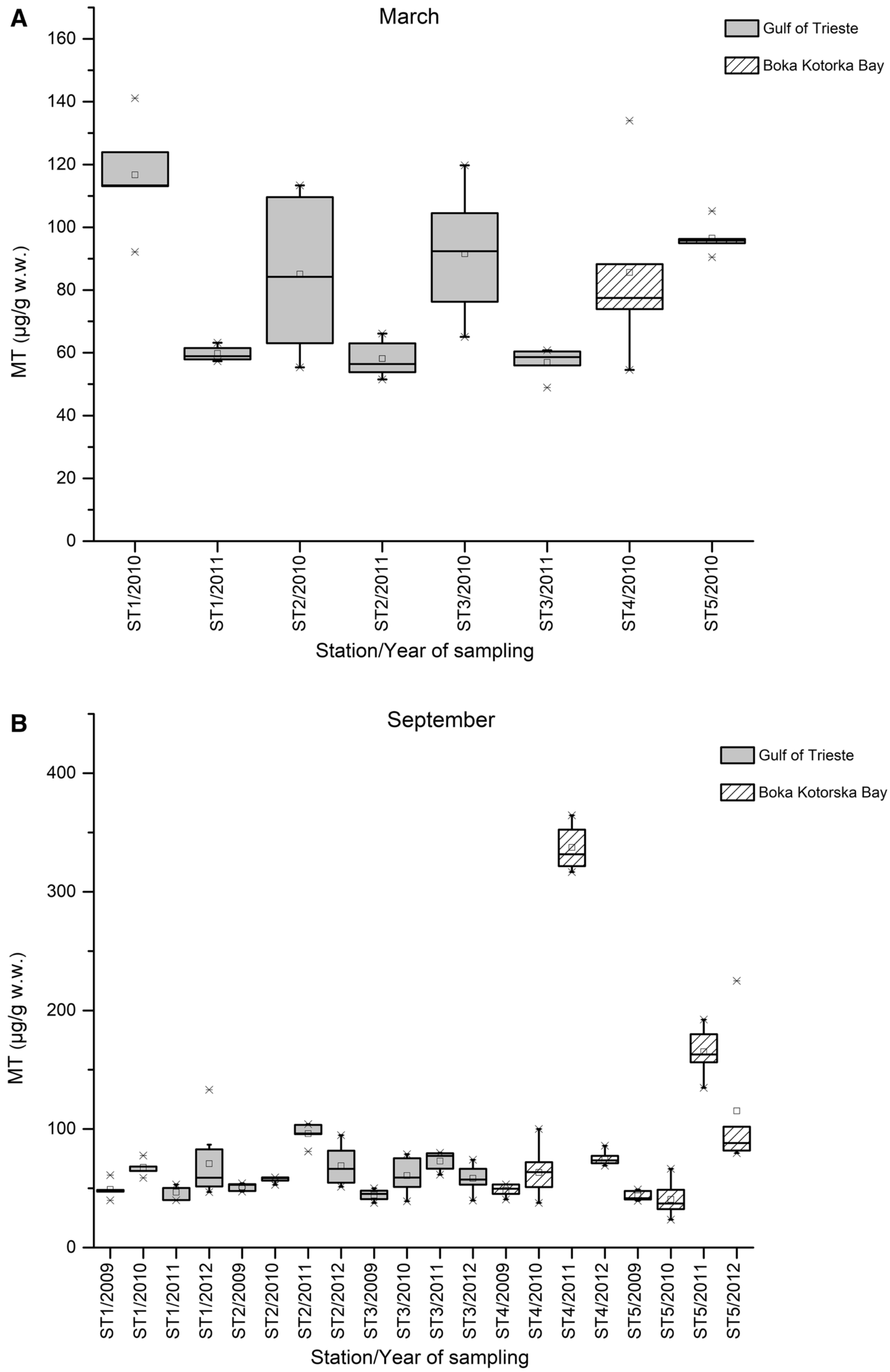


Fig. 2 Metallothionein concentrations in the hepatopancreas of *Mytilus galloprovincialis* during March (**a**) and September sampling (**b**) between 2009 and 2012 in the Gulf of Trieste (ST1, ST2 and ST3) and the Boka Kotorska Bay (ST4 and ST5, Adriatic Sea). Boxes represent the interquartile range between the 25th and 75th percentiles; line inside boxes: median; square: mean; cross: minimum and maximum values; 50 mussels analysed per site

patterns, as shown by elevated MT concentrations and genotoxicity (Nardi et al. 2018).

The health status of mussels was evaluated using the CI, which reflects food availability and physicochemical conditions in a particular environment (Lobel et al. 1991). Other factors such as parasitism and pollution can also have a significant negative impact on the CI (Bignell et al. 2008). During March (Fig. 1), CI values were significantly higher in the Boka Kotorska Bay than in the Gulf of Trieste ($p < 0.05$), probably due to the harsher survival conditions in the colder conditions in the northern Adriatic Sea. The results were reversed in late summer, with

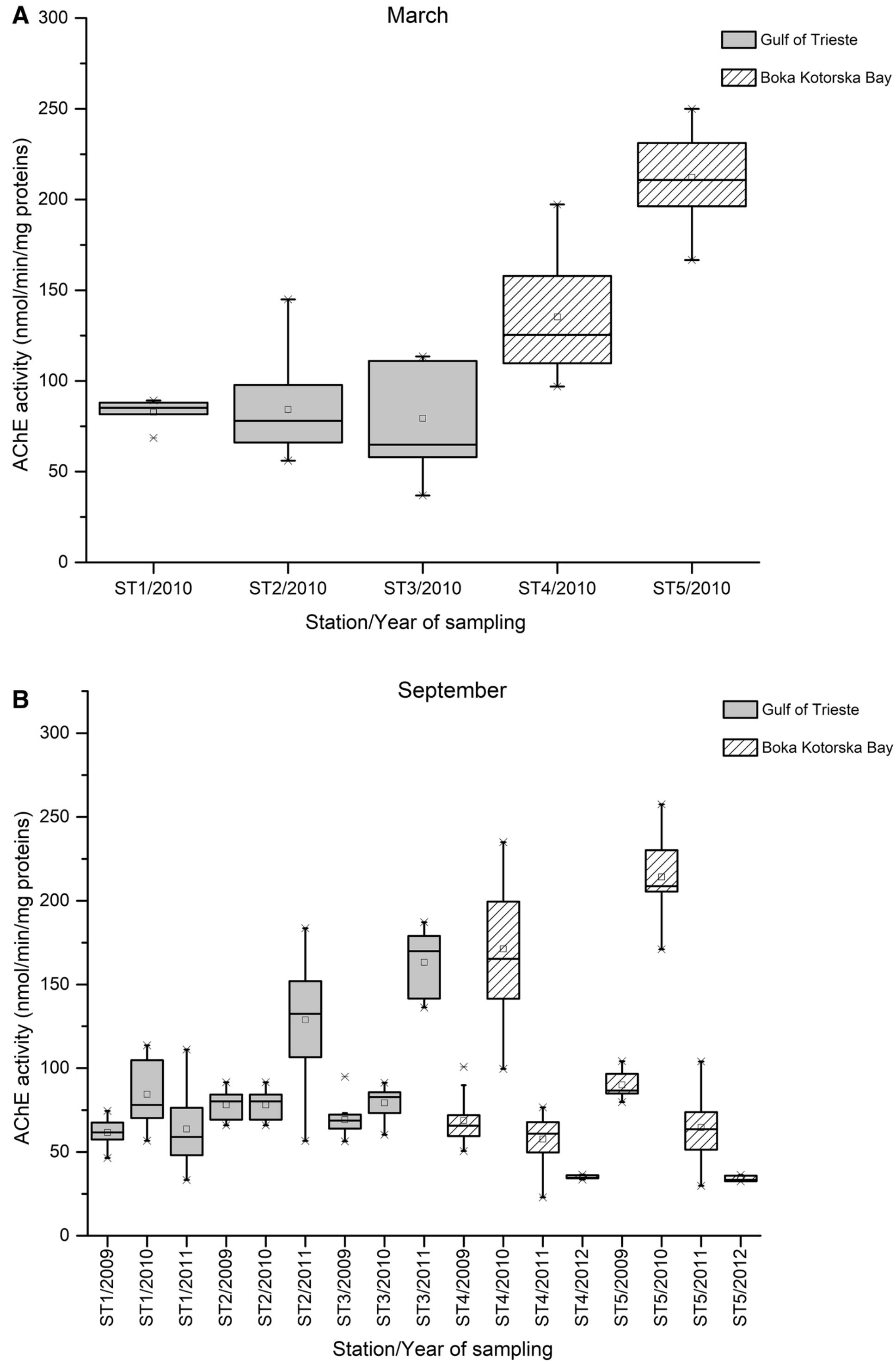


Fig. 3 Activity of acetylcholine esterase (AChE) in *Mytilus galloprovincialis* during sampling in March (**a**) and September (**b**) between 2009 and 2012 in the Gulf of Trieste (ST1, ST2 and ST3) and the Boka Kotorska Bay (ST4 and ST5, Adriatic Sea). Boxes represent interquartile range from the 25th to 75th percentiles; line inside boxes: median; square: mean; cross: minimum and maximum values; 20 mussels analysed per site

mussels from the Gulf of Trieste gaining more storage tissue, growing faster, and showing significantly higher CI values ($p < 0.05$). The growth of Mediterranean mussels is correlated with seawater temperatures, with growth rates slower when seawater temperatures exceed 21 °C, and highest when water temperatures are around 10°C (from March to June) which coincides with highest primary production of the region (Peharda et al. 2007). The increase in CI values from March to September provides evidence of mussels' capability to store the energy needed for the maturation of gonads and spawning by the end of winter. Increased CI is related with the growth of soft tissue and negatively correlated with metal concentrations due to a dilution effect (Lobel et al. 1991).

Studies of MT concentrations are of particular importance in the Adriatic Sea, which is considered to be a hot spot for metal pollution due to especially elevated concentrations of certain metals in sediments and seawater (Kljaković-Gašpić et al. 2010; Joksimović et al. 2016; Bajt et al. 2019). The most prominent pollutants in this region are mercury (Horvat et al. 1999) and organotin compounds (TBT and DBT) (Ščančar et al. 2007). We detected significant differences in MT concentrations between years (higher in March 2010 than March 2011 at all sites, Fig. 2), and between sampling sites (higher in the Boka Kotorska Bay in September 2011; $p < 0.05$). MT induction in the soft tissue of mussels is the consequence of metal pollution, with some metals induced at higher rates (cadmium, copper, and zinc). During the Mytiad study in 2008, researchers found that the mussels at ST4 had the lowest concentrations of metals, while those at ST5 had the highest. ST5 is located near the Bijela shipbuilding yard along the Montenegrin coast, which is a hot spot for nickel, zinc, and chromium pollution (Bajt et al. 2019). Thus, the higher concentrations of MT at ST4, cannot be explained by metal pollution alone, but is instead related to confounding factors such as high seawater temperatures (27.9°C) at the time of sampling. Such temperatures can induce MT uptake as a protective response (Anestis et al. 2010).

The analysis of metal content in mussel tissues in the Gulf of Trieste revealed that concentrations are higher in spring than in autumn due to slower growth during cold periods (Kristan et al. 2014). Environmental concentrations of zinc, lead and copper are moderate at ST1 (Bajt et al. 2019), a pattern that is also reflected in MT concentrations. Meanwhile, the lowest MT concentrations were found at ST3 in all sampling periods. Our findings are in line with previous studies in the region on the progression of MT concentrations over time (Da Ros et al. 2011; Ramšak et al. 2012).

Inhibition of AChE is a major biomarker with particular ecological importance because the neurotoxic action of pollutants causes life-threatening symptoms in animals, significantly reducing their fitness and survival rates (Jemec et al. 2008). AChE inhibition can be the result of exposure to organophosphorus and carbamate pesticides as well as to dioxins and dioxin-like PCBs, PAHs (e.g. benzo(a)pyrene), herbicides (glyphosate), pesticides (e.g. pyrethroids), plasticizers, flame retardants and heavy metals (Fu et al. 2018). AChE activity in March and September 2010 was significantly lower in the Gulf of Trieste than the Boka Kotorska Bay ($p < 0.05$, Fig. 3). In September 2011, mussels from natural beds (ST1, ST4, ST5) had significantly lower AChE activity ($p < 0.05$) than mussels from aquaculture sites at ST2 and ST3 (128.81 ± 37.85 nmol/min/mg protein at aquaculture sites, as opposed to 163 ± 19 nmol/min/mg protein elsewhere). Several AChE inhibitors were found in mussels from May to September 2008 at the same sampling sites. Concentrations of PAHs were in the same range at all sites, while those of organochlorine pesticides, PCBs and endrin were highest in mussels from ST4 (Bajt et al. 2019). The most common mode of action was the inhibition of the catalytic activity of AChE, but pollutants such as dioxins cause downregulation of mRNA biosynthesis (Fu et al. 2018).

Genotoxic agents are hard to detect in seawater; thus the most convenient way to assess their effects is to measure DNA damage in the form of permanent changes such as micronucleus (MN) formation or transient changes measured using Comet assays. MN formation during cell division is a response to genotoxic agents that cause aneugenic and clastogenic effects. MNs represent an index of accumulated genetic damage over the lifespan of the cells, allowing the detection of integrated responses to complex contaminant mixtures and to high levels of PAHs, PCBs, organochlorinated compounds and metals (lead and cadmium) (Bolognesi and Hayashi 2011). We performed MN assays on the gills of mussels collected during September 2009 and March 2010 at all sampling sites. The frequency of MNs varied between 1‰ (ST4 and ST5) and 2‰ (ST1 and ST2) during the warmer September collections. In March 2010, the frequency of MNs ranged from 1.5‰ (ST2) to 5.5‰ (ST4). Sampling sites ST4 and ST5, in the Boka Kotorska Bay, are characterised by higher levels of PAHs and PCBs. Total PAHs ranged between 32.2 and 34.8 µg/kg d.w. (Gulf of Trieste) and 32.4–37.5 µg/kg d.w. (Boka Kotorska Bay). Total PCBs concentrations were between 23 and 38 µg/kg d.w. in the Gulf of Trieste and 74–75 µg/kg d.w. in the Boka Kotorska Bay. Lead concentrations were around 1.1 mg/kg d.w. at all Gulf of Trieste sites and 1.3–1.5 mg/kg d.w. in the Boka Kotorska Bay, while cadmium concentrations were 0.61 mg/kg d.w. in the Gulf of Trieste and 0.57–0.67 mg/kg d.w. in the Boka Kotorska Bay. Concentrations of pollutants with genotoxic activity were measured in mussels at the study sites during spring 2008 (Bajt et al. 2019); Fernández et al. (2011) analysed MNs in *M. galloprovincialis* at several sites with elevated pollutant concentrations along the Spanish coastline and found the highest MN frequency in Cartagena (11.6‰), while rates of up to 5.5‰ have been recorded in the Gulf of Trieste (Kristan et al. 2014). In field studies, MNs have been associated with oxidative stress due to metal pollution, thermal stress in the environment and other factors such as salinity, pH and food availability influencing cell turnover rate and thus modulating the extent of MN formation (Kopecka et al. 2006).

The CI, MT and AChE responses were integrated into the IBRv2 simplified response values for comparison among sampling sites. Results based on summarized biomarker responses showed that sampling sites in the Gulf of Trieste have a lower IBRv2 (3.73–8.46) than in the Boka Kotorska Bay (7.62–10.03), the latter environment being under greater pollutant stress. IBRv2 represents the sum of the deviations between biomarker responses in reference and impacted sites based on robust basal value for each biomarker (Sanchez et al. 2013). The selection of reference sites is not trivial task and depends on expert knowledge and available data on pollutants. In our case, the site with the lowest reported pollution loads was selected as reference site (ST2 in the Gulf of Trieste and ST4 in the Boka Kotorska Bay). The comparison of biomarker responses at the reference and impacted sites allow us to evaluate the extent of detrimental effects. Such an approach can be used for comparisons at the regional scale in large monitoring programmes under MSFD.

We estimated stress caused by certain pollutants in the two environments by evaluating integrated biomarker responses. This comprehensive assessment showed that mussels in the Boka Kotorska Bay study sites are show greater signs of environmental stress than those in the Gulf of Trieste. Moreover, the Boka Kotorska Bay is more vulnerable to pollution and natural stressors due to the very slow exchange of water masses compared to the Gulf of Trieste.

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