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Seasonal dynamics of biomarkers in infaunal clam *Macoma balthica* from the Gulf of Riga (Baltic Sea)

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ABSTRACT

Biomarkers are often regarded as "early warning" signals of environmental pollution; however seasonal changes are mentioned as one of the most important factor that influences the activity of biomarkers. The aim of our study was to assess the importance of seasonal variation of selected contaminant biomarkers in *Macoma balthica* to provide background information for further environmental surveys in the Gulf of Riga. Seasonal variation of biomarkers (acetylcholinesterase (AChE), catalase (CAT), glutathione reductase (GR) and glutathione-*S*-transferase (GST)) was measured in infaunal clam *M. balthica* from the southern part of the Gulf of Riga. The majority of biomarkers (GST, CAT and GR) showed strong seasonal variability; however only CAT and GR were found to be significantly related to environmental factors (near-bottom oxygen, salinity and temperature). Integrated biomarker response (IBR) index indicated that the most stressed condition of *M. balthica* is during August and May. The highest values of IBR were found near the mouth of the River Daugava, suggesting the impact of environmental pollution on the benthic animals.

1. Introduction

The increasing anthropogenic activities are the main factor leading to the increasing levels of contaminants in the aquatic environments. In order to assess water quality, combination of physical, chemical and biological analyses are being traditionally used. Many recent laboratory and field studies in marine and freshwater environments suggest that measurements of enzymatic activity biomarkers are indicative of exposure to chemical pollution (Bocquene and Galgani, 1998; Robillard et al., 2003; Vidal et al., 2002). Biomarkers complement to and enhance reliability on the data of chemical analysis, offering more integral and biologically relevant information on the potential impact of toxic pollutants on the health of organisms (Cazenave et al., 2009; van der Oost et al., 2003). Moreover, effects at the biochemical level are generally used as "early warning" signals to assess the effects of contaminants on organisms, due to the sensitivity, ease of application and specificity to pollution stress of many biomarkers (Livingstone et al., 1992). Therefore, the use of biomarkers can offer an integrated evaluation of the effects of pollutants in organisms and show the "health status" of a system under investigation (Ferreira et al., 2005; Hansen, 2003). The enzymatic activity responses can also be influenced by seasonal changes of both environmental factors and metabolic activities, i.e. those related to food availability, reproductive cycle and gonadic development; in this respect, the understanding of natural changes of biomarkers can be useful for interpretation of field results and to discriminate the onset of biological disturbance from the natural variability (Boccheti and Regoli, 2006).

Infaunal clams *Macoma balthica* are sessile, deposit/suspension feeding organisms, widely distributed in low diversity in the Northern Baltic Sea and abundant in coastal and estuarine areas. *M. balthica* is recognized as an important sentinel organism in the Baltic Sea able to accumulate several classes of pollutants, thus providing a time-integrated picture of their bioavailability (Bocchetti and Regoli, 2006; Lehtonen et al., 2006).

The Gulf of Riga is situated in the Northeastern part of the Baltic Sea with an extensive catchment area. During early 1990s, the Gulf of Riga was described as one of the most polluted parts of the Baltic Sea (Yurkovskis et al., 1993) due to the industrial and agricultural activities in the drainage basin of the Gulf. High concentration of heavy metals, hydrocarbons and other pollutants was observed in the river mouth areas. Afterwards, a considerable decrease has been observed, which is explained both by decline of the agriculture and industry in the Baltic states (Kotta et al., 2008; Ojaveer, 1995). However recent assessments of hazardous substances revealed an alarming increase of DDT, PCB, lead, cadmium and zinc in the molluscs and fish of the Gulf of Riga (HELCOM, 2010), particularly in the southern part of the Gulf, as well as in the vicinity of harbors and shipping routes.

The aim of our study was to assess the importance of seasonal variation of selected biomarkers in *M. balthica* to provide background information for further environmental surveys in the Gulf of Riga. A set of biomarkers was used to represent different types of biological

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responses reacting to different stressors. Acetylcholinesterase (AChE) is an enzyme involved in the synaptic transmission of nerve impulses and is primarily inhibited by neurotoxic compounds, such as organophosphates and carbamate pesticides (Bocquene and Galgani, 1998), but also by other pollutants — heavy metals, detergents, cyanobacterial toxins (Guilhermino et al., 1998; Kankaanpää et al., 2007; Lehtonen et al., 2003). Glutathione-*S*-transferase (GST) is involved in the phase II of metabolism where it contributes to cell survival by detoxification of xenobiotics (Habig et al., 1974; Sherrat and Hayes, 2002). GST can detoxify a number of commonly used pesticides like DDT, atrazine, lindane and methyl parathion either by catalyzing formation of GSH-conjugates or by dehalogenation activity (Sherrat and Hayes, 2002). Glutathione reductase (GR) plays an important role in cellular antioxidant protection and adjustment process of metabolic pathway maintaining adequate levels of reduced cellular GSH by reduction of GSSG to GSH in the NADPH-dependent reaction (Carlberg and Mannervik, 1985; Massy and Williams, 1965). Another antioxidant enzyme — catalase (CAT) is responsible for transformation of reactive oxygen species, i.e. hydrogen peroxide to water and oxygen (Claiborne, 1985; Di Giulio et al., 1989).

2. Materials and methods

2.1. Study area

The Gulf of Riga is situated at the Northeastern part of the Baltic Sea and is quite an enclosed basin, as the water exchange with the sea occurs through two shallow straits. Its surface area is 16.330 km^2 and the volume 424 km^3, while the maximum depth is 62 m and the average depth −26 m (Berzinsh, 1995; Olesen et al., 1999). The Southern and Eastern part of the Gulf receives a large input of freshwater from the main rivers Daugava, Lielupe, Gauja and Salaca, where they supply the dominant part of water runoff (30.2 km^3 or 86% of the total runoff) (Kļaviņš et al., 2002). The salinity of the Gulf is low (5–7 psu) as a result of the weak water exchange and large freshwater impact (Berzinsh, 1995).

The Gulf of Riga is mostly well mixed to the bottom during the winter due to wind-induced mixing of the seasonal cycle. From April to mid-October seasonal stratification restricts vertical water exchange, thus promoting oxygen depletion and storage of nutrients in the bottom water until the water column is remixed in the autumn (Yurkovskis, 2004). The only exceptions are straits and river mouths, where variable salinity stratification can be encountered in all seasons (Stipa et al., 1999). Estuaries of rivers Lielupe and Gauja usually freeze up at the middle of December, and ice cover period lasts 95 days on average. On the River Daugava ice cover formation starts at the beginning of January but is destroyed artificially.

The highest population density is in the Southern part of the Gulf of Riga, where the capital of Latvia, Riga with more than 0.8 million inhabitants, is located. The Western and Eastern coasts of Riga are widely used for recreational purposes.

The Gulf of Riga receives the largest part of nutrients from the rivers Daugava and Lielupe. The majority of nutrients come from agricultural lands in the catchment area and from Riga City municipal wastewaters (e.g. Andrushaitis et al., 1995). Anthropogenic nutrient inputs from land increased until the 1990s when a decline followed. About half of the load of dissolved substances and metals from the territory of Latvia enters via the River Daugava. The highest loads are observed for Zn and Mn and the lowest for Cd (Kļaviņš et al., 2002). The port of Riga is situated on the mouth of the River Daugava, where general cargo, containers and oil products are transhipped.

Stations 167B, 101A and 163B are located opposite the mouth of rivers (167B — estuary of River Lielupe, 101A — River Daugava and 163B — River Gauja) with the depth of 22 m (Fig. 1). The choice of stations was based on the availability of long-term background information — plankton, benthic community, nutrients, heavy metals, recently also few results on hazardous substances.

2.2. Sampling

Molluscs were collected monthly in the Southern part of the Gulf of Riga from May till October 2010 opposite estuaries of the three rivers (Fig. 1). These are transition areas in terms of salinity, since river's waters mix with brackish water from the Gulf of Riga, resulting in a horizontal salinity gradient and a pronounced halocline. During the sampling 30 specimens of *M. balthica* were collected with the Van Veen grab (in May, August with r/v "Varonis" and September with r/v "Aranda") and with bottom dredge (June, July, October) at the depth of 22 m. After sampling *M. balthica* was dissected and foot and digestive gland were removed. The tissues were directly transferred into the vials and stored in liquid nitrogen, but later placed in −80 °C freezer. The shell length of each mollusc was measured. The physicochemical parameters such as salinity, temperature and dissolved oxygen were measured in all stations from May to October 2010 using YSI 6600V2 multiparameter water quality sonde.

2.3. Biomarker analyses

For the analysis of AChE, foot tissues of *M. balthica* were used. Five individuals were pooled per replicate (6 replicates per station). Determination of AChE activity was conducted according to Bocquené and Galgani (1998). The foot tissues (≈0.12 g wet weight) were homogenized in cold 0.02 M phosphate buffer (0.1 % Triton X-100, pH 7.0) in ratio 1:3 (w/v) using pellet pestle (Kontes) for 30 s. During homogenization vials were kept on ice. The homogenate was centrifuged at 10,000 × *g* at 4 °C for 20 min and supernatant (S9 fraction) was taken for measuring AChE activity at 412 nm using microplate reader (TECAN Infinite 200) (Ellman et.al., 1961).

For GR, CAT and GST analysis, digestive gland tissue from *M. balthica* was used. Five individuals were pooled, with six replicates per station. Approximately 0.3 g (wet weight) of digestive gland tissues were homogenized in cold 100 mM potassium phosphate buffer (pH 7.4) in ratio 1:4 (w/v). The homogenate was centrifuged at 10,000 × *g* at 4 °C for 20 min.

GST measurements were performed using modification of the method based on Habig et al. (1974). S9 fraction was diluted with homogenization buffer in ratio 1:30 and the standard reaction mixture contained 20 mM CDNB and 20 mM GSH. Enzyme activity was measured with microplate reader (TECAN Infinite 200) at 340 nm.

CAT activity was determined according to Claiborne (1985) and S9 fraction was diluted in ratio 1:10 (w/v). CAT activity was measured with microplate reader (TECAN Infinite 200) observing decrease of 30 mM H_2O_2 at 240 nm.

For GR determination, S9 dilution of 1:3 (w/v) was made and the activity of GR was assayed following the rate of NADPH (1 mM) oxidation in the presence of GSSG (5 mM). The GR activity was measured with microplate reader (TECAN Infinite 200) at 340 nm.

The quantity of proteins present in homogenate was determined using the method of Bradford (1976) with bovine serum albumin (BSA) as standard for all biomarkers. All measurements of enzymatic activity and protein content were performed in quadruplicate for each replicate.

2.4. Statistical analyses

All data are expressed as the means ± SD and were first tested for normality using Shapiro–Wilk test. As all biomarker data were not normally distributed, Kruskal–Wallis test was used to compare variables between the site and season. Whenever a significant ($p < 0.05$) effect was established on a parameter response, a post-hoc Bonferroni test for multiple comparison between paired means was applied to

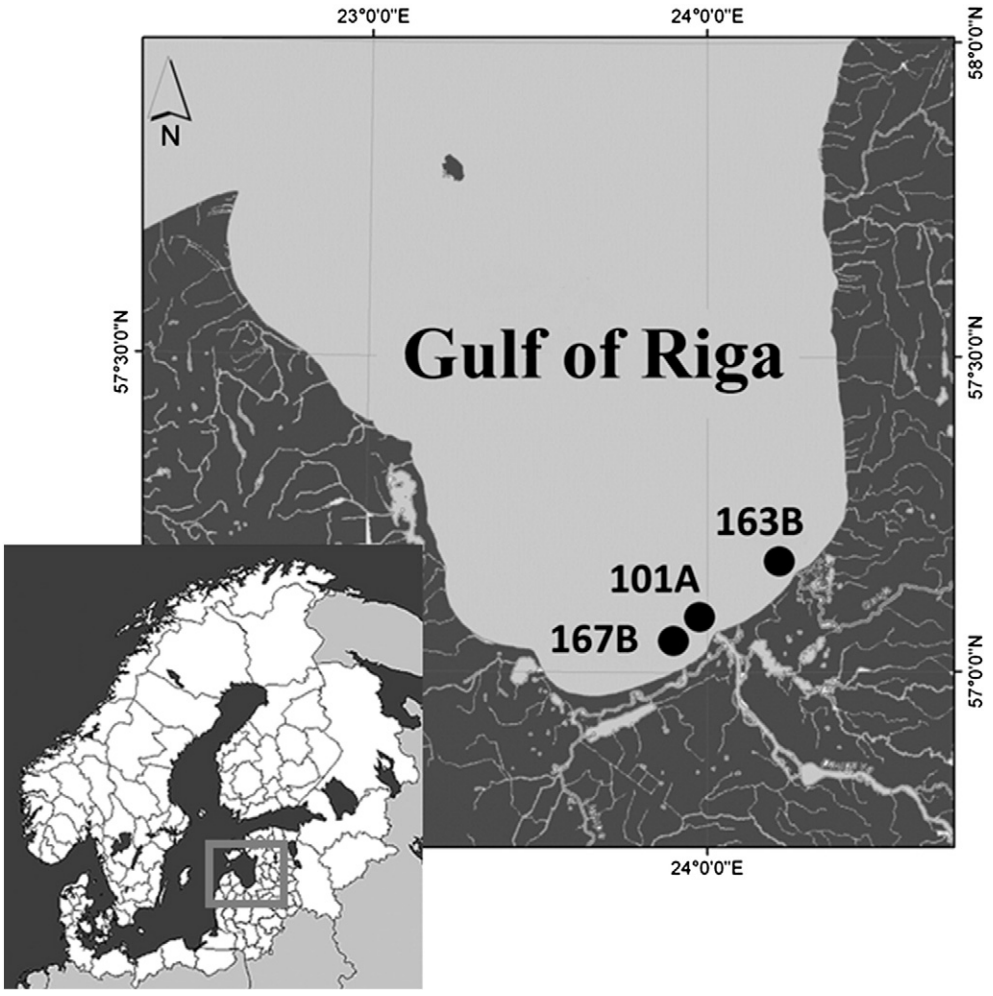

Fig. 1. Location of sampling sites in the Gulf of Riga (167B — estuary of River Lielupe, 101A — River Daugava and 163B — River Gauja).

detect significant differences ($p < 0.05$) between the sites and season (months). Correlation among enzymatic activities and physicochemical parameters were determined for each station with Pearson correlation coefficient ($p < 0.001$). Regression analysis was used to examine the relationship between the biomarkers and environmental factors. All statistical analyses were performed using R-2.14.0 software package.

All the measured biomarker responses were combined into one general "stress index" known as "Integrated Biomarker Response (IBR) index" (Beliaeff and Burgeot, 2002). The procedure described further was used: for each biomarker: (1) calculation of mean and SD for each station; (2) standardization of data for each station: $x'_i = (x_i \text{ mean } x)/s$, where x'_i = standardized value of the biomarker, x_i = mean value of a biomarker from each station, mean x = mean of the biomarker calculated for all the stations, and s = standard deviation calculated for the station-specific values of each biomarker. Result: variance = 1, mean = 0; (3) using standardized data, Z was computed as $+x'_i$ in the case of an activation and x'_i in the case of an inhibition, then the minimum value for all stations for each biomarker was obtained and added to Z; finally the score B was computed as $B = Z + |\min|$ where $B \geq 0$ and $|\min|$ is the absolute value; all the biomarkers were treated this way: (4) calculation of star plot areas by multiplication of the obtained value of each biomarker (B_i) with the value of the next biomarker, arranged as a set, dividing each calculation by 2 and (5) summing all values up: $\{[(B_1 \times B_2)/2] + [(B_2 \times B_3)/2] + \cdots [(B_{n1} \times B_n)/2]\}$; result: IBR (average of different arrangements of biomarkers in the set).

3. Results

3.1. Environmental factors

Seasonal variations in the environmental parameters (temperature, salinity and dissolved oxygen) measured at three sampling stations from May till October 2010, are presented in the Table 1. Water temperature near the bottom was the lowest in May 0.32–0.50 °C with little increase during the summer months. The elevations of water temperature were observed in September and October after mixing of water mass. There was no significant variation of salinity at the bottom layer. The salinity fluctuated within 5.0 to 6.5 ppt depending on the station; all stations presented the highest values

Table 1
Physicochemical parameters (temperature, salinity, dissolved oxygen) in the near bottom water layer during sampling in the three stations.

Sampling-month	Temperature (°C)			Salinity (ppt)			Dissolved oxygen (mg L^{-1})		
	167B	101A	163B	167B	101A	163B	167B	101A	163B
May	0.32	0.33	0.50	5.73	5.77	5.66	8.19	8.00	8.78
Jun	1.83	2.24	1.13	5.87	5.00	5.98	10.23	9.61	10.12
Jul	1.72	2.08	1.65	5.90	5.35	5.89	8.04	7.46	6.59
Aug	1.14	1.74	2.05	5.93	5.83	5.76	5.88	5.82	5.88
Sep	3.84	3.25	4.05	5.97	5.72	5.98	7.24	6.25	6.74
Oct	3.78	7.62	6.02	6.50	6.20	6.07	3.96	5.07	7.05

in October. The dissolved oxygen composes more than 8 mg L^{-1} after the spring mixing and increases in June. Oxygen decrease at the bottom layer was observed during the summer stratification with small oscillation at the end of the sampling.

3.2. Biomarker activity

3.2.1. Acetylcholinesterase activity

The results of AChE activity showed no significant difference between the stations. AChE activity in the foot tissues of *M. balthica* was 26.8 ± 10.1, 20.3 ± 4.0 and 33.3 ± 11.0 nmol/min/mg protein (167B, 101A, 163B, respectively) in May and was gradually decreasing till August, when the lowest concentrations were detected in all stations — 18.8 ± 5.4 (167B), 14.4 ± 4.0 (101A) and 16.9 ± 6.1 (163B) nmol/min/mg protein ($p < 0.05$, Bonferroni test). During September–October the activity of AChE was increasing evenly in Station 163B, while in 101A and 167B decrease was observed in October (Fig. 2). No correlation between AChE activity in *M. balthica* and environmental factors in the stations could be observed, but seasonality was detected only in station 101A ($p < 0.001$).

3.2.2. Glutathione-S-transferase activity

During the seasonal study period GST activity of *M. balthica* showed quite similar pattern for stations 101A and 167B (Fig. 2), while it was significantly different ($p < 0.001$) for station 163B. The lowest values were recorded in May (272 ± 32 and 307 ± 22 nmol/min/mg protein; 167B and 101A, respectively) with a minor fluctuation till July. The GST increased in August, when the highest values (606 ± 132 nmol/min/mg protein) were detected in Station 101A. Comparatively, enzyme activity in Station 163B was increasing until June (522 ± 110 nmol/min/mg protein) and remained quite stable till August. The GST activity decreased at the end of the study period in all stations. Although a significant seasonal impact on GST activity was established (ANOVA: $p < 0.05$), changes in temperature, dissolved oxygen and salinity were not related to the enzyme activity in any station.

3.2.3. Catalase activity

Alike AChE, also CAT activity showed no significant difference between the stations. The highest CAT activity was recorded in May (77.6 ± 11.3 μmol/min/mg protein, station 163B) and June (63.2 ± 11.2 μmol/min/mg protein, station 167B) with decrease till July (Fig. 2). Expressed peak was detected in August, when the highest values were determined in Station 101A (70.2 ± 9 μmol/min/mg protein, respectively). A decline of CAT activity in *M. balthica* was detected from August till October. At the end of the season CAT activity was the lowest (32.5 ± 7.3; 27.2 ± 4.1; 32.9 ± 8.9 μmol/min/mg proteins; Station 167B, 101A and 163B, respectively) and significantly different from that at the beginning of the sampling season ($p < 0.001$, Bonferroni test).

The relationships between all three physicochemical parameters and CAT activity were found to be statistically significant. Negative correlation was detected with temperature ($r = -0.65$; $p < 0.001$) and salinity ($r = -0.29$; $p < 0.001$), while positive with dissolved oxygen ($r = 0.36$; $p < 0.001$). In addition, the seasonal variability of CAT activity was established ($p < 0.001$).

3.2.4. Glutathione reductase activity

The trend of GR activity was dissimilar between the stations, presenting more equal results for station 101A and 167B; however the differences were not statistically significant. The highest activity for station 163B was recorded in May (22.8 ± 5 nmol/min/mg protein) with equable decrease till October (Fig. 2). Comparatively, in the other stations a small variation was observed from May till July when enzyme activity fluctuated from 5.2 to 13.2 nmol/min/mg protein (Station 167B) and 9.6 to 10.8 nmol/min/mg protein (Station 101A). A strongly pronounced peak was detected in August ($p < 0.01$, Bonferroni test) when values were the highest in station 101A and 167B (20.2 ± 9 and 25.3 ± 8 nmol/min/mg protein; respectively). At the end of the sampling period GR activity had reduced and was stable in all stations. The statistical analyses showed that GR activity was significant and correlated negatively with the temperature ($r = -0.39$; $p < 0.001$) and that enzyme activity depends on the seasonal influence ($p < 0.001$).

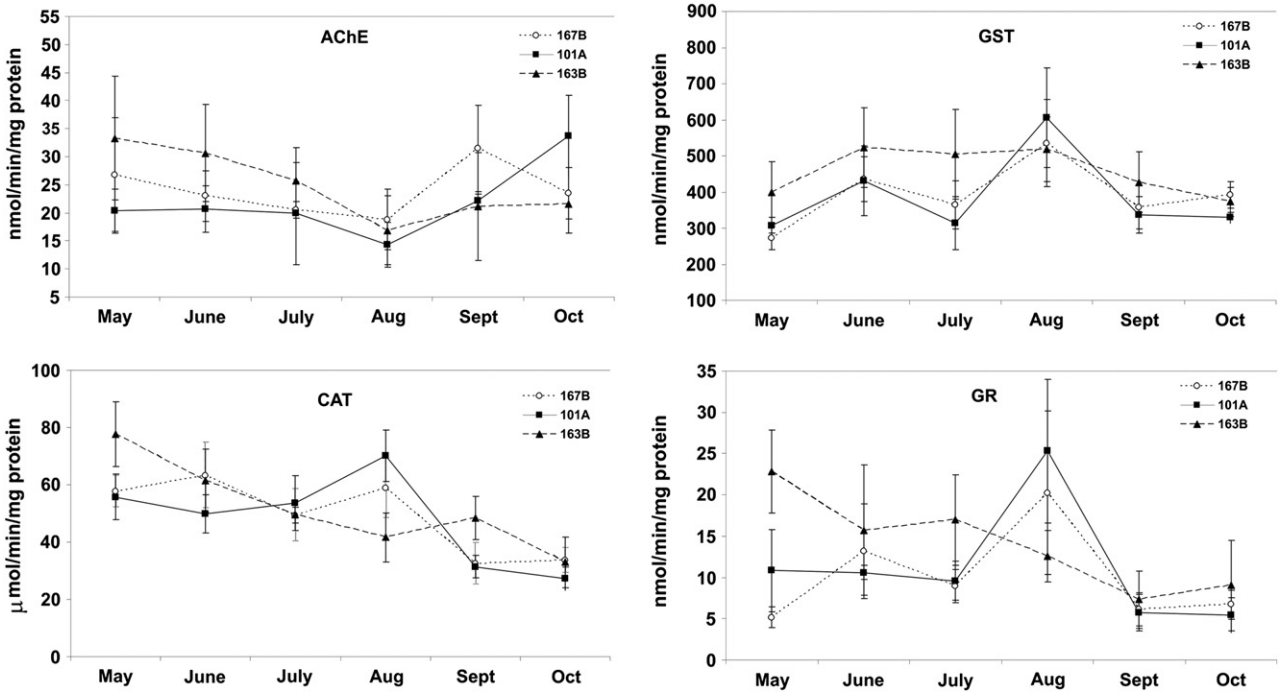

Fig. 2. Seasonal variations in CAT, GR and GST activity (mean ± SD) in the digestive gland tissue and AChE activity in the foot tissue of *M. balthica*.

3.3. Integrated biomarker response

The integrated biomarker responses were based on all measured biomarkers and are shown in Fig. 3. The area in black integrates the IBR for each site and is represented as a star plot. In station 167B the IBR values were the highest between June and August. A similar trend was observed also in station 101A, however in this station IBR shows more expressed integrated stress response of *M. balthica* in August. Different results were calculated for station 163B, where the highest values were obtained in May with decrease till October. Relatively lowest IBR values were determined at the end of the sampling season — September, October in all stations.

4. Discussion

Apart from the pollution, seasonal changes are mentioned as an important factor influencing the biomarker activity (Bocchetti and Regoli, 2006; Dellali et al., 2001; Fitzpatrick et al., 1997; Manduzio et al., 2004; Pfeifer et al., 2005). In our study a strong connection with seasonal variability was established for GST, CAT and GR in all stations. Only AChE did not show seasonal variation. However, relationship between physiochemical parameters and biomarker activity was identified only for some of them.

Impact of the environmental factors on the enzyme activity in molluscs has been reported in many studies. Temperature has been claimed to be the most significant natural factor affecting bivalve AChE (Leiniö and Lehtonen, 2005; Pfeifer et al., 2005; Robillard et al., 2003), CAT, GST (Robillard et al., 2003) as well as GR activity (Verlecar et al., 2008). Also salinity (Pfeifer et al., 2005), oxygen saturation at the bottom level (Leiniö and Lehtonen, 2005) and pH (Robillard et al., 2003) have been mentioned.

As to our study the activity of AChE was the less sensitive to the abiotic factors. The seasonality of AChE activity was established only in one station (101A). Besides, the results did not show statistically significant influence of temperature, salinity or oxygen concentration on the enzyme activity in *M. balthica* in any station of the Gulf of Riga. Similarly to AChE, GST was also not related to physiochemical parameters. Strong thermal stratification, as well as pronounced salinity gradient was established in the estuaries zone during the study period, preventing the mixing and warming of water masses during the summer. Significant warming of near-bottom water was recorded only in October after the breakdown of summer stratification. This could influence the seasonal dynamics of all enzyme activity in the sampling stations. Leiniö and Lehtonen (2005) in their study reported of higher AChE activity during the summer period, probably due to higher water temperatures during the summer months, while in our study such pronounced trend was not established. Comparably, in our study the AChE activity was higher in May and was decreasing steadily until August. An elevated activity of GST, GR and CAT were also recorded in August, especially in the station 101A, suggesting that enzyme activity could be affected by phytoplankton composition, namely, blooms of potentially toxic cyanobacteria. The accumulation of cyanobacteria toxins into biota (Kankaanpää et al., 2002; Sipiä et al., 2001a, 2001b), as well as toxin impact on the enzyme activity has been reported in many studies concerning the Baltic Sea. Significant changes of AChE and CAT activity were found in bivalves after exposure to nodularin or nodularin-containing cyanobacterial extract (Lehtonen et al., 2003; Pflugmacher et al., 2007). The influence of nodularin on the GST activity was also recognized in the organisms of different trophic levels (Davies et al., 2005; Kankaanpää et al., 2007; Pflugmacher et al., 2007). The long-term data from the studies of phytoplankton in the Gulf of Riga shows that usually the highest proportion of cyanobacteria in biomass occurs during July–August (Jurgensone et al., 2011), when mass development of potentially toxic cyanobacteria blooms can be found (Seppälä and Balode, 1999). The high concentrations of nodularin (270–540 ng/g dw) detected in digestive glands of *M. balthica* from the Gulf of Riga (unpublished data) confirms the influence of cyanobacterial toxins on enzymatic activity of molluscs.

CAT and GR are antioxidant enzymes, involved in the enzyme system preventing the cellular damage caused by reactive oxygen species (ROS). Our studies showed that these enzymes are strongly affected by the environmental parameters. Temperature and salinity had a negative impact on CAT activity in *M. balthica* while dissolved oxygen — positive. On the contrary, GR activity negatively correlated only with the temperature. In addition to that the seasonal variability of both enzyme activities was established. The antioxidant enzyme GR in *M. balthica* showed similar seasonal trend as GST activity, however there was no significant correlation between them.

The seasonal influence on GR activity in mussels has been reported by Manduzio et al. (2004), Bocchetti and Regoli (2006). Verlecar et al. (2008) observed significant connection between the temperature and GR activity in green-lipped mussel *Perna viridis* from the Arabian Sea. As to our study, negative correlation between the temperature and seasonal variability of enzyme activity has also been detected. In the *M. balthica* higher CAT and GR activity was detected in spring (May, June) when the bottom temperature was the lowest. High CAT activity in bivalves of the Baltic Sea has been reported at the end of the spring (Kopecka et al., 2006; Leiniö and Lehtonen, 2005) and also for Mediterranean mussel *Mytilus galloprovincialis* (Bocchetti and Regoli, 2006). Phytoplankton blooms of diatoms are typical in spring, thus increasing the food availability for molluscs. This affects also their metabolism, as oxidative changes are reported to be typical responses in bivalve molluscs during the periods of more intense feeding activities (Regoli et al., 2002). In the Gulf of Riga the highest phytoplankton biomass has been recorded in May (Jurgensone et al.,

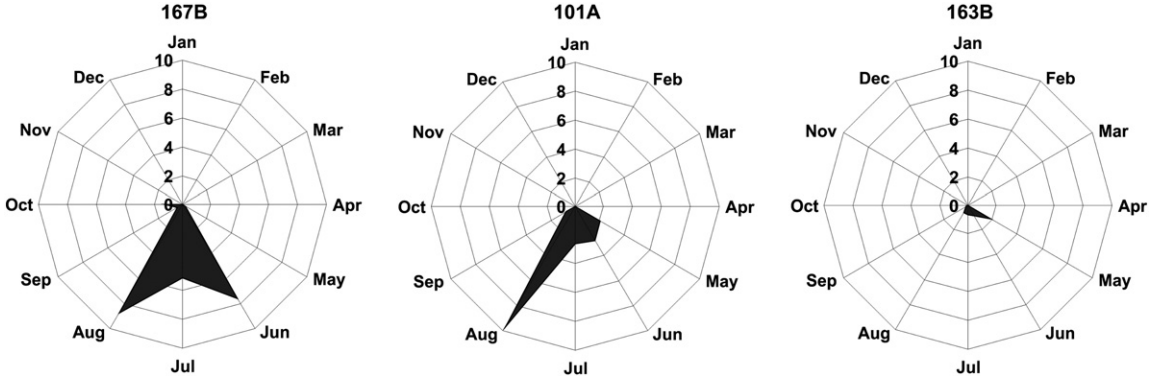

Fig. 3. Seasonal variations in the integrated biomarker response index (IBR) calculated for all stations using 4 measured biomarkers in *M. balthica*.

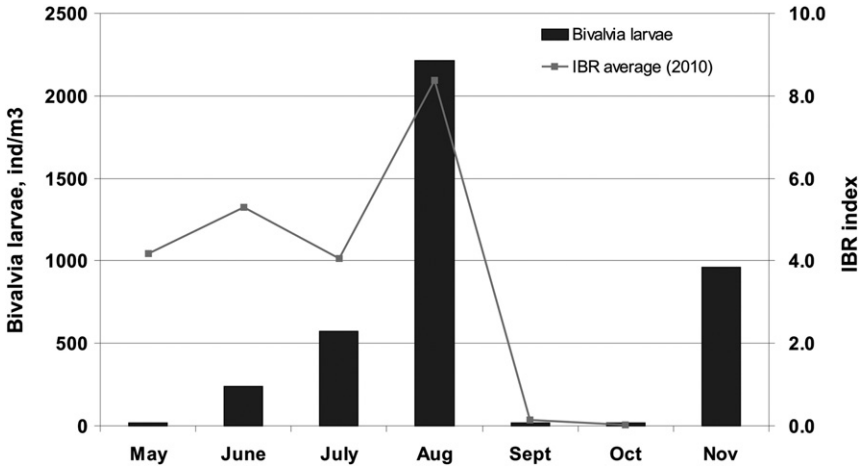

Fig. 4. Relationship between seasonal integrated biomarker response index (IBR) and amount of bivalve larvae in plankton.

2011), but higher sedimentation rates of suspended matter in June (data from Latvian Institute of Aquatic Ecology). This could explain higher enzyme activity in *M. balthica* at the end of the spring.

The integrated biomarker response index (IBR) has been used in many studies to assess toxically-induced stress level of populations (Broeg and Lehtonen, 2006; Damiens et al., 2007; Lehtonen et al., 2006) or generally describe the "stress period" of the population (Leiniö and Lehtonen, 2005). As to our study, two stations (167B and 101A) presented higher IBR during the summer period reaching the highest peak in August, but also May showed higher IBR response (163B), indicating that these months could be stressful for *M. balthica* in the Southern part of the Gulf of Riga. As discussed previously, food availability (phytoplankton blooms in spring and summer) has strong connection to oxidative stress in bivalves. Many studies have shown impact of cyanobacterial toxins on the biomarker activity in molluscs, also for *M. balthica*. In the Gulf of Riga, the mass development of cyanobacteria varies from year to year, but still the most intensive blooms are observed from July till September (Jurgensone et al., 2011). This shows that toxin production of cyanobacteria and also diatom abundance has to be taken into account when assessing IBR of the study area.

There is close functional relationship between the reproduction cycle and oxidative stress in bivalves (Filho et al., 2001). In the Northern Baltic Sea *M. balthica* spawns once a year: April–May, while from March to early May it spawns in the South of the Baltic Sea (Bonsdorff and Wenne, 1989). In contrast, *M. balthica* from the Southern limit of its distribution (Gironde Estuary, France) is known to spawn twice a year: in late spring/early summer and again from September to November (Bachelet, 1986). Günther et al. (1998) has reported about mass occurrence of *M. balthica* larvae in midsummer from Wadden Sea. Our results show that increased IBR could also have connection with the spawning period. The average IBR from all stations increased in August significantly, while large amounts of bivalve larvae were also detected in the summer months (Latvian Institute of Aquatic Ecology) (Fig. 4). As the *M. balthica* has a planktonic life stage of 2–5 weeks, spawning in July or August could affect the biomarker activity.

The lowest values of IBR were calculated at the end of the sampling period (September–October), indicating that this could be the best sampling period for biomarker activity during the vegetation season.

In the estuary of the River Gauja (163B) IBR was less pronounced than in the estuaries of the River Daugava (101A) and Lielupe (167B). The highest values of IBR were found near the mouth of the River Daugava, suggesting the impact of the environmental pollution on the benthic animals.

5. Conclusions

The results of this study allow to set the "baseline" level of selected biomarker responses in *M. balthica* in the Gulf of Riga in order to be able to identify the pollution related events in the future. It may be assumed that the seasonal variability of biomarkers integrates the characteristic variation of reproductive status, food availability and environmental conditions in the study area, therefore seasonal variability should be taken into account when the environmental quality is assessed. The results showed that neurotoxic biomarker AChE exhibited the lowest seasonal variability. AChE and detoxification enzyme GST were not affected by abiotic environmental factors, opposite to oxidative stress biomarkers GR and CAT. However further research is required to estimate the year-to-year variability.

Integrated biomarker response is useful when the seasonal estimation of biomarkers is performed. According to our results September–October is the best period for sampling of *M. balthica* for the assessment of hazardous substances and their effects, because the IBR indicates the most stable biomarker levels at this period.

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