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Biomarker Responses in the Mussel (*Mytilus edulis)* and the Flounder *(Platichthys flesus)* in the Klaipeda-Butinge Area (Baltic Sea)

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Abstract:

In spring and autumn 2001 and 2002, acetyl cholinesterase activity, metallothionein level, micronuclei, and apoptosis were measured in blue mussel (*Mytilus edulis*) and flounder (*Platichthys flesus*) (additionally, bile PAH-metabolites were measured in flounder) collected along a suspected pollution gradient in the coastal zone of Nemirseta, Butinge and Palanga. Biomarkers in flounder from the Klaipeda offshore (reference site) zone were analyzed in December 2001. The Palanga zone was originally considered as coastal reference site, but an oil spill occurred in the Butinge oil terminal in November 2001, causing contamination of this area. The results of this multi-biomarker study shows that lowest level of genotoxicity and cytotoxicity exists in organisms sampled from offshore and from Palanga locality in spring 2001. The highest biomarker response was observed in Butinge area. After the oil spill an increase in genotoxicity and cytotoxicity was detected in mussels and flounder from the Palanga and Butinge localities. Bile PAH-metabolite concentrations in flounder were higher in the polluted sites compared to the reference site. In addition to spatial differences, seasonal variations in some biomarkers (e.g. metallothionein) were also marked. The study demonstrates that biomarker analysis is a cost-effective and rapid approach to detect ecotoxic effects of environmental pollution *in situ*. The endpoints are well characterized and can easily be recognized and the techniques are convenient to use in field samplings by following standard procedures and protocols.

Keywords: biomarkers, Baltic Sea, AChE, apoptosis, micronuclei, metallothioneins, PAH metabolites, blue mussels, flounder

Introduction:

The ecological conditions in Lithuanian coastal zone of the Baltic Sea depends mainly on fresh water outflow from the Curonian lagoon. Annual water inflow from the lagoon into the Baltic Sea is approximately 28 km^3 at an average rate of 890 m^3/s. In average 250 days per year, the currents from Curonian lagoon flows northward and approximately for 75 days marine water flows into Curonian lagoon. The average salinity levels range between 5 and 7 PSU in a surface layer and between 7 and 8 PSU near the bottom.

Due to predominance of northward currents, zones with a highly contaminated bottom sediments are located mainly in a north direction from the Klaipeda city. The bottom sediments in the coastal area by the Butinge and Nemirseta are heavily polluted by Cu, Pb, Ni, Zn and Hg and PAH compounds. The Baltic Sea at Palanga town is comparatively unpolluted (Jokšas, Stakeniene, 1997).

The main objective of the study was assessment of biomarker responses fish and mussel species in regard to season, sampling year, locality and sex and elaboration of methodology for the exploration of the biomarker approach in biomonitoring systems.

Flounder and blue mussels were used as target species taking into consideration their ability to accumulate hazardous compounds in tissues, also to transform petroleum hydrocarbons into cancerogenic, mutagenic and cytotoxic metabolites. Since both species are linked directly to human food, the assessment of exposure and effect biomarkers are extremly relevant.

Material and Methods:

Sampling

The blue mussel (*Mytilus edulis*) and flounder *(Platichthys flesus)* were collected along a suspected pollution gradient in Lithuanian coastal zone by the Palanga, Nemirseta, and Butinge in June and September 2001 and 2002. Samples from offshore were collected in December 2001 and 2002. Additionall material for the micronuclei analysis was sampled in June 2003.

The original habitat by the Palanga on the Lithuanian coast (due to lower level of pollution) was selected as a reference site. Nemirseta locality is affected by pollution from Nemunas River basin (population over 5 mln). The Butinge site is under the influence of municipal sewage discharge as well as contamination from Mažeikiai oil refinery plant and Butinge oil terminal (Fig.1).

The samples for the assessment of acetyl cholinesterase activity, metallothionein and micronuclei levels were collected from fish and mussels. In addition, apoptosis, EROD and PAH-metabolites were studied in flounder tissues.

Slides for the analysis of micronuclei were prepared during sampling campaigns. The collected tissues for the investigation of other parameters were stored in cry vials in liquid nitrogen, or deep frozen at -20^0 C (PAH metabolites) and latter were shipped to partnership laboratories.

Biomarker analysis

Micronuclei

Mussels - the gill cell suspension was prepared in a drop of 3:1 ethanol acetic acid and smeared on clean microscopic slides. After that the slides were air-dried, fixed in methanol for 10 min and stained with 5% Giemsa solution in phosphate buffer pH=6.8. The stained slides were analyzed

under the light microscope Olympus BX51 at a final magnification of 1000. 2000 cells with intact cytoplasm were scored in each studied specimen of mussels.

Flounder - a drop of blood from caudal vessels of fish was directly smeared on slides. The slides were air-dried, fixed in methanol for 10 min and were stained with 10% Giemsa solution for 8 min. The frequency of micronuclei was evaluated by scoring at a 1250× magnification of 5,000 mononucleated intact erythrocytes in each fish specimen.

Micronuclei were identified according to following criteria:

- spherical or ovoid-shaped extra nuclear bodies in cytoplasm
- a diameter in mussels of between 1/3 and 1/20, in fish - 1/3-1/50 that of the main nucleus
- bodies are non-refractory
- color, texture and optical features resemble the nucleus
- completely separated of the main nucleus.

The statistical analysis in mussels and fish was done using PRISM statistical package (ANOVA and Mann-Whitney U-test).

AChE activity

Analysis was conducted according to ICES TIMES No. 22 (Bosquene and Galgani, 1998) using deep frozen muscle tissues. AChE activity was measured using a 96-well micro plate reader (412 nm).

Metallothionein content

For mussels, the digestive gland of several (5-8) individuals was pooled to obtain sufficient amount of tissue (0.5 g wet wt), with 2-5 parallel samples per station and date. For flounder, pieces of liver were analysed from either individual fish (about 30%) or pooled samples (2-7 individuals). Mainly female fish were studied, with only 8 out of a total of 114 samples being males. The samples were homogenised on ice in sucrose-TRIS buffer with leupeptin (3,0 µl ml^{-1}), PMSF (1,5 µl ml^{-1}) and β-mercaptoethanol (0,1 µl ml^{-1}) in 1:4 (*w*/*v*) using a motor-driven teflon/glass Potter homogeniser. The homogenates were centrifuged at 30,000 x *g* for 20 min at 0-4 °C. 1 ml of supernatant was taken, 1,05 ml EtOH (99%, -20°C) and 80 µl of chloroform were added and the samples were mixed with a vortex for 2 min. The sample was centrifuged in a swinging rotor at 6,000 x *g* for 10 min at 0-4 °C. The supernatant was collected and its volume measured using a pipette (usually 1,8 ml). 40 µl of 37% HCl, 10 µl of RNA (1 mg 10 μl^{-1}), and 3 x sample volume of EtOH (ca. 5,4 ml) were added. After storing the samples at –20 °C for 1 h (protein precipitation), they were centrifuged at 6,000 x *g* for 10 min (horizontal rotor). The supernatant was discarded and the pellet treated with 2 ml washing solution (17,4 ml 87% *v*/*v* EtOH,+ 0,2 ml 1% *v*/*v* chloroform + 2,4 ml of 12% *v*/*v* sucrose–TRIS buffer). The sample was again centrifuged at 6,000 x *g* for 10 min at 0-4 °C, the supernatant discarded and the pellet dried under a nitrogen gas stream for 10 min. The dry pellets were added with 150 µl of 0,25 M NaCl and 150 µl of 1 N HCl containing 4 mM EDTA and mixed with vortex with a glass stirrer. 4,2 ml of DTNB dissolved in phosphate buffer was added to the samples and they were centrifuged at 3,000 x *g* for 5 min at room temperature. The absorbance of the sample was measured at 412 nm and plotted against the concentration (nmol ml^{-1}) to obtain $\varepsilon_{GSH.}$ Metallothionein content of the sample was calculated from

$$[[(\overset{MT}{ABS_{412}}/\varepsilon_{GSH})/21]*8{,}600]*4{,}5*4$$

AChE activity

Analysis was conducted according to ICES TIMES No. 22 (Bosquene and Galgani, 1998) using deep frozen muscle tissues. AChE activity was measured using a 96-well microplate reader (412 nm).

Apoptosis

Apoptotic cells in gills of mussels were identified according to morphological features, which has been described in human (Fenech et al., 2003).

PAH-metabolites

In female of flounder, bile PAH-metabolites were measured by total fluorescence. Samples were analysed according to Aas et al. (1998) with slight modifications. Bile samples were diluted 1:1000 with 48% ethanol which was sufficient dilution for most of the samples. Diluted samples were measured with a Hitachi F-4500 Fluorescence spectrophotometer. Slit width was set at 2.5 nm. Samples were screened only for pyrene type metabolites at the excitation-emission wavelength pair 341/383 nm.

Results

Assessment of biomarker responses in flounder and blue mussels was performed within the frame of EU BEEP project. The responses were measured in regard to season, sampling year, localities and sex.

Micronuclei

Analysis of micronuclei frequency in blue mussels inhabiting three studied localities in eastern part of the Baltic Sea showed that the values in June was significantly lower than in September and varied from 1,2‰ to 2.72‰. The range of variation in September was from 2.93 to 3.85‰ (Fig. 2). Statistically significant differences were detected between sampling years (June 2001 and 2002 - F=6.844; September 2001 and 2002 – F=7.974), as well as between those mussels, collected in June and September (in 2001 – F=8.664; in 2002 – F=10.57). P values in all cases were less than 0.0001. No statistically significant differences of MN frequency were in a whole mussel cohort, sampled from three Lithuanian localities (F=1.622, P=0.1996). Although, the gradient of MN frequency (F=2.748; P=0.0221) was observed in mussels, which were studied in June 2001 and 2002 (Table 1).

Table 1. Results of analysis of variance of micronuclei frequency in blue mussels from Lithuanian coast (ANOVA)

Source of variability	Sum of squares	df	Mean squares	F	P-level
Sampling stations	11.28	2	5.642	1.622	0.1996
Year (June 2001 and 2002)	32.17	5	6.434	2.748	**0.0221**
Year (September, (2001 and 2002)	18.37	5	3.674	1.078	0.3761
Season (June and September 2001)	97.43	5	19.49	5.187	**0.0003**
Season (June and September 2002)	82.58	5	16.52	8.294	**<0.0001**

The comparison of data on MN frequency in mussels collected in June 2001 and 2002 showed an increase (P<0.0001) of genotoxicity in molluscs, which were sampled in 2002 from Palanga area. An accidental oil spill in Butinge oil terminal was occurred in November 2001 and study area by the Palanga was covered by oil.

The highest induction of micronuclei in erythrocytes of flounder from all three studied stations was observed in September 2001. The frequency of micronuclei reached 1.45 ‰ in Palanga and 1.34‰ in Nemirseta site. These values were the highest in flounder from all other localities (of 12 localities) we have studied in the Baltic Sea (Fig. 3).

Comparatively high values of MN was detected after the oil spill in flounder inhabiting Palanga and Butinge zones in spring 2002. In comparison to June 2001, the frequency increased significantly ($P<0.0001$) (Fig. 3; Table 2).

The lowest frequency of MN (0.04‰) was observed in fish from the Lithuanian offshore zone in December 2002. Comparatively low level of genotoxicity was found in flounder collected from Lithuanian coastal area in June 2001 and 2003 and in September 2002 (Fig. 3).

Table 2. Significance of differences in MN frequencies in flounder
(*June 2001 and 2002; Palanga 2002, Butinge 2002 – after oil spill*)

Palanga 01	**Butinge 01**	**Nemirseta 02**	**Palanga 02**	**Butinge 02**	
ns	ns	ns	**	**	**Nemirseta 01**
	ns	ns	***	***	**Palanga 01**
		ns	***	***	**Butinge 01**
			***	***	**Nemirseta 02**
				ns	**Palanga 02**

There was a significant influence of sampling time, sampling locality and sex on the frequency of MN in flounder from Klaipeda-Butinge site. The differences were found between MN induction in fish from Lithuanian coast and from Wismar site (Table 3).

Table 3 Results of analysis of variance of micronuclei in erythrocytes of flounder from Klaipeda-Butinge site and comparison with other studied sites in the Baltic Sea (ANOVA)

Source of variation	**P** value	**R** squared
Sampling stations	0.2708	0.01083
Sampling season	**<0.0001**	0.1053
Sampling year (June 2001/2002)	**<0.0001**	0.3217
Sampling year (September 2001/2002)	**<0.0001**	0.5745
Fish sex	**0.0206**	0.1076
Sampling sites (Klaipeda-Kvadofjarden)	0.1676	0.01605
Sampling sites (Klaipeda-Gdansk	0.1042	0.02162
Sampling sites (Klaipeda-Wismar	**0.0005**	0.05646

Metallothionein content

In *M. edulis*, the highest MT induction was found in mussels from the Nemirseta station in September 2001, with the mean value (322±26 μg g wet wt^{-1}) being significantly higher compared

to the original reference site Palanga (209±32µg g wet wt^{-1})($F_{2, 5}$=5.64, p<0.05). No other significant differences could be seen between the stations within each sampling campaign (Fig. 4). Season had a significant effect on MT levels, with lower levels observed in September compared to June (pooled stations: ($F_{1, 35}$=15.77, p<0.001). Interannual differences between seasons were also observed, with levels in June 2001 being lower compared to June 2002 ($F_{1, 17}$=21.95, p<0.001) and values in September 2001 slightly higher compared to September 2002 ($F_{1, 15}$=4.58, p<0.05).

In *P. flesus*, seasonal variability was very strong, with almost twice higher MT levels in September (665±140 µg g wet wt^{-1}) compared to June (381±82 µg g wet wt^{-1}) during both study years (years pooled: $F_{1, 112}$=179.1, p<0.001) (Fig. 5). Both seasonal samplings varied according to year with the June 2001 levels being markedly higher compared to June 2002 ($F_{1, 58}$=8.84, p<0.01), while the levels measured in September 2001 were lower compared to September 2002 ($F_{1, 52}$=7.86, p<0.01).

AChE activity

Data on AChE activity available only in mussels collected in June and September 2002. Lower level of AChE induction was observed in mussels sampled in September from all three studied stations (Fig. 6).

Apoptosis

Similar incidences of apoptosis were observed in gill cells of mussels collected in all sampling campaigns and from all three stations. The highest values of apoptotic cells were registered in *M. edulis* inhabiting Nemirseta and Butinge localities in September 2002 and in June 2001 (Fig.7).

PAH- metabolites

There were no clear trends in bile PAH-metabolites in female flounders, except the levels were in general lowest in samples from autumn 2002 (Fig. 8). However, in samples collected in spring the levels of PAH-metabolites were lowest in Palanga. In autumn samples in 2001 and 2002 the concentrations of PAH-metabolites were quite similar in different sampling stations (Fig. 8).

Discussion

Fish and mussels have often been considered as the “sentinel” organisms in aquatic environmental health assessment. These organisms are distributed in practically all zones of the aquatic habitat, have a great commercial and recreational value. An extension ecotoxicological studies with fish and mussels by elaboration and standartization of some ecotoxicological tests should be a great interest in incidental or operational oil, or other hazardous compound spills from human activities related to transport or processing of products (Pietrapiana et al., 2002).

Clean-up campaign after oil spill in Lithuanian coast wasn‘t efficient and significant increase of genotoxicity was registered in flounder and blue mussels. It is well known that crude oil and its products are very important pollutants of marine environment. Polycyclic aromatic hydrocarbons present in oil may be quite effectively accumulated by aquatic organisms (Baussant et al., 2001). The highest level of an original PAH concentrations in liver have been observed in Atlantic cod 3 days after the start of exposure (Aas et al., 2000). There are well-established associations between neoplastic and preneoplastic lesions in fish livers and pollution by PAHs of their habitats (Woodhead et al., 1999).

Since oil contains potentially genotoxic components (Klekowski et al.,1994), the elevation in MN frequency in *M. edulis* and *P. flesus* from Lithuanian coast after oil spill, is an obvious response to action of genotoxic substances of the spilled oil. As direct confirmation of the statement, the

increase of PAH-metabolites in bile the same individuals of flounder was also shown. Moreover, the AChE activity was higher in fish catched in June 2002.

Anaphase aberrations in fish embryos were correlated with concentrations of PAHs within the oil trajectory following the Exxon Valdez oil spill in Prince William Sound in March 1989 (Hose, Brown, 1998). Elevated levels of cytogenetic damage was observed earlier in molluscs inhabiting marine port and oil terminal areas of the Baltic Sea (Baršienė, Baršytė Lovejoy, 2000; Baršienė, 2002). DNA adducts have been more frequently detected in marine organisms 12-17 months after the Sea Empress oil spill (Harvey et al., 1999). Increased values of MN could also be related to an overall genetic instability, as cells with unstable karyotype tend to compensate by the way of chromosome elimination and MN formation (Philippe et al., 1993).

Many known environmental genotoxins are lipophilic aromatic compounds that contain three or more fused benzene rings. The liver is one of major site of their metabolic activation to give highly reactive genotoxic metabolites (Stegeman 1981). DNA breaks usually appear as a result of action of these highly reactive metabolites, as well as result of DNA recombination, or replication during mitosis. Studies on PAH genotoxicity in fish present a model where in benzo[a]pyrene-treated zebrafish 74% of mutation occurred in G:C base pair substitutions (Maria et al., 2002; Gravato, Santos, 2002; Amanuma et al., 2002). The majority of DNA lessions and a single-strand breaks are quickly repaired. However, double-strand breaks usually results in cytogenetic damage, like structural chromosomal injures, formation of micronuclei or changes in chromosome number (Eastman, Barry, 1992).

Extensive chromosomal rearrangements, such as micronuclei, are a recognized consequence of genome instability (Fenech et al., 1999*).* The micronucleus (MN) test is the most widely used *in vivo* test in assessment of cytogenetic damage. The test allows to evaluate the influence of genotoxic compounds at a low concentrations and to assess of dose-response relationships of both DNA reactive and non-DNA reactive genotoxins (e.g. aneugens). Micronuclei originate from chromosome fragments or whole chromosomes, which lag at cell division due to lack of centromeric region, damage in centromeric region, or defect in cytokinesis (Kirsch –Volders et al., 2000; Fenech et al., 2003).

Data of the present study showed the comparatively low levels of genotoxicity and PAH-metabolites in flounder in autumn 2002. Whilst, that increase of mettallothionein content was registered at the same time. The explanation of the findings might be: a) the spring and summer 2002 in Lithuania was exceptionally hot and dry; b) due to unusual ecological conditions, the migration of flounder to a deeper and less contaminated areas was marked (Repecka, personal communication); c) at the same time in sedentary *M. edulis* the values of MN were at the same level as in September 2001.

The differences observed in MT levels in *M. edulis* and *P. flesus* collected during the same season in different years may be caused by slightly different times of sampling, with varying hydrographical conditions. However, they may also be caused by variability in the degree of bioavailability metals between the years. In *P. flesus*, the elevating concentration in MT content towards autumn may be related to various natural factors, both biotic (e.g. changes in reproductive stage, feeding etc.) and abiotic (e.g. temperature, oxygen conditions, freshwater runoff), and are not necessarily related to increased metal load from the environment. This is corroborated by the fact that an opposite trend, i.e. decreasing MT levels in autumn, was observed in *M. edulis*.

In overall the results of the present study have points on seasonal variations of biomarker responses in studied marine organisms. In poikilotherm organisms is very important to identify the baseline level of biomarker response. It is known, that baseline level can vary depending upon age, sex, season, temperature, oxygen and other factors. All these factors must be taken into account when using biomarker assays under the field conditions at different times of the year. It is known, that these factors vary with reproductive status and reproductive timing variations across the country, ecosystems etc. Moreover, even when samplings performed in the same season from

different sites of the sea/country, the baseline and induced by pollutants levels of biomarker responses may differ significantly (Dixon et al., 2002).

Conclusions

Selection of relevant assays for the assessment of ecotoxicity *in situ* should be based on sensitivity of biomarker response regarding ecological conditions, season, expected target organisms and tissues, as well as on biological peculiarities of populations inhabiting exposed sites.

Micronuclei assay is a simple, cost effective and rapid method to detect genotoxic effects of pollution. The endpoint is well characterised and can easily be recognised. MN test allows to evaluate the influence of genotoxic compounds at a low concentrations and to assess of dose-response relationships of both DNA reactive and non-DNA reactive genotoxins (e.g. aneugens).

The clear response of marine organisms to exposure of contaminants (for example - crude oil) could be easily assessed in gill cells of mussels, as well as in kidney and liver cells of fish, which are under the direct influence of contamination. The cells can be obtained easily in field samplings and do not need to be centrifuged, cultivated, frozen in nitrogene etc. The gills, pieces of kidney and liver can be fixed and stored for a long time. The permanent slides allow to make additional analysis of genotoxicity or cytotoxicity (apoptotic, polynucleated cells, nuclear buds etc)

Cytotoxic effects in cells could be very efficient additional parameters for the evaluation of environmental genotoxicity. Such parameters don't need any additional techniques and could be scored on the same slides as micronuclei. Standard procedures of the MN test are easily learned by new researchers, and don't require expensive facilities.

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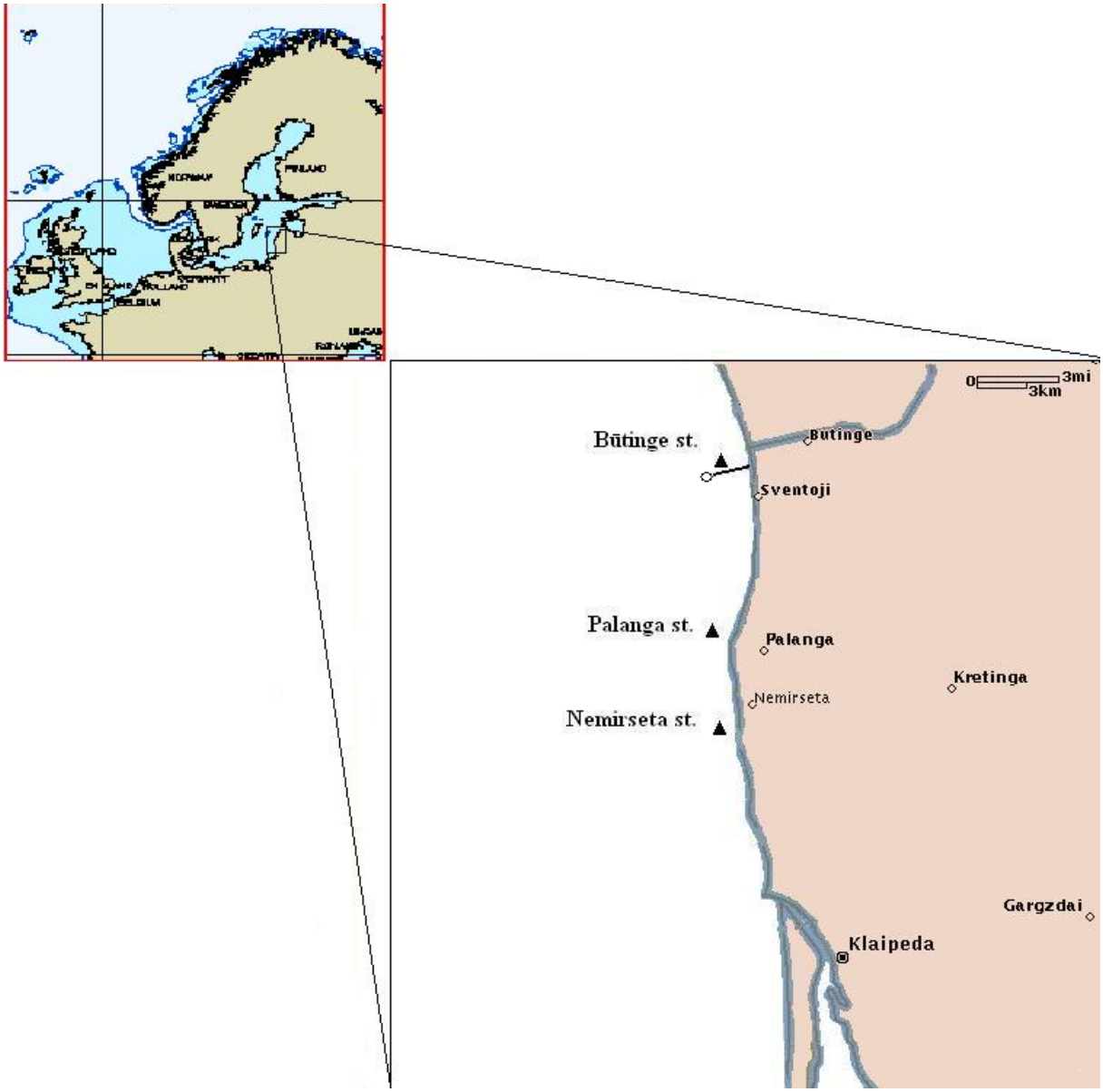
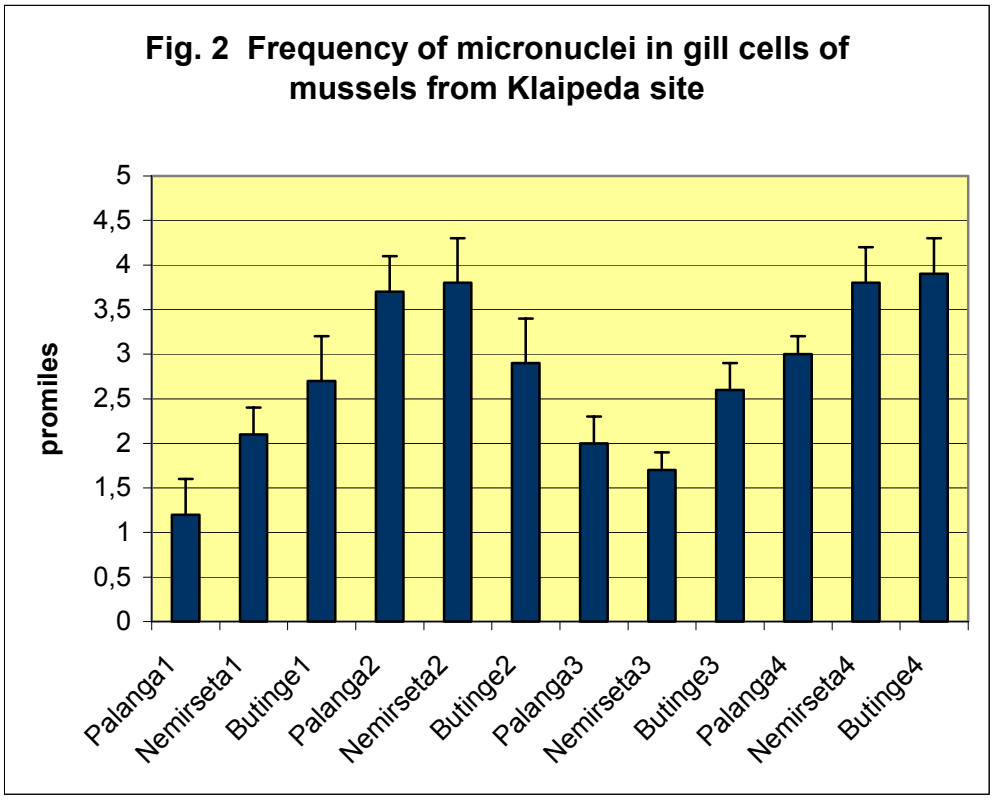


Fig. 1 Sampling stations in Klaipeda-Butinge site



Palanga 1 – sampling in June 2001
Palanga 2 – sampling in September 2001
Palanga 3 – sampling in June 2002
Palanga 4 – sampling in September 2002

Fig. 3 Frequency of micronuclei in erythrocytes of flounder from Lithuanian coastal area

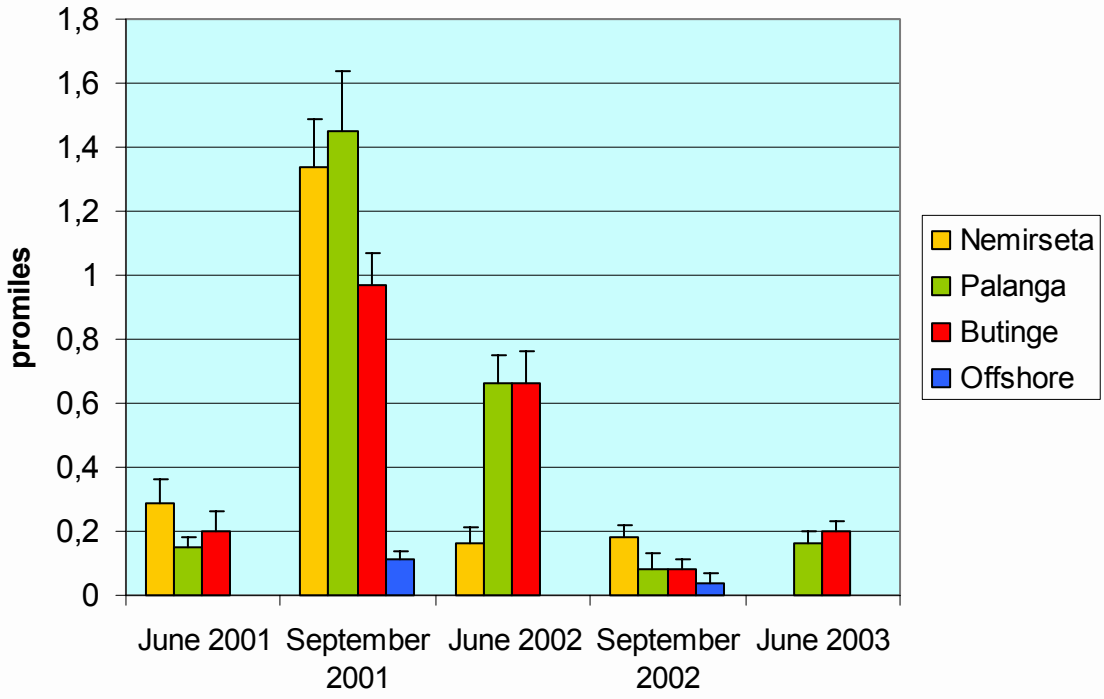


Fig. 4 Metallothionein level in soft tissues of mussels

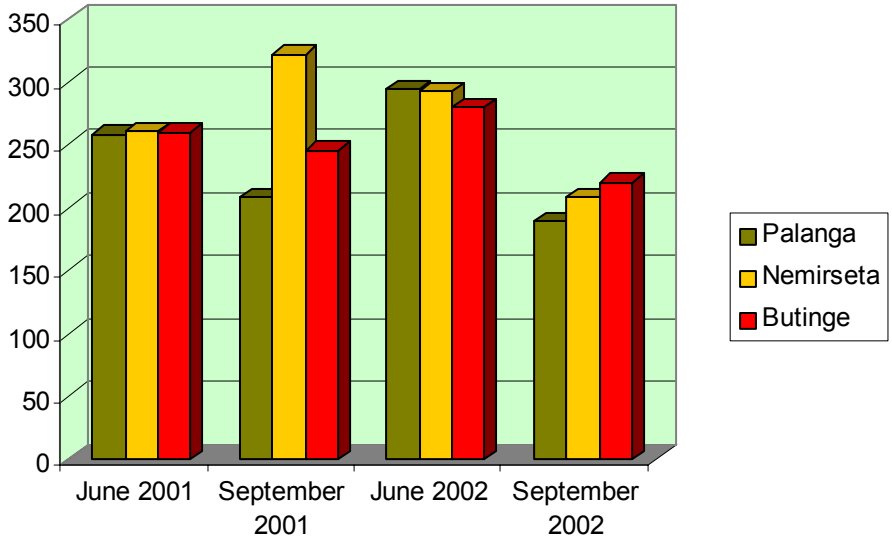


Fig. 5 Metallothionein level in muscle tissues of flounder

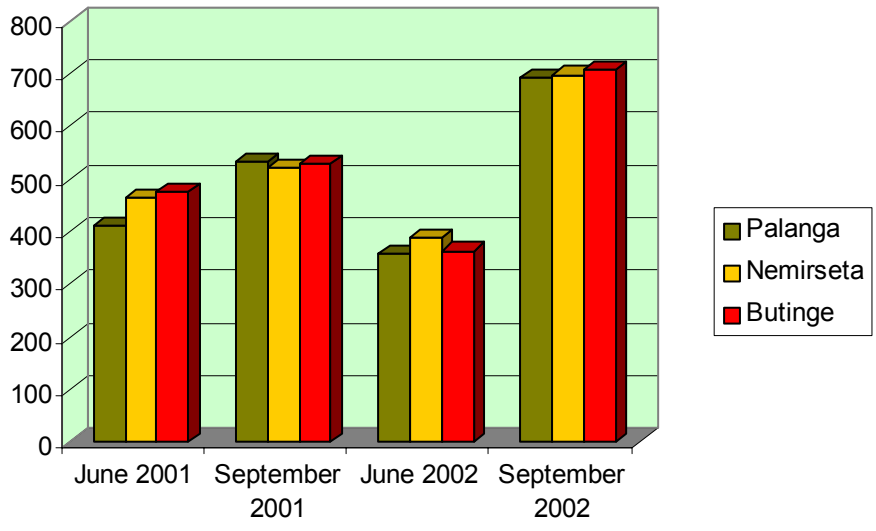


Fig. 6 AChE activity in gills of mussels, collected in 2002

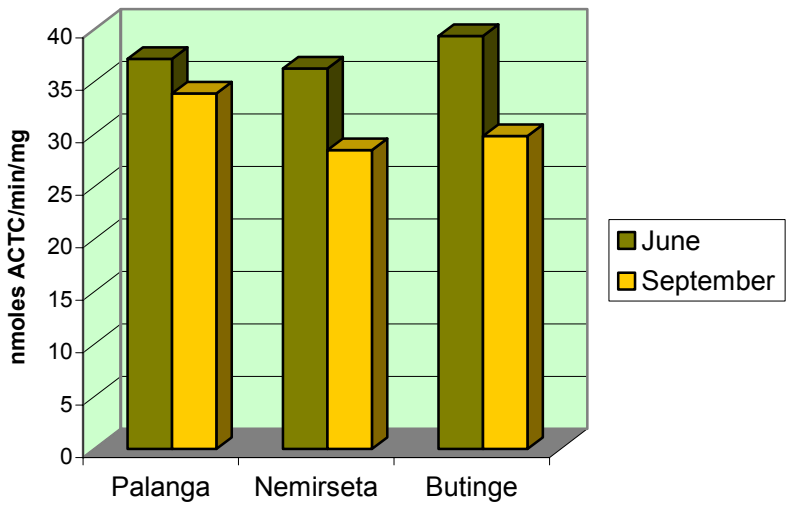


Fig 7. Frequency of apoptotic cells in gills of mussels from Klaipeda-Butinge site

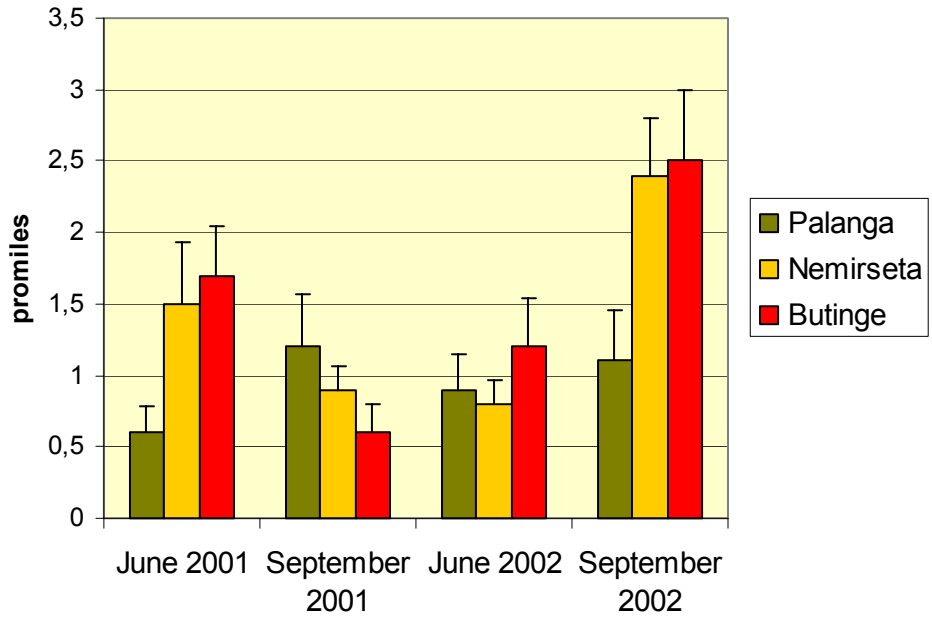


Fig. 8. PAH-metabolites in the bile of flounder from Klaipeda-Butinge site