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Measurements of biomarker levels in flounder (*Platichthys flesus*) and blue mussel (*Mytilus trossulus*) from the Gulf of Gdańsk (southern Baltic)

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Abstract

In the framework of the EU funded BEEP project a set of biomarkers, gross morphometric indices and tissue concentrations of selected organic pollutants were measured in flounder (*Platichthys flesus*) and mussels (*Mytilus trossulus*) collected twice a year (April and October) from three sites in the inner Gulf of Gdańsk between autumn 2001 and spring 2003. In flounder, seasonal differences in most biomarkers were observed, but no correlations with tissue pollutant levels could be found. In mussels, highly variable levels in biomarker responses were seen, but no clear seasonal or spatial trends, directly related to tissue concentrations, could be established. The observed biomarkers distribution the study sites are probably mostly caused by interannual, seasonal and individual variability and, in case of flounder, possibly by exchange of stocks between the sampling sites.

Keywords: Baltic Sea; Biomarkers; Gulf of Gdańsk; Flounder; Mussel; Organic pollutants

1. Introduction

The aquatic environment is affected by different types of chemicals toxic to biota that originate from both natural (e.g. heavy metals and various polycyclic aromatic hydrocarbons (PAHs)) and anthropogenic sources (e.g. PAHs, polychlorinated biphenyls (PCBs), pesticides and heavy metals). Many organic contaminants and heavy metals enter readily the food chain and tend to bioaccumulate, while some of them are rapidly metabolised (McCarthy and Shugart, 1990; Livingstone et al., 1992; Livingstone, 1993).

Traditionally, in order to monitor the effects of contaminants on biota analysis of tissue concentrations of selected compounds are carried out. A different approach is to measure the specific biological effects that different types of substances produce (Walker, 1995). Biomarkers are an integral part of this approach (McCarthy and Shugart, 1990; Peakall, 1992) and many of them are recommended tools for assessing the impacts of pollution on marine organisms (ICES WGBEC, 2004). Specifically, effects at the biochemical level are generally used as "early warning" signals for assessing the effects of contaminants on organisms (Haux and Förlin, 1988). This is due to the sensitivity, ease of application, low cost and specificity to pollution stress of many biomarkers (Livingstone et al., 1992; WHO, 1993).

Between 2001 and 2004, a pan-European EU funded research programme titled "Biological Effects of

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Environmental Pollution in Marine Coastal Ecosystems'' (BEEP) was carried out. A part of the BEEP project comprised of biomonitoring activities in different parts of the Baltic Sea (Lehtonen and Schiedek, 2006). The Gulf of Gdańsk in the southern part of the Baltic was selected as one of the five Baltic Sea study sites. The area is under a marked influence of inflow from the large Vistula River and sewage discharge from the Gdańsk–Sopot–Gdynia urban agglomeration consisting of a population of over one million people. In regard to biomarkers in fish and bivalve species only a limited number of studies have so far been performed in this area (Draganik et al., 1996; Kopecka and Pempkowiak, 2002, 2003, 2004; Napierska and Podolska, 2003a,b, 2005; Kopecka et al., 2004).

A suite of biomarkers reflecting exposure to and effects of contaminants at different biological levels were selected for this study. In regard to detoxification of organic contaminants the activity of CYP1A1, measured commonly as ethoxyresorufin-*O*-deethylase (EROD) activity, belonging to Phase I (biotransformation) enzymes, and glutathione-*S*-transferase (GST) involved in Phase II (conjugation) enzymes (Sijm and Opperhuizen, 1989; Stegeman et al., 1992) were chosen. Induction of EROD activity indicates not only exposure to organic chemicals such as PAHs, PCBs and dioxins (Livingstone, 1993; Bucheli and Fent, 1995) but may also precede effects manifested at various levels of biological organisation (Whyte et al., 2000; van der Oost et al., 2003). GSTs catalyse the conjugation of the tripeptide glutathione (GSH) with xenobiotics (e.g. PAHs and PCBs) (Clark, 1989; Stegeman et al., 1992) and metals (Pellerin-Massicotte, 1994; Regoli and Principato, 1995). Catalase (CAT) is a hematin-containing enzyme that reduces hydrogen peroxide (H_2O_2) to water and is widely used as a biomarker of oxidative stress (Winston and Di Giulio, 1991; Stegeman et al., 1992). Chemical analyses of fish bile have proved suitable to study the uptake of hydrocarbons and chlorinated organic compounds (Ariese et al., 1993). The levels of PAH metabolites in bile can be estimated as fluorescent aromatic compounds (FAC) (Ariese et al., 1993; Vuontisjärvi et al., 2004; Vuorinen et al., 2006). Inhibition of acetylcholinesterase (AChE) activity is commonly applied as a biomarker of exposure to organophosphorus and carbamate compounds used as active agents in many pesticides (Bocquené et al., 1990; Peakall, 1992; Escartin and Porte, 1997), but it responds also to other compounds including metals, detergents, hydrocarbons and algal toxins (Payne et al., 1996; Guilhermino et al., 1998; Lehtonen et al., 2003). Metallothioneins (MT) constitute a family of low-molecular weight, cysteine-rich proteins induced by a wide variety of metal ions (e.g. Cd, Cu, Zn, Hg, Co, Ni, Bi and Ag) (Stegeman et al., 1992; Roesijadi, 1992; Viarengo et al., 1999, 2000). Vitellogenin (VTG) is a high-molecular weight lipophosphoprotein which, in vertebrates, is synthesised in the liver and regulated by 17β-estradiol (Melancon et al., 1992; Kleinkauf et al., 2004a,b,c). Plasma concentrations of VTG in male fish have been used as a sensitive biomarker of exposure to a variety of compounds that produce estrogenic effects, recently including also PCBs and PAHs (van der Oost et al., 2003). Lysosomes are a morphologically heterogeneous group of membrane-bound sub-cellular organelles (Mayer et al., 1992) that play a key role in the catabolism of cellular components, intracellular transport of macromolecules and in the uptake and sequestration of organic and inorganic pollutants and their metabolites (Köhler and Pluta, 1995). The frequency of micronuclei (MN) is among the most widely used methods to assess of cytogenetic damage. The MN test enables the evaluation of the influence of genotoxic compounds at low concentrations and the assessment of dose–response relationships of both DNA reactive genotoxins and non-DNA reactive aneugens (Baršienė et al., 2004). Gross morphometric indices including condition factor (CF), gonadosomatic index (GSI) and hepatosomatic index (HSI) are potentially indicative of toxicant effects, providing information on energy reserves and the ability of individuals to tolerate chemical pollution challenge or other kinds of environmental stress (Mayer et al., 1992; van der Oost et al., 2003).

The use of biomarkers in selected indicator species (''biomonitors'') could be standardised to provide a database for future monitoring programmes (Goksøyr et al., 1996). Flounder (*Platichthys flesus*) is a widely distributed species in northern European coastal waters, including the Baltic Sea. This bottom-dwelling fish that prefers fine-grained to sandy sediments is regarded as a good indicator of pollution effects (Goksøyr et al., 1996; Burgeot et al., 2001). The blue mussel (*Mytilus* spp.), a sedentary suspension-feeding species is a cosmopolitan, dominant member of coastal and estuarine communities and therefore extensively used as a sentinel or indicator species in environmental monitoring programmes (e.g. Widdows and Donkin, 1992). In the Gulf of Gdańsk the mussel populations consist mainly of *Mytilus trossulus*. The species above were selected as target organisms for this study.

Samples were obtained during four seasonal sampling campaigns between October 2001 and April 2003. By applying this sampling scheme the suitability of biomarkers in establishing the ''health status'' of selected sites in the Gulf of Gdańsk was assessed using the flounder and mussel as biomonitoring organisms. To assess the level of contamination in the study area, concentrations of PCBs, DDTs, HCB, HCH and polybrominated diethyl ethers (PBDEs) were analysed from selected flounder tissue samples.

2. Materials and methods

2.1. Study area and sampling

The bathymetry and bottom sediments of the Gulf of Gdańsk are variable. The coastal areas are sandy while the open-sea part is situated at the edge of a deep sedimentary basin. The Inner Puck Bay forms a unique separate shallow basin in the western part of the gulf (Fig. 1).

The samples were collected following the general sampling strategy and procedures of the BEEP project Baltic Sea component. Flounder and mussels were collected from three sites along a suspected pollution gradient based on previous information on local contaminant levels (Pazdro, 2004) (Fig. 1): (1) Mechelinki, regarded as the most polluted of the three sites, located close to the sewage outflow from the Gdynia Waste Water Treatment Plant; (2) Sobieszewo, close to the mouth of the Vistula River; (3) Sopot, a recreational and tourist area. Bi-annual samplings were performed in October (2001 and 2002) and April (2002 and 2003).

Water depth, temperature and salinity at the sampling sites were measured during all sampling campaigns (Table 1). Mussels (shell length range 35 ± 5 mm) were collected aboard r/v "Oceania" using a drag net towed at the speed of 2.5–3 knots for 15–20 min. The mussels were kept in aerated seawater prior to dissection of target tissues aboard the vessel within 2–3 h from collection. Tissue samples were placed in cryovials, snap-frozen in liquid nitrogen and stored in an ultrafreezer (−80 °C). Flounder (size range 24–30 cm) were collected by local fishermen using gillnets. Within 2 h the catch was transferred to 30 l tanks filled with aerated seawater and transported to the laboratory, where the individuals were dissected during the same day. Total length and weight as well as liver, gonad and spleen weights were determined for each individual. The dissected tissues were frozen in cryovials in liquid nitrogen and stored at −80 °C (unless stated otherwise; see below) until the biomarker analyses. Samples for the different analyses were distributed among the BEEP Baltic Sea partner laboratories according to their analytical expertise. In regard to flounder the assessment of biomarker responses focuses on data obtained from females.

2.2. Biomarker measurements

AChE. The method of Ellman et al. (1961) modified for microplate readers by Bocquené and Galgani (1998) was used for the measurement of AChE activity in the muscle (flounder) or gill (mussels) tissue. Details of the method are described in Kopecka et al. (2004).

EROD. The procedure of Burke and Mayer (1974) modified for microplate readers using the S9 fraction (Galgani and Payne, 1991; Stagg and McIntosh, 1998) was applied for the determination of EROD activity in the liver tissue of flounder.

GST and CAT. For the measurement of GST activity (flounder: liver, mussels: digestive gland) the procedure of Habig et al. (1974) was followed. The method described by Claiborne (1985) was used for the determination of CAT activity (flounder: liver, mussels: gills). The analyses of GST activity was performed as a microplate reader assay (GENios, TECAN). CAT activity was measured using a spectrophotometer (DU®-62, Beckman).

LMS. Lysosomal membrane stability was assessed histochemically from liver (flounder) and digestive gland (mussels) tissue sections according to a standard operation procedure described in detail by Köhler et al. (2002).

MT. The analysis of MT was performed according to the method of Viarengo et al. (1997), using liver (flounder) or digestive gland (mussels) tissue. In flounder, the analyses were mostly performed on individual fish but occasionally (15% of cases) tissues from two individuals had to be pooled. In mussels, the analyses were always carried out using 5–8 pooled individuals. For more methodological details, see Leiniö and Lehtonen (2005).

MN. The analysis of MN in the hematocytes of flounder and the gill cells of mussels was performed according to Baršienė et al. (2004, 2006a,b).

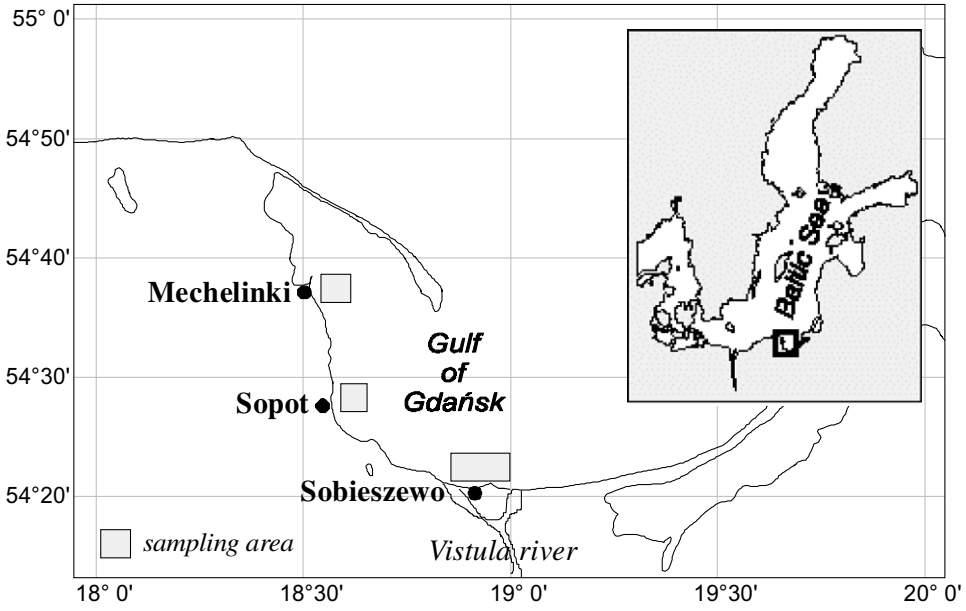

Fig. 1. Map of the BEEP sampling sites in the Gulf of Gdańsk.

Table 1
Background information on the sampling stations in the Gulf of Gdańsk

Campaign	Sampling date	Station	Coordinates		Depth (m)	Temperature (°C)	Salinity (PSU)
			Latitude	Longitude			
Autumn 2001	19.10[a]/07.10[b]	Mechelinki	54°37.4N	18°36.6E	20	14.8	6.84
	18.10[a]/06.11[b]	Sobieszewo	54°24.8N	18°52.8E	28	10.2	7.24
	26.10[a]/06.10[b]	Sopot	54°28.3N	18°40.3E	10–25	14.1	6.81
Spring 2002	25.04[a]/21.03[b]	Mechelini	54°37.9N	18°34.5E	16	3.2	7.07
	24.04[a]/23.03[b]	Sobieszewo	54°24.6N	18°53.4E	29	3.3	7.01
	19.04[a]/24.03[b]	Sopot	54°29.1N	18°39.7E	17	3.2	7.06
Autumn 2002	16.10[a]/14.11[b]	Mechelinki	54°36.2N	18°33.5E	11	9.4	nd
	28.10[a]/15.11[b]	Sobieszewo	54°24.3N	18°52.5E	22	8.5	nd
	25.10[a]/23.10[b]	Sopot	54°28.9N	18°39.9E	16	nd	nd
Spring 2003	22.04[a]/16.04[b]	Mechelinki	54°36.5N	18°33.3E	16	7.0	nd
	24.04[a]/14.04[b]	Sobieszewo	54°24.9N	18°53.4E	25	nd	nd
	06.05[a]/16.04[b]	Sopot	54°28.6N	18°40.5E	14	nd	nd

nd = No data available.
[a] Flounder sampling.
[b] Mussel sampling.

VTG. Concentrations of VTG in the plasma of flounder were quantified by means of an indirect competitive ELISA using a polyclonal rabbit antibody against perch (*Perca fluviatilis*) kindly provided by Prof. Lars Förlin (Gothenburg University). A detailed method description is found in Gercken et al. (2006).

PAH metabolites. Fluorescent aromatic compounds (FACs) were measured in the bile of flounder using the fixed wavelength fluorescence method (FF) according to Vuontisjärvi et al. (2004) and Vuorinen et al. (2006).

Protein. Protein in the S9 fraction used for calculating the specific activity of AChE, EROD, GST and CAT was measured by the method of Bradford (1976) with BSA (bovine serum albumin, Sigma) as the standard (see also Kopecka et al., 2004).

2.3. Gross morphometric indices

Gross morphometric indices were calculated for each individual flounder according to the following formulas:

$$\text{condition factor(CF)}: \ (W_T/L^3) \times 100;$$
$$\text{hepatosomatic index(HSI)}: \ (W_H/W_T) \times 100;$$
$$\text{gonadosomatic index(GSI)}: \ (W_G/W_T) \times 100,$$

where W_T = total wet weight (g), L = total length (cm), W_H = liver weight (g) and W_G = gonad weight (g).

2.4. Analysis of organic contaminants in flounder muscle tissue

Flounder muscle tissue samples were analysed for the following organic contaminants: dichlorodiphenyltrichloroethane (DDT) and its metabolites, polychlorinated biphenyls (PCB), hexachlorocyclohexanes (HCH), hexachlorobenzene (HCB), and polybrominated diphenyl ethers (PBDE). Analyses were performed on composite samples of 2–3 individuals selected according to station, sampling occasion and observed biomarker response established as the individuals having the highest and the lowest GST activities. Details of the methodology are given in Zebühr (1992) and Koslowski et al. (1994).

2.5. Statistical treatment of data

Relationships between the different biomarker responses in female and male flounder and seasonal differences (spring and autumn) were evaluated by Student's *t*-test (significance level at $p < 0.05$), except for MN for which the non-parametric Mann–Whitney *U*-test was applied. Seasonal and geographical differences in biomarker responses in flounder and mussels were assessed by the ANOVA rank Kruskal–Wallis non-parametric test designed for data sets with $n < 30$ ($p < 0.05$). Linear Pearson correlations were used to examine dependencies between the biomarkers ($p < 0.05$). Principal component analysis (PCA) was performed on a flounder data set consisting of four biomarkers (AChE, EROD, GST and CAT) and the three gross morphometric indices (CF, HSI and GSI) measured in 20 specimens collected at each of the three sites during all four sampling campaigns (a total of 1680 data points). All statistical analyses were performed using STATISTICA® 5.0 software package.

3. Results

3.1. Flounder

3.1.1. Biomarker ranges, and geographical, seasonal and gender differences

AChE. Mean activity of AChE in females ranged between 68.8 (Mechelinki, October 2002) and 187.1 nmol min^{-1} mg $protein^{-1}$ (Mechelinki, October 2001)

(Fig. 2). Statistically significant differences in AChE activity between the sampling stations could be observed only in October 2001. No differences were found between the sampling seasons (two autumns and two springs) ($p > 0.05$). Among all sampling campaigns significant differences were observed in Mechelinki and Sobieszewo. No significant differences between females and males could be found ($p = 0.3076$).

EROD. EROD activity was 3–20 times higher in spring compared to autumn, depending on site and year (Fig. 2). In autumn, mean EROD activity in females ranged between 102.4 (Sopot, October 2001) and 212.3 pmol min^{-1} mg $protein^{-1}$ (Mechelinki, October 2001) while in spring the range was from 1197.8 (Sobieszewo, April 2002) to 2198.0 pmol min^{-1} mg $protein^{-1}$ (Sopot, April 2002). Significant differences between the study sites were recorded in three out of four sampling campaigns. Significant seasonal variability was observed also between autumn and spring seasons at each sampling site ($p < 0.001$) and between all sampling campaigns at each station. In males the EROD activity was generally higher than in females, being significantly higher in males collected during sampling campaigns of October 2002 and April 2003 ($p < 0.001$).

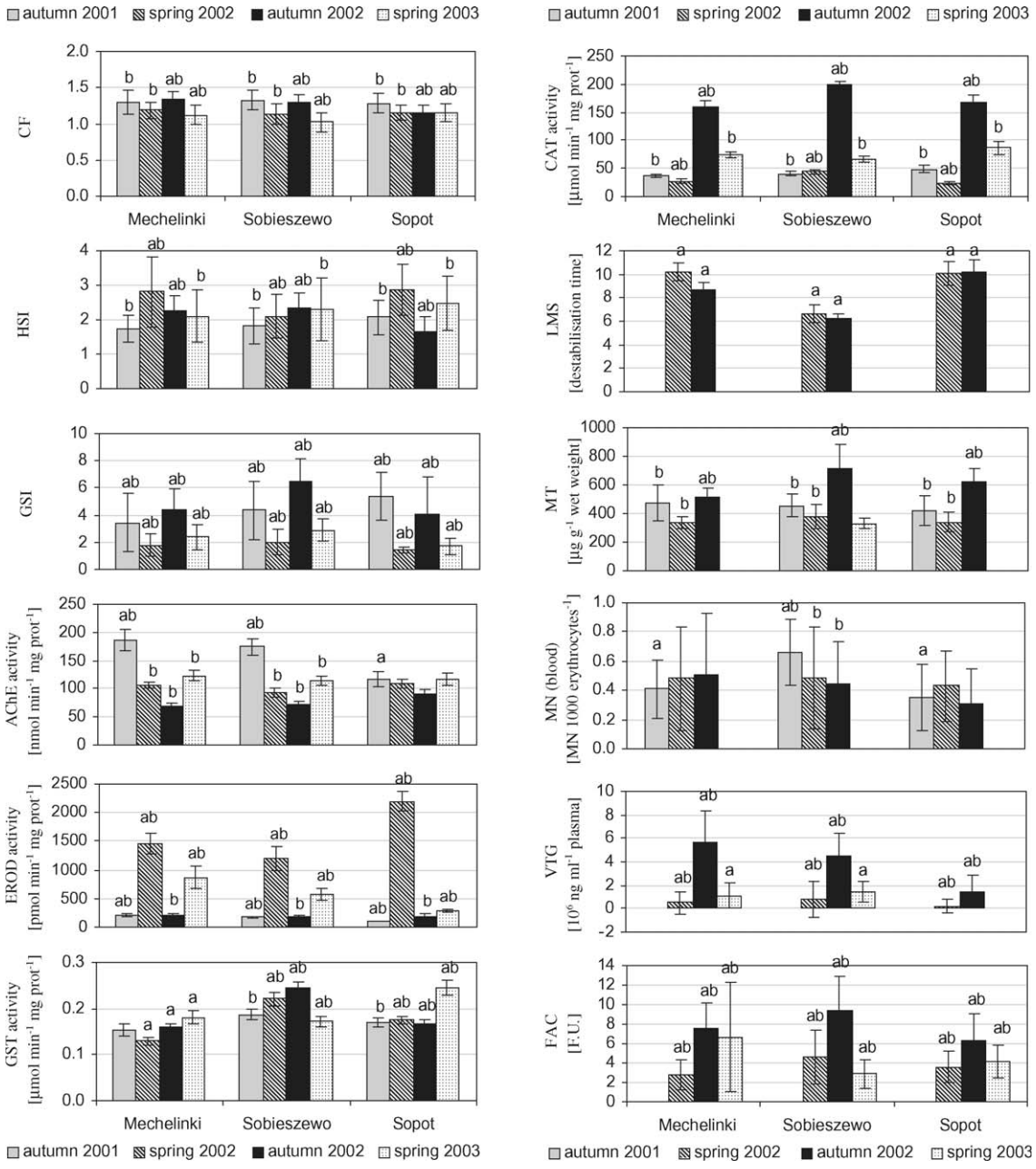

Fig. 2. *Platichthys flesus.* Mean values (±SD) of the different biomarkers measured in female flounder at the three study stations in the Gulf of Gdańsk during 2001–03. Statistically significant differences (Kruskal–Wallis ANOVA, $p < 0.05$ at least) between the sampling sites are indicated by "a" and seasonal differences within each site by "b".

GST. Mean GST activity in females ranged between 0.130 (Mechelinki, April 2002) and 0.245 μmol min^{-1} mg $protein^{-1}$ (Sopot, April 2003) (Fig. 2). Significant geographical differences were observed except in samples collected in October 2001. Seasonal variability between autumn and spring samples was significant at station Sopot ($p < 0.01$) and between all the sampling campaigns at stations Sobieszewo and Sopot. GST activity in males was always significantly higher compared to females at all sampling sites during all campaigns ($p < 0.001$).

CAT. Mean activity of CAT in females ranged from 24.3 (Sopot, April 2002) to 199.5 μmol min^{-1} mg $protein^{-1}$ (Sobieszewo, October 2002) (Fig. 2). The highest activities were measured in October 2002 at all sampling sites. Significant differences between the sites could be observed in April 2002 and October 2002. Seasonal variability was significant between the autumn and spring at each sampling site ($p < 0.001$) and between all sampling campaigns at each station. CAT activity was always significantly higher in males compared to females ($p < 0.001$).

LMS. LMS in female flounder was measured only in spring and autumn 2002. Mean LMS ranged from 6.20 (Sobieszewo, April 2002) to 10.28 min (destabilisation time) (Sopot, October 2002) (Fig. 2). Significant differences between the study sites could be observed both in autumn and spring. No significant seasonal differences were found ($p > 0.05$).

MT. Average concentration of MT in pooled liver samples from females ranged from 331.5 (Sobieszewo, April 2003) to 719.9 μg g^{-1} wet wt (Sobieszewo, October 2002) (Fig. 2). Statistically significant differences between the sampling sites were observed only in autumn 2002. Significant differences in MT levels between the autumn and spring samplings were found at each sampling site ($p < 0.001$).

MN. Mean MN frequency in females ranged from 0.31 to 0.66 MN 1000 $erythrocytes^{-1}$ (Fig. 2). The lowest MN levels (0.31–0.43 MN 1000 $erythrocytes^{-1}$) were detected in individuals from station Sopot while higher responses were observed at stations Sobieszewo (0.44–0.66 MN 1000 $erythrocytes^{-1}$) and Mechelinki (0.41–0.65 MN 1000 $erythrocytes^{-1}$). At station Mechelinki an apparent increase in MN frequency during the study period could be observed but the trend was not statistically significant. The MN values in flounder collected from Sobieszewo in October 2001 differed significantly from those recorded at stations Sopot and Mechelinki in October 2001 and 2002 (from $p = 0.0116$ to $p < 0.0001$). A significantly elevated level of genotoxicity was observed in individuals caught at station Mechelinki (April 2003) compared to those inhabiting the Sopot locality.

VTG. Mean concentrations of VTG in the blood plasma of females ranged from 0.2×10^6 (Sopot, April 2002) to 5.7×10^6 ng ml^{-1} (Mechelinki, October 2002) (Fig. 2). The levels differed intensely between the sampling sites according to season. VTG concentrations in females in Sopot were markedly lower than in Mechelinki and Sobieszewo both in October 2002 and April 2003. The levels showed significant seasonal variability ($p < 0.001$) and also between all sampling campaigns at each sampling site. In males the VTG levels were several orders of magnitude lower than in females, ranging from 216 (Mechelinki, April 2002) to 5291 ng ml^{-1} (Sopot, October 2002).

PAH metabolites in bile. Mean concentration of FACs in the bile of females ranged from 3 to 9 F.U. (fluorescence units) (Fig. 2). The levels were highest at all stations in October 2002 compared to both spring campaigns. Statistically significant differences were observed between the sampling sites during each campaign and between the sampling seasons in Mechelinki ($p < 0.05$), Sobieszewo ($p < 0.01$), Sopot ($p < 0.001$) and between all sampling campaigns.

3.1.2. Morphometric condition indices

CF. In females, the mean CF ranged from 1.02 (Sobieszewo, April 2003) to 1.34 (Mechelinki, October 2002) (Fig. 2). Significant differences between the stations occurred in autumn 2002 and spring 2003 between the different sampling seasons (two autumns and two springs) in Mechelinki and Sobieszewo ($p < 0.001$) and in Sopot ($p < 0.05$), and between all sampling campaigns at all and each sampling station. The CF did not differ significantly between the sexes ($p = 0.1897$).

HSI. In females, the mean HSI ranged between 1.66 (Sopot, October 2002) and 2.87 (Sopot, April 2002) (Fig. 2). Significant geographical differences in HSI could be observed in April 2002 and October 2002. Variability between the autumn and spring samples was significant at all stations ($p < 0.01$) except for Sobieszewo ($p > 0.05$). In males the HSI was significantly lower compared to females at all sampling sites during all campaigns ($p < 0.001$).

GSI. In females, the mean GSI ranged between 1.46 (Sopot, April 2002) and 6.43 (Sobieszewo, October 2002) (Fig. 2). Statistically significant differences between stations during each campaign, between seasons ($p < 0.01$) and among all sampling campaigns at all and each sampling stations were observed. In males the GSI was significantly lower than in females at all sites and during all campaigns ($p < 0.001$).

3.1.3. PCA on selected biomarkers and gross morphometric indices

Similar to the results obtained using ANOVA, seasonal (Fig. 4a) and year-to-year variability (Fig. 4b) could be seen in PCA performed on selected biomarkers and gross morphometric indices. Differences between the sampling sites were also observed (Fig. 4c, Table 2). The separation of station Sopot in autumn 2001 and spring 2003 was mainly caused by variation in the stage of gonad maturity, indicated by differing GSI. In spring 2002, station Sobieszewo was separated from the other two by the activity of GST and CAT; higher levels of PCBs and DDTs in muscle tissue were also recorded in specimens from Sobieszewo during this sampling campaign (Fig. 5).

3.1.4. Tissue levels of contaminants

In October 2001, the levels of ∑PCB and ∑DDT in the muscle tissue of flounder were relatively similar at all the study stations (145–165 and 348–492 ng g^{-1} lipid wt, respectively) (Fig. 5). In April 2002, concentrations of ∑PCB were elevated and markedly higher in specimens from Sobieszewo (235 ng g^{-1} lipid wt) compared to Sopot and Mechelinki where no change could be seen compared autumn 2001 (155 and 157 ng g^{-1} lipid wt, respectively). At the same time, markedly higher ∑DDT concentrations were also observed in Sobieszewo (875 ng g^{-1} lipid wt) compared to Mechelinki (351 ng g^{-1} lipid wt) and Sopot (507 ng g^{-1} lipid wt). Tissue levels of HCH did not show any spatial or temporal variability (17.8–20.8 ng g^{-1} lipid wt). HCB levels were stable in April 2002 (17.9–20.8 ng g^{-1} lipid wt) but had been up to 2–3 times higher in the previous autumn 2001 at Sobieszewo (38.3 ng g^{-1} lipid wt) and Sopot (46.1 ng g^{-1} lipid wt).

In October 2001 the levels of PBDE were twice higher at station Sopot (11.3 ng g^{-1} lipid wt) compared to the two other sites (5.0–5.8 ng g^{-1} lipid wt), but in April 2002 a quite opposite pattern could be seen at stations Sobieszewo and Mechelinki with 3–4 times higher levels to those measured in autumn (17.2 and 20.8 ng g^{-1} lipid wt, respectively) but no change in specimens collected from Sopot (12.2 ng g^{-1} lipid wt).

Correlations between biomarker responses and tissue contaminant concentrations were analysed separately for the spring and autumn campaigns (Table 3). The most interesting positive correlations were observed between total organic contaminant loads (∑POP) and CAT activity in October 2001 ($r = 0.90$), and with FAC ($r = 0.56$) and GSI ($r = 0.68$) in April 2001. Also, negative correlations were observed between total contaminant levels and AChE activity (indicating inhibition) in October 2001 ($r = -0.72$) and CF ($r = -0.56$) in April 2002. Since ∑PCB and ∑DDT formed the major bulk of ∑POP correlations with the respective groups were generally recorded (e.g. with CAT in autumn 2001 and GSI in spring 2002). Detoxification enzymes EROD and GST showed positive correlations only with HCB and ∑HCH, respectively, in April 2002. The levels of VTG in females correlated with ∑PCB ($r = 0.67$) and ∑PBDE ($r = 0.63$) in April 2002. However, some biomarkers showed a negative correlation with tissue contaminant levels, most notably EROD with ∑HCH in autumn 2001 and GST with ∑PCB in spring 2002.

3.2. Mussels

3.2.1. Biomarker ranges, and geographical and seasonal differences

AChE. Mean AChE activity in mussels ranged from 15.1 (Mechelinki, October 2001) to 38.1 nmol min^{-1} mg $protein^{-1}$ (Sopot, October 2001) (Fig. 3). Between-site variability was statistically significant only in October 2001 but almost significant also in spring 2002 ($p = 0.066$). No marked differences between the sampling campaigns could be observed when the stations where either pooled or treated separately.

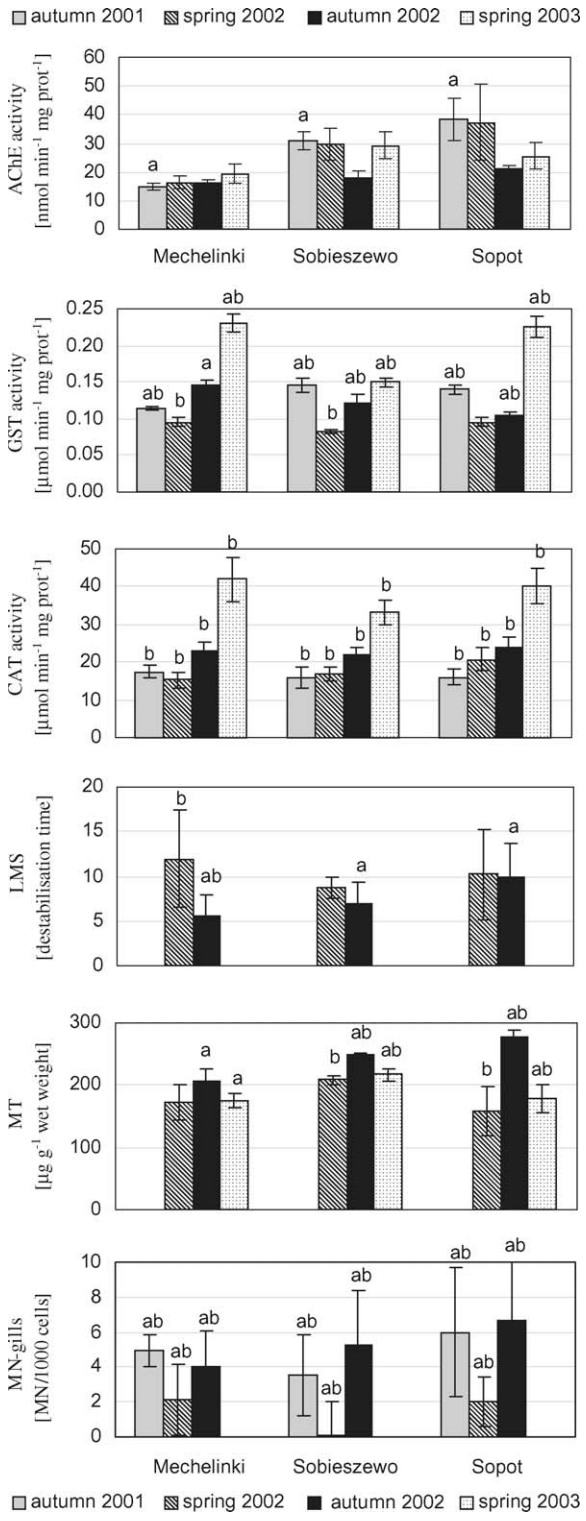

Fig. 3. *Mytilus trossulus.* Mean values (±SD) of the different biomarkers measured in mussels at the three study stations in the Gulf of Gdańsk during 2001–03. Statistically significant differences (Kruskal–Wallis ANOVA, $p < 0.05$ at least) between the sampling sites are indicated by "a" and seasonal differences within each site by "b".

GST. Mean GST activity in mussels ranged from 0.082 (Sobieszewo, April 2002) to 0.230 µmol min^{-1} mg pro-

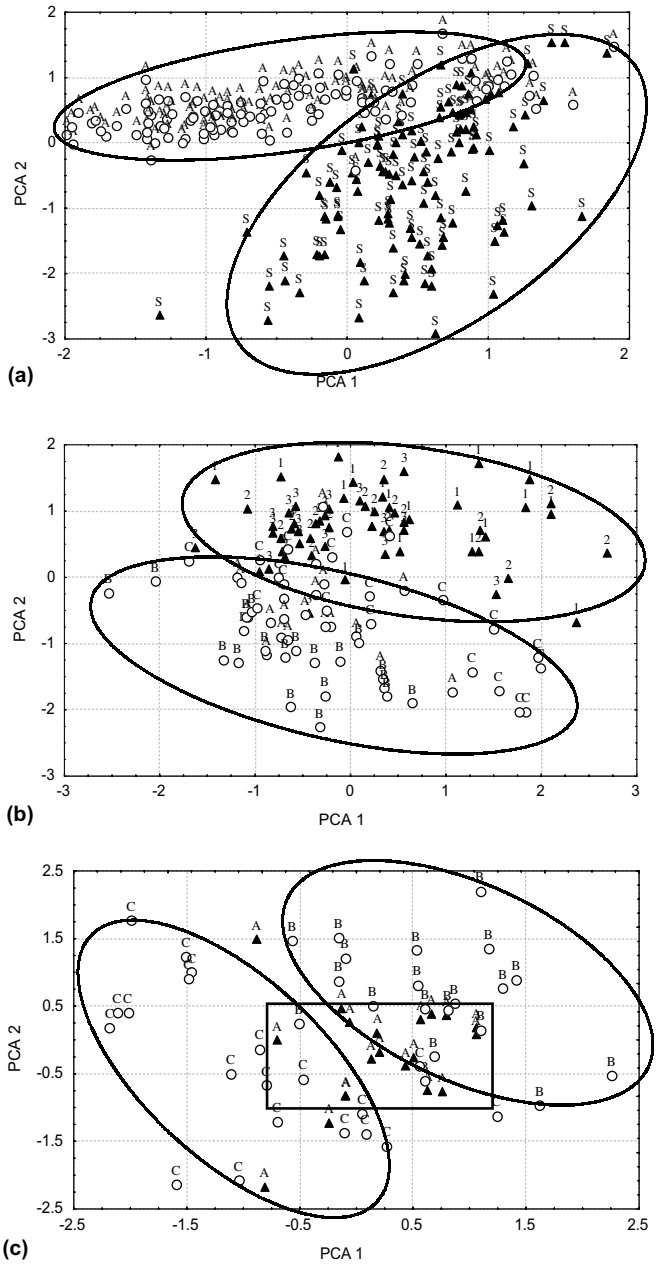

Fig. 4. *Platichthys flesus.* Selected PCA graphs showing (a) differences between the sampling sites during all sampling campaigns (A – autumns (October 2001 and 2002); S – springs (April 2002 and 2003)), (b) interannual variability (autumn 2001 and 2002) between the sampling sites (autumn 2001: 1 – Mechelinki, 2 – Sobieszewo, 3 – Sopot; autumn 2002: A – Mechelinki, B – Sobieszewo, C – Sopot), and (c) differences between the three sampling sites in autumn 2002 (A – Mechelinki, B – Sobieszewo, C – Sopot).

tein^{-1} (Mechelinki, April 2003) (Fig. 3). Significant differences between the sampling sites were observed in all cases except for spring 2002. Seasonal variability in GST activity was marked both in pooled or separately treated stations.

CAT. Mean CAT activity ranged from 15.2 (Mechelinki, April 2002) to 41.9 μmol min^{-1} mg protein^{-1} (Sobieszewo, April 2003) (Fig. 3). In April 2003 the activities were high at all sites in general. Mean CAT activities measured in spring (April 2002 and 2003) were significantly higher than those recorded in autumn (October 2001 and 2002; $p = 0.0045$). No between-site differences could be observed while seasonal variability was great within all sampling sites.

MT. Mean levels of MT ranged from 158.2 (Sobieszewo, April 2002) to 275.8 μg g^{-1} wet wt (digestive gland) (Sobieszewo, October 2002) (Fig. 3). Significant between-site variability in MT levels was recorded in all cases except for spring 2002 and also seasonal differences occurred between all sites and at each study site, except for Mechelinki.

MN. In autumn 2001 and 2002 the mean levels of MN reached 6.0 MN 1000 cells^{-1} in mussels from stations Sopot and Mechelinki (Fig. 3). In samples collected in early spring 2002 (March 21–24) the mean frequency of MN was significantly lower than in those obtained in autumn (October–November 2001) and practically identical at all stations. Geographical differences in MN levels were observed during all sampling campaigns except for spring 2002 and also seasonal variability occurred at each site.

LMS. Mean LMS showed a maximum of 11.70 and a minimum of 5.45 min (destabilisation time) in April and October 2002, respectively (Fig. 3). At station Mechelinki a significant difference in LMS could be noted between the two seasonal campaigns. Statistically significant differences between the sampling sites were recorded only in autumn 2002 while seasonal variability was significant when all stations were pooled and at station Mechelinki alone.

4. Discussion

4.1. Factors influencing biomarker levels in flounder in the Gulf of Gdańsk

Various biotic and abiotic factors are well known to affect biomarker responses. In the Baltic Sea seasonality in various environmental factors is more distinct than in

Table 2
Platichthys flesus; principal component analysis (PCA) on data of selected biomarkers and morphometric condition indices (see text)

Campaign assessed	Explained variability (%)			PC assignment		
	PC1	PC2	PC3	PC1	PC2	PC3
Autumn 2001	39.3	17.0	15.0	Station Sopot separated by GSI	CAT	EROD
Spring 2002	26.2	21.0	19.3	Station Sobieszewo separated by GST and CAT	CF,HSI	GSI
Autumn 2002	38.6	22.1	–	Stations Mechelinki, Sobieszewo and Sopot separated by GSI	EROD,GST,CAT	
Spring 2003	45.0	17.5	–	Station Sopot separated by CF, GSI, HSI and GST	AChE,EROD	

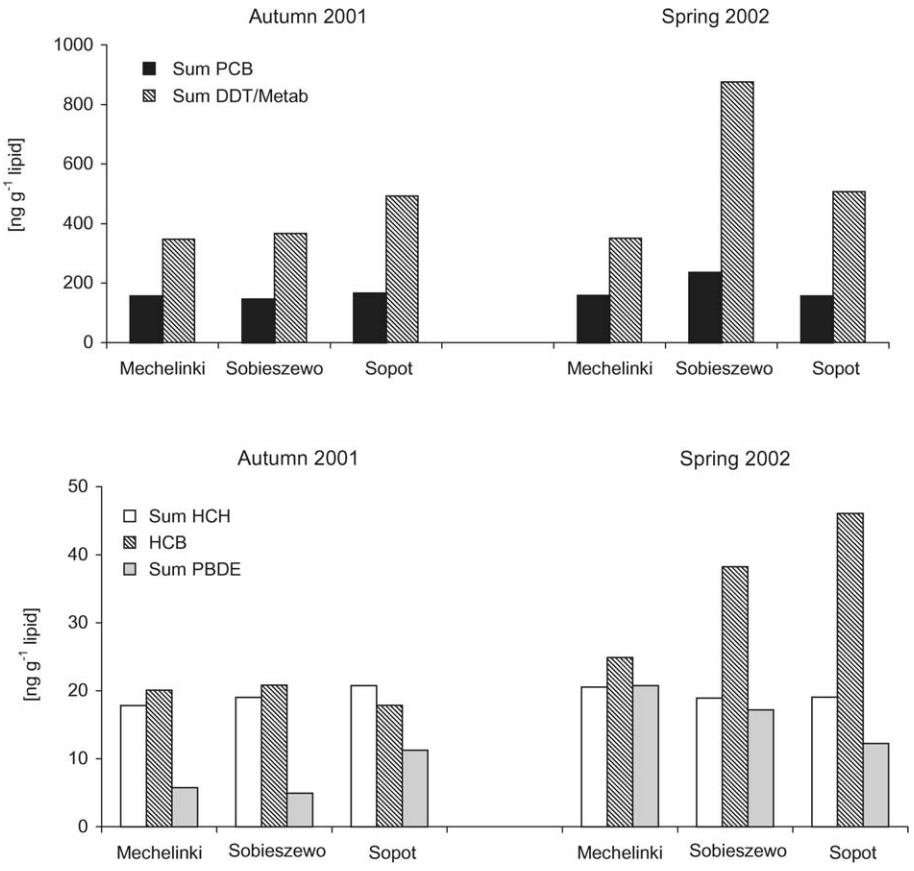

Fig. 5. *Platichthys flesus.* Levels of selected POPs (ng g^{-1} lipid wt) in the muscle tissue at the three study stations in the Gulf of Gdańsk in autumn 2001 and spring 2002.

Table 3
Platichthys flesus; Pearson's linear correlation coefficients on biomarkers and gross morphometric indices vs. concentrations of selected POPs (PCBs, HCHs, HCBs, DDT metabolites and PBDE (ng g^{-1} lipid)) in the muscle tissue of individual flounders collected in autumn 2001 (A2001) and spring 2002 (S2002)

Pearson correlation coefficient between parameter and tissue contaminant level												
Contaminant	∑PCB		∑HCH		HCB		∑DDT		∑PBDE		∑POPs	
Sampling	A2001	S2002	A2001	S2002	A2001	S2002	A2001	S2002	A2001	S2002	A2001	S2002
Parameter												
CF	0.17	**−0.71**	0.03	**−0.75**	**0.68**	−0.37	−0.12	−0.51	0.41	−0.13	0.03	**−0.56**
HSI	0.14	−0.39	0.16	0.11	0.15	0.26	0.26	−0.36	0.27	**−0.58**	0.24	−0.36
GSI	0.59	**0.87**	0.55	**−0.68**	0.20	0.13	0.47	**0.62**	0.34	0.51	0.55	**0.68**
AChE	**−0.74**	0.42	−0.47	−0.33	−0.33	0.19	−0.66	0.19	−0.43	0.01	**−0.72**	0.24
EROD	−0.66	−0.24	**−0.89**	−0.03	−0.37	**0.63**	−0.34	0.00	−0.54	−0.41	−0.51	−0.02
GST	0.25	**−0.57**	−0.23	**0.62**	0.48	−0.33	0.24	−0.14	0.28	−0.40	0.27	−0.24
CAT	**0.70**	−0.39	0.46	**0.63**	0.33	−0.45	**0.95**	−0.21	0.63	**−0.54**	**0.90**	−0.27
MN	−0.11	**−0.57**	−0.02	0.33	−0.05	−0.19	−0.23	−0.47	−0.28	−0.41	−0.12	−0.50
MT		0.33		−0.24		−0.11		0.40		0.30		0.39
VTG		**0.67**		**−0.55**		0.03		0.38		**0.63**		0.44
LMS		−0.40		0.38		0.07		−0.47		−0.45		−0.45
FAC		**0.62**		−0.51		0.24		**0.53**		0.15		**0.56**

Statistically significant correlations ($p < 0.05$) are given in bold. In autumn 2001 $n = 9$, in spring 2002 $n = 14$. Note: in regard to AChE and LMS a negative correlation indicates a higher response at higher tissue concentrations.

temperate areas; therefore, their influence on certain biomarker responses can be expected to be greater.

Dizer et al. (2001) observed the mean AChE activity in flounder collected in November from the Pomeranian

Bay (southern Baltic Sea) to vary between 60 and 102 nmol min^{-1} mg $protein^{-1}$, which falls within the range reported in the present study. Differences in AChE activity observed here could partly be explained by the high near-bottom temperatures recorded in autumn 2001 (10–14 °C) compared to autumn 2002 and springs 2002 and 2003 (see Table 1), because temperature can affect both contaminant concentrations and physiological activity of fish (Hogan, 1970; Bocquené et al., 1990; Bocquené and Galgani, 1998). Parallel to the present work, Napierska and Podolska (2005) studied seasonal (autumnal) differences in AChE activities in flounder from the Gulf of Gdańsk. Also they recorded a markedly higher AChE activity in autumn 2001 compared to the autumns of 2002 and 2003, attributing the interannual variability to differences in water temperature.

The seasonal variability in EROD activity in female flounder observed in this study has also been reported in the North Sea (Broeg et al., 1999) with seasonal values in the same range as those measured here (autumn: 20–850 pmol min^{-1} mg $protein^{-1}$, spring: 100–2900 pmol min^{-1} mg $protein^{-1}$). Draganik et al. (1996) measured substantially higher EROD activities (up to 1898 pmol min^{-1} mg $protein^{-1}$) in flounder from the Gulf of Gdańsk in February 1996, which suggests that pollution levels in the area might have been higher during that time. However, it is possible that these results obtained in February are more comparable to those of April (2002) in the present study when high values were also measured (station means between 1198 and 2198 pmol min^{-1} mg $protein^{-1}$) rather than October 2002 when much lower values were recorded (station means between 179 and 211 pmol min^{-1} mg $protein^{-1}$). The observed differences in EROD activity can be caused by variable stages of gonad maturity or HSI, which has been proposed by Whyte et al. (2000) as an "exposure index" to contaminants. In the Baltic the flounder spawns in winter, and the high EROD activity recorded here in spring can therefore be interpreted as resulting from spawning stress as noted elsewhere (Eggens et al., 1995, 1996; Kirby et al., 1999; Rotchell et al., 1999). Immature flounder have been recorded to show higher EROD activity than mature ones (Eggens et al., 1995; Draganik et al., 1996). In the present study the length range of the flounders studied was 24–30 cm and the specimens can therefore be considered mature. Conclusively, seasonal variability related mainly to the reproductive cycle affects markedly the levels of EROD activity in flounder from the Gulf of Gdańsk.

In the present study the spatial pattern of EROD activity was inconsistent. This may reflect the migratory nature of flounder and the mixing of stocks exposed to pollution in different areas, and differential gonad stage development of individuals. The data also imply that males have a higher EROD activity than females. In flounder, but also in other species, Förlin et al. (1984) reported similar patterns of a higher mono-oxygenase activity and total cytochrome P450 content in mature males compared to mature females. Gonadal sex steroids have been suggested to play an important role in the regulation of gender-dependent differences in cytochrome P450 mediated metabolism of xenobiotics in fish (Förlin et al., 1984). Conclusively, the observed seasonal differences indicating strong season-dependent temporal trends in EROD activity imply that the timing of sample collection is a crucial factor when this biomarker is used in monitoring programmes.

In regard to seasonal levels of VTG in mature female flounder, Kleinkauf et al. (2004c), measured very low concentrations of VTG in summer compared to winter from the Mersey Estuary (UK). In this study, 3–4 times higher VTG levels in female flounder at the Mechelinki and Sobieszewo stations were observed in autumn 2002 compared to those collected in Sopot. It is also noteworthy that HSI in females was markedly higher at the aforementioned sites during the period.

Bresler et al. (1999) observed seasonal changes in hepatic GST activity in flounder from the German North Sea but found no spatial variability. In Norwegian fjords, Beyer et al. (1996) reported GST activity in the flounder to range from 0.7 to 1.1 μmol min^{-1} mg $protein^{-1}$, which is at a substantially higher level than the values recorded in the present study. Also Vigano et al. (2001) reported slightly higher activities (mean 0.320 μmol min^{-1} mg $protein^{-1}$) in a laboratory experiment using flounder from the northern Adriatic Sea. Possible reasons for these differences include the effects of higher salinity and/or, in the case of the latter study, smaller individual size of specimens from the Adriatic Sea compared with flounder from the Gulf of Gdańsk.

In regard to CAT activity and LMS in the Baltic flounder little or no seasonal data is available for relevant comparisons. Broeg et al. (1999) reported a wider range but generally higher lysosomal destabilisation times in flounder from the North Sea (spring samples: 2–35 min, autumn samples: 3–30 min) compared to those determined here (spring 2002: 6.2–10.3 min, autumn 2002: 6.6–10.2 min). LMS data obtained for flounder from the two main regions, the Baltic and North Seas, are not markedly influenced by season but mainly by the sampling location. The lowest LMS values reported here during the spring and autumn campaigns of 2002 were recorded in flounder from Sobieszewo, the site close to the mouth of the Vistula River, indicating a deteriorated condition of the flounder population residing in this area.

Although no real between-site differences could be detected in the levels of MT they were almost twofold higher in specimens collected in autumn compared to spring at all study stations. Similar seasonal variability in MT levels was recorded in flounder collected during the BEEP project from the Lithuanian coast (Baršienė et al., 2006a). Thus, although differential exposure to pollutants during the season and the migration behaviour of the species likely play a major role, the ubiquitous seasonal changes observed are probably strongly related to endogenous metal homeostasis regulation during different stages of the reproductive cycle.

Comparison of MN frequency in flounder from different localities of the Gulf of Gdańsk revealed a comparatively low level of genotoxicity in individuals inhabiting the original "reference" station Sopot (0.31 MN 1000 erythrocytes^{-1}) and an elevated response in individuals from Mechelinki and Sobieszewo (up to 0.66 MN 1000 erythrocytes^{-1}). Genotoxicity of numerous organic substances in aquatic environment is associated with particulate matter while others are distributed in the water-soluble fraction (Claxton and Houk, 1998). Elevated levels of MN were detected in flounder and mussels even 8 months after the crude oil spill at the Būtingė oil terminal (Baršienė et al., 2004). Following the "Exxon Valdez" oil spill in Prince William Sound in March 1989, anaphase aberrations in fish embryos showed a correlation with concentrations of PAHs within the oil trajectory (Hose and Brown, 1998). In addition, more frequent chromosomal aberrations and malformations have been observed in cod (*Gadus morhua*) and pollock (*Pollachius virens*) embryos from an area contaminated by an oil spill (Longwell, 1977). In regard to PAH metabolites in bile similar levels (3–9 F.U.) were reported by Baršienė et al. (2006a) in flounder off the open-sea Lithuanian coast suggesting that PAH contamination is at a similar level in the two areas.

Results from the PCA illustrate some of the difficulties in distinguishing the influence of contamination from that of natural variability in the levels of many biomarkers. The potential impact of pollution can often be seen during one season (e.g. in spring or autumn) or during one sampling campaign, but when the biomarker levels of a two-year or longer study are evaluated the effects of contamination are often masked by seasonal and/or year-to-year variability.

The selected data on flounder used here for PCA indicates that Sopot, the site considered the least contaminated (Pazdro, 2004), is completely separated from the more contaminated site Sobieszewo, while station Mechelinki is partly separated from the two others (Fig. 4c). The separation between Mechelinki and Sobieszewo is much less clear. In general, the PCA results suggest that flounder probably is a less suitable organism for geographically small-scale monitoring (point sources) because, due to stock mixing, the results may represent specimens originating from a potentially large area. Therefore, to prevent stock mixing effects on the detection of pollution it can be recommended that the distances between the sampling sites for flounder should be sufficiently large.

The data obtained from the analyses of organic contaminants from flounder muscle tissues did not follow the expected pollution gradient basing on which the study sites in the Gulf of Gdańsk were originally selected. Because of the lack of clear contaminant gradients and small differences in concentrations observed between the sites the data are difficult to link straightforwardly to the biomarker responses observed.

PCBs and PAHs have been reported to occur only in moderate concentrations in the sediments of the Gulf of Gdańsk (Kowalewska and Konat, 1997; Konat and Kowalewska, 2001; Pazdro, 2004). Pazdro (2004) reported the lowest $\sum$PAH, $\sum$PCB and DDT concentrations at the Sopot site compared to a reference site outside the Hel Peninsula. Albalat et al. (2002) and Potrykus et al. (2003) reported high concentrations of $\sum$PAH, $\sum$PCB and DDTs in mussels as well as organotins in mussels and flounder in the vicinity of the Vistula River mouth and the Gdynia harbour (close to station Mechelinki). Different PAHs, PCBs and chlorinated pesticides have also been detected in mussels from this area (Potrykus et al., 2003). However, the tissue concentrations measured in flounder collected during this study do not agree well with these previous results.

A possible explanation for the observed variability in biomarker responses in flounder is that the concentrations of POPs in the sediments and biota of the Gulf of Gdańsk can in general be considered low in comparison with other marine coastal areas of industrialised regions (Bouloubassi and Saliot, 1991; Van Zoest and Van Eck, 1993; Fava et al., 2003; Potrykus et al., 2003; Pazdro, 2004) and therefore elicit only low biomarker responses. Furthermore, a statistically significant value of a correlation coefficient indicates that the hypothesis on the dependence between a contaminant and a given biomarker cannot be excluded, i.e. they may or may not be dependent. In this study, tissue concentrations of some important contaminant groups, e.g. PAHs and heavy metals, were not determined. However, the statistically significant correlation between FACs in bile and $\sum$PCB suggests that high levels of PAHs in the environment, revealed by the elevated metabolite concentrations, accompanied high tissue levels of PCBs. Finally, the CF of specimens was negatively correlated with most tissue contaminants, indicating a deteriorating influence of contaminant loads on the general health of fish.

4.2. Factors influencing biomarker levels in mussels in the Gulf of Gdańsk

In mussels from the southern and south-eastern Baltic Sea, comparable mean AChE activity levels to those recorded in the present study have been observed off the Lithuanian coast (25-37 nmol min^{-1} mg protein^{-1}; Baršienė et al., 2006a), but in the Wismar Bay (German Baltic coast) a much wider activity range was recorded (16–85 nmol min^{-1} mg protein^{-1}) depending strongly on season and location (Schiedek et al., 2006). Romèo et al. (2003) observed no seasonal variability in the AChE activity of the mussel *Mytilus galloprovincialis* in the NW Mediterranean. However, seasonal variability in AChE in *M. edulis* was apparent in the 3-year monitoring programme elucidating the effects of the "Erika" wreck off Brittany (Bocquené et al., 2004). In the Baltic Sea, where seasonal variations in the marine environment are more extensive than in temperate seas, greater variability can be anticipated. AChE activity in mussels from the northern Baltic Sea have been shown to have marked seasonal variability likely related to several abiotic (temperature, salinity) and

biotic (food abundance, reproductive stage) factors (Leiniö and Lehtonen, 2005), which themselves are characteristic for each geographical sub-region of the Baltic.

Dizer et al. (2001) noted no spatial differences in AChE activity of mussels collected from several coastal sites in the western Baltic Sea. In a nearby region, the Wismar Bay, inhibition of AChE activity in mussels followed the gradient of increasing tissue load of organochlorines (Schiedek et al., 2006), but this was not the case in two areas in the southern coast of Finland (Lehtonen et al., in press). In the present study, the low levels observed in AChE activity can in some cases be caused by unknown pesticides not measured in this study but may as well be attributed to other substances that have been shown to affect AChE activity (Payne et al., 1996; Guilhermino et al., 1998; Lehtonen et al., 2003). Whatever the type of contamination, the markedly lower AChE activities observed in mussels at Mechelinki compared to Sopot, both in autumn 2001 and spring 2002, indicate apparent biological effects at that site.

The mean GST activity of mussels ranged from 0.082 to 0.230 μmol min^{-1} mg $protein^{-1}$. In addition to pollution and seasonal variability also salinity may affect the levels of GST activity in mussels. In the low-salinity (6 PSU) Gulf of Finland (northern Baltic Sea), Leiniö and Lehtonen (2005) recorded a seasonal range from 0.162 to 0.472 μmol min^{-1} mg $protein^{-1}$ from sites considered relatively clean. In their study area, the seasonal pattern in GST activity was complex (e.g. poor correlation with changes in temperature) and evidently modulated by several abiotic and biotic factors. In the south coast of Ireland, Fitzpatrick et al. (1997) did not observe any significant seasonal changes in GST activity in mussels. No differences between sampling sites or any clear temporal seasonal trends for GST activity in mussels were observed from the western Mediterranean Sea by Porte et al. (2001), neither were they found in a lake and rivers in the Tucholski Landscape Park (central Poland) by Petushok et al. (2002). Seasonal changes in CAT activity in mussels have been reported by Solé et al. (1995), Regoli (1998), Narbonne et al. (1999) and Leiniö and Lehtonen (2005), all showing a higher CAT activity in spring compared to autumn, similar to this study. Bresler et al. (1999) observed no differences in GST activity levels of mussels measured from "clean" and polluted stations in the German part of the North Sea. The same was observed also for CAT activity in mussels off the Salento Peninsula (Italy) by Lionetto et al. (2003). However, both these authors suggest these findings to be caused by biochemical adaptation to chronic exposure to pollutants. In laboratory experiments, Suteau et al. (1988), Fitzpatrick et al. (1997) and Pempkowiak et al. (submitted for publication) recorded no response in GST activity in mussels exposed to various pollutants. In the present study neither GST nor CAT activities showed differences between the study sites, which could be interpreted as a homogenous distribution of contamination in the study area. However, most other results obtained in this study suggest different pollution levels at the sampling stations during each campaign and/or the effect of biotic and abiotic factors.

Baršienė et al. (2006a) reported MT levels in mussels off Lithuania to range from 180 to 310 μg g^{-1} wet wt (digestive gland), corresponding to those recorded in the present study. In the Wismar Bay the levels of MT were much lower (119–190 μg g^{-1} wet wt; Schiedek et al., 2006). In the Gulf of Finland the seasonal range consists of even higher MT levels (239–359 μg g^{-1} wet wt; Leiniö and Lehtonen, 2005). Since bioavailability of metals is well known to be salinity-dependent it is probable that the observed regional differences in MT levels in the Baltic Sea are, in addition to actual metal concentrations in the environment, also related to variability in the salinity regime. This further stresses the importance of taking into account regional differences and their effects on biomarker responses for correct interpretations.

In the present study it is of interest to note that while at the "reference" station Sopot the difference between the MT level in mussels measured in autumn was almost two-fold higher compared to the levels measured in spring, the corresponding seasonal differences at the two more contaminated sites were much smaller, while significantly higher MT levels were recorded in Sobieszewo in spring. It appears that the mussels inhabiting these two locations regarded contaminated may suffer from metal or oxidative stress (Viarengo et al., 2000) but the exact causal agents or mechanisms of action are difficult to pinpoint with the data available.

The highest mean value of MN frequency (6 MN 1000 $cells^{-1}$) recorded here was the highest incidence recorded in mussels compared to all other populations studied in the Baltic Sea (Baršienė et al., 2006b). In the autumn samples very high frequencies of MN were detected in mussels from Sobieszewo and Sopot localities while in early spring 2002 a low response was measured at all study stations. During the latter period the water temperature ranged between 3.2–3.5 °C, and, as a result, very low mitotic activity (less than one mitosis per 1000 cells) and comparatively low amounts of micronucleated cells (from 2.07 to 2.11 MN 1000 $cells^{-1}$) were registered in the gills of the mussels. In autumn 2001 mussels were collected twice (in October and November) from station Sobieszewo and the results confirmed the influence of water temperature on the frequency of MN in mussels with a higher incidence in specimens collected in October compared to November (Baršienė et al., 2006a). In the soft-bottom clam *Macoma balthica* high levels of cells with abnormal chromosome sets and high prevalence of tumours in the gill and digestive system have recently been reported in the Gulf of Gdańsk (Thiriot-Quievreux and Wołowicz, 2001; Sokołowski et al., 2004). Relationships between elevated tissue concentrations of heavy metals (As, Ag, Cd, Pb, Cu and Zn) in *M. balthica* and symptoms of environmental adversity in this area have been demonstrated (Sokołowski et al., 2004).

5. Conclusions

In this study carried out in the inner part of the Gulf of Gdańsk the differences observed in a number of biomarker responses are largely attributable to natural physiological processes and/or variability in environmental hydrographic and climatic conditions during the season. However, they are at least partially caused by pollution due to e.g. differential exposure conditions between the locations and mobilisation of accumulated toxicants within the organism during specific physiological states (e.g. starvation, reproduction), meanwhile the different sensitivity of species and individuals to pollutants also contributes to the observed variability. According to the currently available data on the levels of anthropogenic contaminants the Gulf of Gdańsk on the whole is not considered as a heavily polluted area. Yet, the combination of biological effects and tissue contaminant data obtained in the present study could in many cases detect geographical differences related to the nature and degree of pollution within the study area.

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