Relationship between biomarker responses and contaminant concentration in selected tissues of flounder (*Platichthys flesus*) from the Polish coastal area of the Baltic Sea

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Abstract

Previous studies in the Gulf of Gdańsk discussed the responses of selected enzymatic biomarkers to the contaminant gradient in fish and mussels. In the present study, flounder muscle and liver tissues were analyzed for polychlorinated biphenyls (PCB congeners: 28, 52, 101, 118, 138, 153 and 180), organochlorine pesticides (HCHs, HCB and DDTs), and trace metals (Pb, Cd, Zn, Cu, Hg, Cr). An attempt was made to identify the relationship between the measured enzymatic biomarker responses (cholinesterases, malic enzyme, isocitrate dehydrogenase, glutathione S-transferase) and contaminant concentrations in selected flounder tissues. The observed differences in enzymatic biomarker levels suggest that chronic exposure to low-concentration mixtures of contaminants may be occurring in the studied area. However, no conclusive evidence was found of a clear link between the biomarker responses and contaminant concentrations in flounder tissues.

1. Introduction

Over the past fifty years there have been substantial inputs of POPs into the Baltic Sea from numerous sources. These include industrial discharges of organochlorines in effluents from pulp and paper mills, runoff from farmland, the antifouling paints used on ships and boats, and dumped

wastes. Although recent monitoring data indicate that loads of certain hazardous substances have been reduced over the past ten years, problems do persist (HELCOM 2002). Concentrations of dioxins and PCBs in marine ecosystems declined in the 1980s, but this decrease leveled off in the 1990s. The concentration of dissolved trace metals in the Baltic Sea is many times higher than in the northern Atlantic, and these elements can still be found in high concentrations in certain marine organisms (HELCOM 2003). For endocrine disrupting substances and new contaminants like flame retardants, a full assessment of their levels or effects is not possible owing to the lack of monitoring data.

It has been suggested that the biological effects of such contamination may be more severe in brackish water systems than in marine systems. Laboratory studies have indicated that the sensitivity of aquatic animals to contaminants is closely related to the disruption of water and ion regulation in them (Cleveland et al. 1991, Dave et al. 1993). The results of the EU BEEP Project clearly indicated that the present contaminant concentrations in the different parts of the Baltic Sea are eliciting biological responses in various species, and in some cases are leading to chronic stress (Baršienė et al. 2006, Lang et al. 2006, Schiedek et al. 2006, Vuorinen et al. 2006). The Gulf of Gdańsk is one of the anthropogenically most severely stressed sites in Polish marine areas because of the great impact of the Vistula River, the second largest river in the Baltic Sea drainage area. Field-testing of a battery of biomarkers in this location showed responses of selected enzymatic biomarkers to a contaminant gradient in both fish and bivalves (Napierska & Podolska 2005, Kopecka et al. 2006, Napierska et al. 2006).

Many substances can affect the physiological processes of living organisms through the induction or suppression of enzymatic reactions (Stegeman et al. 1988, Galgani et al. 1992, George 1994, Escartín & Porte 1996, Kirby et al. 2000). The neurotoxic effects of carbamates or organophosphates can be evaluated by measuring the inhibition of cholinesterase (ChE) activity; however, many authors have also linked the inhibition of ChE activity to the toxic effects of heavy metal exposure (Payne et al. 1996, Bocquené et al. 1997, Guilhermino et al. 1998, Dethloff et al. 1999, Sturm et al. 1999). Widespread pollutants such as PAHs, PCBs and dioxin-like compounds usually induce phase II biotransformation glutathione S-transferase (GST) activity (Gubbins et al. 2000, Lenártová et al. 2000, Porte et al. 2000, Xu et al. 2001). Far fewer studies have described changes in enzyme activity that reflect basic metabolism in relation to exposure to contaminants. The malic enzyme (ME) that occurs in the cells of the metabolically more active tissues in crustaceans and bivalves may play an important role in pyruvate and Krebs cycle intermediate metabolism (Paynter et al. 1985,

Brodey & Bishop 1992a,b). It has been suggested that extramitochondrial malic enzyme could be one of the enzymes involved in the anaplerotic supply of Krebs cycle intermediates in skeletal muscle (Świerczyński 1980, Biegniewska & Skorkowski 1983, Konradt & Braunbeck 2001). Isocitrate dehydrogenase (IDH) is an enzyme that participates in the citric acid cycle. Both NADP-dependent enzymes have the ability to regenerate cellular NADPH, which is a necessary cofactor in antioxidant and detoxification systems.

In earlier publications, the authors presented the results of enzyme biomarker measurements (ChEs, GST and EROD) in flounder from the Polish coastal area of the Baltic Sea (Napierska & Podolska 2005, Napierska et al. 2006). In the present study, the results of previous measurements of ChE and GST activity and new data on contaminant concentration in selected tissues of flounder are analyzed using the new GLM model. The activities of two other enzymes (ME, IDH) are also discussed. An attempt is made to establish a relationship between all the measured biomarker responses and contaminant concentrations in selected flounder tissues.

2. Material and methods

2.1. Sample collection and handling

Flounder were sampled in the southern Baltic Sea in September 2001, 2002 and 2003. The fish were caught at three sites regarded as 'contaminated' in the Gulf of Gdańsk (C1 – Mechelinki, C2 – Sopot, C3 – the Vistula Mouth) and from a site on the open sea coast, considered to be 'clean' and designated as the reference region (REF – Łeba) (Figure 1). Detailed descriptions of the study area in relation to contamination can be found in the authors' earlier publications (Napierska & Podolska 2005, Napierska et al. 2006). Thirty fish were collected at each site (15 males and 15 females) during each sampling; only fish over 20 cm in length were taken. The liver and muscle of each fish were excised and immediately frozen at −80°C for biochemical analysis. Dissections were performed within 1 h of capture. The total body length (cm) weight (g), age, sex and gonad developmental stage of each fish were recorded. The age was determined from otoliths, and the gonad stages were classified according to Maier's scale. Fulton's formula was used to determine the body condition factor, CF:

$$\mathrm{CF} = \frac{\mathrm{w}}{\mathrm{l}^3} \times 100,$$

where w – total weight and l – length of the fish.

Somatic indices for liver (HSI) and gonads (GSI) were determined as follows:

$$\mathrm{HSI} = \frac{w_l}{w_f} \times 100$$

and

$$\mathrm{GSI} = \frac{w_g}{w_f} \times 100,$$

where w_l – weight of the liver, w_g – weight of gonads and w_f – weight of fish.

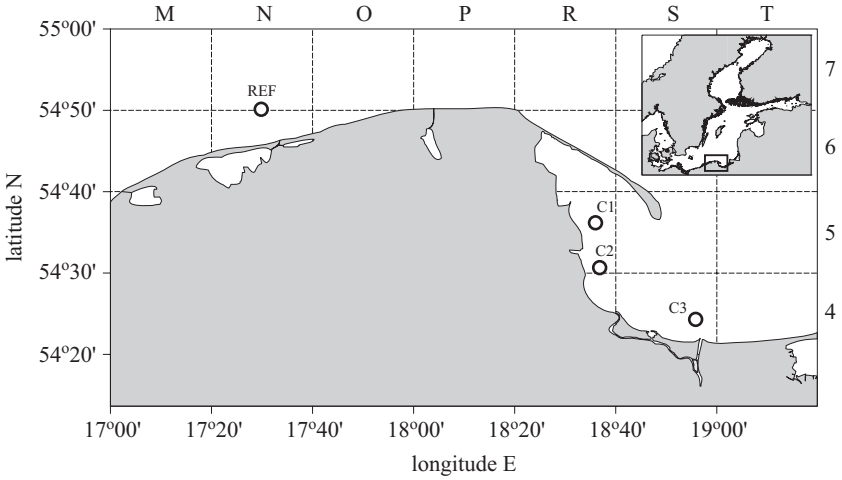

Figure 1. Location of flounder (*Platichthys flesus*) sampling sites: REF – 'clean' reference site, C1, C2, C3 – contaminated sites (C1 – Mechelinki, C2 – Sopot, C3 – Vistula Mouth)

2.2. Preparation of tissue homogenates and enzyme activity determination

The methods of extraction of ChEs and GST were described earlier (Napierska & Podolska 2005). ChE activities were determined using the method described by Ellman et al. (1961) and adapted for use with a microplate reader (Bocquené & Galgani 1998). The enzyme kinetics was monitored at 412 nm for three minutes. The standard reaction mixture (final volume 0.380 cm^3) contained 0.02 M phosphate buffer (pH 7.0), 0.5 mM DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) and 2.6 mM ACTC (acetylthiocholine chloride) or BCTC (butyrylthiocholine chloride).

GST measurements were performed using a modification of the method described in Habig et al. (1974). The enzyme activity was tracked

spectrophotometrically with a microplate reader at 340 nm. The standard reaction mixture (final volume 0.210 cm^3) contained 0.1 M phosphate buffer (pH 7.4), 1 mM CDNB and 1 mM GSH.

For IDH and ME, extraction was performed on 500 mg muscle tissue using a 0.02 M phosphate buffer (pH 7.0). The tissue was homogenized in buffer (1:4, w:v) and centrifuged at 10 000 g for 20 minutes at 4°C. An aliquot of the supernatant (the S9 fraction) was stored at −80°C and used in the assay. IDH and ME were assayed spectrophotometrically as described in Gronczewska et al. (2003) by monitoring the changes in absorbance at 340 nm as a result of the appearance of NADPH. The standard reaction mixture for the ME assay contained 50 mM Tris-HCl at pH 7.5, 0.5 mM NADP, 10 mM L-malate and 1 mM $MnSO_4$. The standard reaction mixture for the IDH assay contained 50 mM Tris-HCl at pH 7.5, 0.5 mM NADP, 5 mM isocitrate and 1 mM $MnSO_4$.

Protein concentration was determined as described by Bradford (1976) using the Protein Kit II from Bio-Rad laboratories and bovine serum albumin as the protein standard.

2.3. Chemical analysis

The flounder muscle tissue from the females from one sampling site was pooled and analyzed for polychlorinated biphenyls (PCB congeners: 28, 52, 101, 118, 138, 153 and 180), organochlorine pesticides (HCHs, HCB and DDTs) and trace metals (Pb, Cd, Zn, Cu, Hg, Cr). The same pooling procedure was applied to the muscle tissue from the males, and to the male and female liver tissue. Liver and muscle tissue was lyophilized and extracted with hexane to obtain a fat containing the accumulated organochlorine compounds. This extract was cleaned with sulfuric acid (di Muccio et al. 1990). DDTs and PCBs were determined by capillary gas chromatography with electron capture detection (UNEP/IOC/IAEA 1988). Trace metals were determined by atomic absorption. The fish tissue was mineralized with nitric acid in microwave ovens. The zinc and copper concentrations (> 1 mg kg^{-1}) were determined by the flame method, and the concentrations of lead, cadmium, chromium and copper (< 1 mg kg^{-1}) were determined by the flameless method in a graphite furnace. The mercury concentration was measured by the vapor generation method in a gold amalgam mercury analyzer (AMA 254) (UNEP/FAO/IAEA/IOC 1984a,b). The Testing Laboratory of the Sea Fisheries Institute works in compliance with a quality system (the laboratorys competence is confirmed by Accreditation Certificate No. AB 017).

2.4. Statistical analysis

The significance of the differences between average concentrations of trace metals and organochlorine compounds in the muscle tissue and liver of flounder sampled at the clean and contaminated sites were tested with the non-parametric Mann-Whitney U-test. The significance level was taken to be $p < 0.05$. Next, the data were analyzed using generalized linear models (GLM) (McCullagh & Nelder 1989). The calculations were performed using the GenStat software package (GenStat 2002). Enzymatic activity (AChE, BChE, GST, IDH, ME) was modeled as being dependent on the area, year of sampling, the biological parameters of the fish (body length, condition factor, sex, gonad maturity stage) and contaminant concentrations as the explanatory variables. The values of the dependent variables were log-transformed. Separate models were fitted for each of them:

$$\mathrm{y} = \mathrm{area} + \mathrm{year} + \mathrm{sex} + \mathrm{gonad\ stage} + \mathrm{LT} + \mathrm{CF} + \mathrm{Cd} + \mathrm{Cr} + \mathrm{Cu} +$$

$$+ \mathrm{Hg} + \mathrm{Pb} + \mathrm{Zn} + \mathrm{DDTs} + \mathrm{HCHs} + \mathrm{PCBs} + \mathrm{HCB} + \mathrm{error}$$

where y (dependent variable) – enzymatic activity in muscle tissue (AChE, BChE, IDH, ME) and liver (GST); LT – total length of fish; CF – body condition factor; Cd + Cr + Cu + Hg + Pb + Zn + DDTs + HCHs + PCBs + HCB – contaminant concentrations in fish tissues (muscle or liver). The concentrations of contaminants in fish muscles were used for modeling the muscular enzymatic activity (AChE, BChE, IDH, ME), and the contaminant levels in fish liver for hepatic GST. The area has 4 factor levels (REF, C1, C2, C3), the year 3 levels (2001, 2002, 2003), the sex 2 levels (males, females) and the gonad stage 4 levels (2, 3, 4, 8).

The error term was assumed to be normally distributed with zero mean and constant variance. Corner point parameterization was used, i.e., factor effects for level one were assumed to be zero for all factors. Thus, the factor effect for the other levels can be regarded as the difference between the effect at a given level and the effect at level one. The significance of factors and variables was tested, and only significant terms were left in the final model. Similarly, factor levels that did not produce a significantly different response of the enzymatic activity were grouped into new factor levels. The tests were performed by deletion, i.e., only those terms whose deletion did not result in a significant increase in deviance (i.e., the GLM measure of discrepancy between modeled and observed values) were left in the model, and the F-test was used. The model assumptions and performance were evaluated by analyzing residuals.

3. Results

3.1. Biological parameters

Table 1 shows the biological parameters of the examined fish. Fish length ranged from 23 to 41 cm. The fish sampled at Łeba (the 'clean', reference site) were usually longer, with a higher condition factor in comparison to the fish sampled at the other sites. The HSI and GSI index values were usually also the highest in fish from this area.

Table 1. Biological parameters recorded in the examined flounder (*Platichthys flesus*)

Sex	Year	Area	Date of Sampling	n	Body length min	Body length max	CF	SE	HSI	SE	GSI	SE
male	2001	REF	20.09.2001	15	25	30	1.36	0.03	2.07	0.11	ND	–
		C1	21.09.2001	15	25	29	1.24	0.03	1.75	0.09	ND	–
		C3	14.09.2001	15	24	27	1.38	0.03	1.65	0.12	ND	–
	2002	REF	4.09.2002	15	25	30	1.29	0.03	2.41	0.11	0.62	0.08
		C1	25.09.2002	15	25	27	1.19	0.03	1.84	0.10	0.79	0.09
		C2	10.09.2002	15	25	28	1.20	0.02	1.61	0.13	0.32	0.04
		C3	12.09.2002	15	25	31	1.21	0.04	1.73	0.08	0.60	0.10
	2003	REF	5.09.2003	15	26	31	1.39	0.05	2.03	0.08	0.28	0.03
		C1	4.09.2003	15	23	30	1.31	0.05	1.73	0.13	0.17	0.02
		C2	11.09.2003	15	25	29	1.20	0.03	1.44	0.10	0.24	0.02
		C3	10.09.2003	15	25	28	1.22	0.02	1.56	0.08	0.23	0.03
female	2001	REF	20.09.2001	15	25	32	1.54	0.03	2.38	0.13	ND	–
		C1	21.09.2001	15	25	31	1.35	0.03	1.73	0.07	ND	–
		C3	14.09.2001	15	25	28	1.49	0.03	1.93	0.11	ND	–
	2002	REF	4.09.2002	15	26	39	1.36	0.04	2.76	0.13	2.46	0.15
		C1	25.09.2002	15	25	30	1.28	0.02	1.70	0.08	2.02	0.19
		C2	10.09.2002	15	26	31	1.30	0.02	2.05	0.12	2.20	0.19
		C3	12.09.2002	15	25	29	1.27	0.03	1.96	0.08	2.98	0.21
	2003	REF	5.09 2003	15	25	41	1.48	0.05	2.50	0.13	2.77	0.23
		C1	4.09 2003	15	24	28	1.35	0.03	1.83	0.12	1.46	0.12
		C2	11.09 2003	15	25	33	1.34	0.04	1.85	0.16	2.27	0.17
		C3	10.09 2003	15	26	31	1.33	0.03	1.81	0.11	2.05	0.10

ND – not determined.

Table 2 presents the mean enzymatic activities of the biomarkers (IDH, ME) with standard error. The highest activity was recorded in fish sampled at the reference site in 2001. Generally, IDH activity was higher in males. The results of measurements for AChE, BChE and GST are presented in detail in Napierska & Podolska (2005).

Table 2. Mean enzymatic activity of IDH and ME [nmol min^{-1} mg $protein^{-1}$] with standard errors (SE) in the examined flounder (*Platichthys flesus*)

Sex	Year	Area	n	IDH	SE	ME	SE
male	2001	REF	15	52.75	3.00	11.85	1.37
		C1	15	39.39	3.00	10.79	1.06
		C3	15	46.39	3.21	10.56	0.73
	2002	REF	15	40.38	3.80	6.44	0.38
		C1	15	43.44	2.53	6.33	0.41
		C2	15	48.68	5.11	6.68	0.52
		C3	15	44.62	4.13	7.61	0.37
	2003	REF	15	36.59	2.57	6.87	0.33
		C1	15	34.69	3.06	7.28	0.35
		C2	15	45.95	1.80	8.10	0.41
		C3	15	39.60	3.39	7.40	0.41
female	2001	REF	15	50.99	4.30	14.94	1.99
		C1	15	31.73	1.15	13.72	1.32
		C3	15	46.24	4.07	9.31	0.53
	2002	REF	15	30.99	2.04	4.97	0.33
		C1	15	36.81	2.59	6.84	0.27
		C2	15	41.74	2.19	6.37	0.41
		C3	15	35.70	1.89	7.02	0.32
	2003	REF	15	36.93	2.00	6.82	0.36
		C1	15	35.80	1.11	6.00	0.30
		C2	15	40.14	2.41	6.49	0.23
		C3	15	29.71	2.50	7.01	0.31

3.2. Concentrations of trace metals and organochlorine substances

Table 3 sets out the concentrations of trace metals and the major organochlorine substances in the pooled samples of muscle tissue and liver of flounder.

Cd levels in the fish muscles (0.001–0.03 mg kg^{-1} dry weight (d.w.)) were lower than those in the liver (0.15–1 mg kg^{-1} d.w). Cr concentrations did not usually exceed 0.2 mg kg^{-1} d.w. in either of the tissues examined. Like the Cd levels, Cu concentrations were lower in the muscles (0.1–1.4 mg kg^{-1} d.w.) than in the liver samples (28–66 mg kg^{-1} d.w.). Hg levels lay at 0.09 –0.3 mg kg^{-1} d.w. in muscle tissue and 0.04–0.13 mg kg^{-1} d.w. in liver. Pb concentrations ranged from 0.03 to 0.23 mg kg^{-1} d.w. in muscles and 0.07 to 1.32 mg kg^{-1} d.w. in liver. In the muscle tissue samples, the Zn level was lower (20.8–28.1 mg kg^{-1} d.w.) than in the liver samples (87 –157 mg kg^{-1} d.w.). DDT levels in muscles were almost twice as high in males than in females, the highest values of 11 μg kg^{-1} wet weight (w.w.)

Table 3. Concentration of trace metals and major organochlorine substances in selected tissues of male (a) and female (b) flounder (*Platichthys flesus*)

a – male flounder

				Trace metals [mg kg^{-1} dry weight]						Organochlorine substances [μg kg^{-1} wet weight]			
Sample	Year	Area	n	Cd	Cr	Cu	Hg	Pb	Zn	$\sum$DDT	$\sum$HCH	$\sum$PCB	HCB
muscle	2001	REF	1	0.028	0.056	0.760	0.112	0.034	23.500	3.487	0.835	1.404	0.007
		C1	1	0.004	0.057	0.430	0.167	0.,066	22.640	9.793	0.587	5.578	0.005
		C3	1	0.006	0.090	0.460	0.169	0.053	21.580	4.934	0.364	2.265	0.004
	2002	REF	1	0.007	0.026	0.670	0.136	0.142	22.160	8.922	0.739	5.378	0.022
		C1	1	0.008	0.051	1.390	0.193	0.103	24.020	8.990	0.572	5.929	0.021
		C2	1	0.001	0.072	0.620	0.139	0.171	23.230	10.308	0.662	6.633	0.026
		C3	1	0.034	0.082	0.860	0.308	0.186	24.970	10.958	0.492	8.178	0.029
	2003	REF	1	0.006	0.050	0.380	0.148	0.146	23.540	7.242	1.010	4.647	0.033
		C1	2	0.006	0.066	0.285	0.095	0.183	28.051	8.329	0.804	6.518	0.019
		C2	2	0.010	0.041	0.350	0.130	0.161	25.572	7.391	0.443	5.031	0.028
		C3	2	0.006	0.056	0.353	0.141	0.092	26.053	10.905	0.948	6.696	0.027
liver	2001	REF	1	0.927	0.076	40.210	0.043	0.258	88.770	22.118	4.854	9.045	0.007
		C1	1	0.668	0.048	29.380	0.083	0.230	112.710	44.880	3.970	28.590	0.009
		C3	1	0.659	0.030	38.860	0.079	0.499	108.600	37.315	3.783	20.129	0.005
	2002	REF	1	0.876	0.178	34.280	0.059	0.124	89.840	27.495	4.836	32.955	0.029
		C1	1	0.999	0.057	38.700	0.081	1.320	114.960	28.099	2.089	25.952	0.028
		C2	1	0.163	0.100	37.140	0.089	1.202	107.750	27.990	3.012	23.880	0.019
		C3	1	0.908	0.023	42.010	0.128	0.514	111.900	43.012	2.135	31.375	0.027
	2003	REF	1	0.942	0.021	37.610	0.076	0.083	87.430	26.258	3.537	19.021	0.028
		C1	2	0.378	0.070	28.246	0.046	0.299	90.546	19.509	2.203	17.220	0.020
		C2	2	0.559	0.155	39.100	0.065	0.413	109.874	29.566	2.877	22.066	0.026
		C3	2	0.620	0.031	45.284	0.067	0.282	115.166	31.019	3.256	20.981	0.030

Table 3. (*continued*)

b – female flounder

Sample	Year	Area	n	Trace metals [mg kg^{-1} dry weight]						Organochlorine substances [μg kg^{-1} wet weight]			
				Cd	Cr	Cu	Hg	Pb	Zn	$\sum$DDT	$\sum$HCH	$\sum$PCB	HCB
muscle	2001	REF	2	0.010	0.072	0.490	0.093	0.056	23.644	2.104	0.459	0.980	0.005
		C1	1	0.014	0.190	0.532	0.172	0.066	22.804	3.843	0.252	2.617	0.004
		C3	2	0.008	0.053	0.470	0.174	0.025	22.760	3.449	0.110	1.812	0.006
	2002	REF	1	ND	0.181	0.730	0.143	0.081	20.750	4.763	0.882	2.397	0.025
		C1	1	0.007	0.128	0.810	0.160	0.183	25.990	4.234	0.358	2.830	0.041
		C2	1	0.010	0.083	0.620	0.180	0.180	24.400	8.102	0.651	6.363	0.019
		C3	1	0.001	0.032	0.570	0.233	0.137	22.760	5.998	0.297	3.992	0.033
	2003	REF	1	0.008	0.063	0.390	0.160	0.075	24.420	3.546	0.771	2.379	0.029
		C1	2	0.007	0.154	0.318	0.093	0.108	25.376	5.881	0.499	4.642	0.025
		C2	2	0.004	0.136	0.476	0.127	0.230	23.780	6.558	0.533	4.059	0.026
		C3	2	0.002	0.011	0.099	0.145	0.094	23.621	6.266	0.610	4.426	0.029
liver	2001	REF	2	0.812	0.055	46.515	0.039	0.173	142.497	17.780	4.428	9.434	0.005
		C1	1	0.422	0.026	39.640	0.069	0.150	150.160	67.135	5.945	36.258	0.009
		C3	2	0.482	0.037	44.400	0.069	0.160	156.800	55.933	4.324	30.047	0.005
	2002	REF	1	0,552	0.126	30.890	0.047	0.262	108.910	17.944	3.948	13.882	0.024
		C1	1	0,580	0.135	43.350	0.055	1.270	152.840	25.229	1.611	19.350	0.032
		C2	1	0,147	0.090	37.270	0.068	0.827	125.760	55.019	4.172	57.975	0.027
		C3	1	0,196	0.020	42.230	0.066	0.399	143.830	49.162	3.162	32.607	0.032
	2003	REF	1	0.821	0.018	66.430	0.075	0.070	124.680	29.781	5.333	17.228	0.025
		C1	2	0.570	0.018	43.997	0.056	0.182	115.391	19.306	2.174	14.580	0.024
		C2	2	0.299	0.033	32.482	0.040	0.191	122.338	46.558	4.687	32.096	0.026
		C3	2	0.631	0.020	41.781	0.057	0.244	123.765	50.091	4.547	35.876	0.023

ND – not determined; n – number of pooled samples.

being recorded at site C3 (2002 and 2003). The highest concentration of DDTs in liver (67 μg kg^{-1} w.w.) was recorded in females caught at C1 in 2001. HCHs ranged from 0.1 to 1 μg kg^{-1} w.w. in muscle tissue and from 1.6 to 6 μg kg^{-1} w.w. in liver. PCB concentrations were from 1 to 8.2 μg kg^{-1} w.w. in muscles and from 9 to 58 μg kg^{-1} w.w. in liver. PCB values in muscle and liver tissues were lowest at the reference site in 2001. In both these tissues, HCHs did not usually exceed 0.03 μg kg^{-1} wet weight.

Table 4 gives the significance of the differences between the average concentrations of contaminants at the sampling sites. Concentrations of DDTs and PCBs in muscle and liver tissues were significantly higher ($p < 0.05$) at the contaminated than at the clean sites. The concentration of Pb in fish liver differed significantly and was higher at the contaminated sites ($p = 0.014$), whereas Cd levels in liver were higher at the clean sites than at the contaminated ones ($p = 0.006$).

Table 4. Average concentrations of contaminants (in mg kg^{-1} d.w. for metals, and μg kg^{-1} w.w. for organochlorine substances) in muscle and liver of flounder (*Platichthys flesus*) at the 'clean' (REF) and contaminated sites (C1 –C3). Statistically significant differences (Mann-Whitney U-test, $p < 0.05$) between sampling sites are given in bold

	Muscle tissue			Liver		
	'clean' (n = 7)	contaminated (n = 23)	p	'clean' (n = 7)	contaminated (n = 23)	p
Cd	0.011*	0.008	0.212	**0.822**	**0.518**	**0.006**
Cr	0.075	0.081	0.962	0.079	0.056	0.471
Cu	0.570	0.540	0.245	42.656	38.992	0.886
Hg	0.132	0.164	0.144	0.056	0.070	0.135
Pb	0.089	0.127	0.144	**0.162**	**0.511**	**0.014**
Zn	23.002	24.225	0.107	107.021	122.649	0.335
$\sum$DDT	**5.011**	**7.246**	**0.037**	**23.562**	**39.364**	**0.006**
$\sum$HCH	0.783	0.511	0.061	4.489	3.372	0.086
$\sum$PCB	**2.864**	**4.848**	**0.010**	**16.927**	**28.061**	**0.005**
HCB	0.020	0.021	0.564	0.020	0.021	0.413

* n = 6

3.3. Biomarker activity models

The analysis of residuals did not reveal marked departures from the assumptions of the GLM. The normal probability plot of errors for all models deviated only slightly from linear and appeared to be acceptable.

Table 5. Parameter estimates with standard errors (SE) for AChE (a), BChE (b), GST (c), IDH (d) and ME (e) activity models for flounder (*Platichthys flesus*) (2001–2003)

	Parameter/Factor		Estimate	SE	p	Explained variance [%]
a)						
AChE*	Intercept		7.98	0.46	< 0.001	41.1
	Area:	REF and C2	0.00	Aliased		
		C1 and C3	−0.28	0.05	< 0.001	
	Year:	2001	0.00	Aliased		
		2002+2003	−0.59	0.07	< 0.001	
	Sex:	females	0.00	Aliased		
		males	−0.22	0.05	< 0.001	
	Body length		−0.05	0.01	< 0.001	
	Condition factor		−0.50	0.17	0.003	
	Muscle Pb		−1.80	0.53	< 0.001	
b)						
BChE*	Intercept		5.18	0.41	< 0.001	22.8
	Area:	REF	0.00	Aliased		
		contaminated	−0.31	0.07	< 0.001	
	Year:	2001+2003	0.00	Aliased		
		2002	0.31	0.06	< 0.001	
	Sex:	females	0.00	Aliased		
		males	−0.19	0.06	< 0.001	
	Gonad stages:	2, 4, 8	0.00	Aliased		
		3	−0.23	0.06	< 0.001	
	Body length	−0.03	0.01	0.016		
	Muscle Pb	−2.64	0.52	< 0.001		
c)						
GST*	Intercept		3.44	0.04	< 0.001	80.6
	Area:	REF	0.00	Aliased		
		contaminated	0.29	0.04	< 0.001	
	Year:	2001	0.00	Aliased		
		2002	0.41	0.05	< 0.001	
		2003	1.01	0.03	< 0.001	
	Sex:	females	0.00	Aliased		
		males	0.20	0.03	< 0.001	
	Liver Pb		0.32	0.06	< 0.001	
	Liver PCBs		0.01	0.00	< 0.001	
d)						
IDH	Intercept		2.27	0.47	< 0.001	25
	Area:	REF and C2	0.00	Aliased		
		C1	−0.29	0.04	< 0.001	
		C3	−0.16	0.04	< 0.001	
	Year:	2001	0.00	Aliased		
		2002+2003	−0.18	0.05	< 0.001	
	Body length		−0.02	0.01	0.011	
	Condition factor		0.37	0.11	0.001	

Table 5. (*continued*)

	Parameter/Factor		Estimate	SE	p	Explained variance [%]
	Muscle Cu		0.26	0.06	< 0.001	
	Muscle Zn		0.06	0.01	< 0.001	
	Muscle DDTs		0.11	0.03	< 0.001	
	Muscle HCHs		−0.33	0.09	< 0.001	
	Muscle PCBs		−0.11	0.04	0.004	
e)						
ME	Intercept		2.01	0.25	< 0.001	41.7
	Year:	2001	0.00	Aliased		
		2002+2003	−0.56	0.04	< 0.001	
	Condition factor		−0.26	0.10	0.008	
	Muscle Zn		0.03	0.01	< 0.001	

* The results of previous measurements of AChE, BChE and GST activity (Napierska & Podolska 2005, Napierska et al. 2006) and new data on contaminant concentration in selected tissues of flounder were analyzed using the new GLM model.

The model of AChE activity explained 41% of the variance. The following factors and variables were significant: area, year, sex, body length, condition factor and Pb concentration in fish muscles. Table 5a gives the parameter estimates. The highest area effects were noted at REF and C2. These areas were grouped into a new factor level since they were not significantly different. The effects of C1 and C3 did not show significant differences and were also merged into a single factor level. The year effect of 2001 differed significantly ($p < 0.001$) from the other year effects. The year effects for 2002 and 2003 were not significantly different and were grouped into one level. Estimated AChE activity was higher in females than in males ($p < 0.001$). Enzymatic activity correlated negatively with body length and condition factor. The concentration of Pb in the muscle tissues of flounder had a significant ($p < 0.001$), negative impact on AChE activity.

The BChE activity model accounted for 23% of the variance. The area, year, sex, gonad stages, body length and the Pb concentration in fish muscles were significant. Table 5b provides the parameter estimates. The effects of sites designated as contaminated (C1–C3) were significantly lower than those of the reference site. Contaminated sites were merged into one factor level, since their effects did not differ significantly. The highest year effect was estimated for 2002, while the effects of 2001 and 2003 were not significantly different and were grouped together. As in the model of AChE activity, the sex effect was higher in females ($p < 0.001$). The effects of gonads at stages 2, 4, and 8 were higher than the effect of gonad stage 3. No significant differences were recorded between stages 2, 4,

and 8, so these effects were grouped into a new factor level. BChE activity was negatively correlated with body length. Pb concentration in muscles of fish had a significant ($p < 0.001$), negative impact on the response variable.

The model of GST activity explained over 80% of the variance. Area, year, sex and the Pb and PCB concentrations in the liver were highly significant ($p < 0.001$). Table 5c sets out the parameter estimates. Estimated GST activity was higher at the contaminated sites (C1–C3) than at the reference one. The effects of the contaminated sites were not significantly different and were merged. The year effect increased linearly, reaching its highest level in 2003. The sex effect was higher in males. Concentrations of Pb and PCBs in fish liver had a significant, positive impact on GST activity.

The IDH model explained 25% of the variance. The following factors and variables were significant: area, year, body length and condition factor, as well as the concentrations of Cu, Zn, DDTs, HCHs, and PCBs in muscle tissue. Table 5d supplies the parameter estimates. The highest area effect was found for REF and C2. As the effects for these sites were not significantly different, they were merged. The effect of C1 was lowest. The year effect was highest for 2001. The effects of the subsequent years did not differ significantly and were grouped into one level. IDH was correlated negatively with body length but positively with the condition factor. Concentrations of HCHs and CBs in muscle tissue had significant, negative impacts on IDH. Cu, Zn, and DDT concentrations in fish muscles had significant, positive impacts on the response variable.

In the model fitted for ME activity, the area effect was not significant. The model accounted for 41.7% of the variance. The year effect for 2001 was significantly higher ($p < 0.001$) than the effects of the subsequent years, which were merged since they were not significantly different. ME was negatively correlated with the condition factor. The concentration of Zn had a significant ($p < 0.001$), positive impact on ME activity. Table 5e gives the parameter estimates.

4. Discussion

In their earlier publications the authors showed that samples from the polluted sites associated with the Gulf of Gdańsk area had substantially lower muscular ChE levels and higher hepatic GST levels than did those from the reference site (Napierska & Podolska 2005, Napierska et al. 2006). In the present analyses, the biomarker activity models include contaminant concentrations as explanatory variables. The concentration of Pb in the muscle tissue of flounder had a significant, negative impact on the modeled

cholinesterase activity (both AChE and BChE) and the concentrations of Pb in fish liver had a significant, positive impact on GST activity.

The inhibitory effect of heavy metals (including Pb) on AChE activity is well known (Gill et al. 1991, Bocquené et al. 1997, Dethloff et al. 1999). Hg and Pb cations inhibited AChE in muscle extracts of fathead minnows (Payne et al. 1996). Toxic effects in the tropical fish *Hoplias malabaricus* exposed in vivo to lead were reported by Rabitto et al. (2005), where cholinesterase activity was inhibited in muscle after dietary doses of Pb. According to Szabó et al. (1992), the catalytic properties of AChE can be inhibited by changes in the conformational structure of the enzyme secondary to metal-protein bonding.

Although the lead concentration in the flounder muscle analyzed in this study was higher at the contaminated sites than at the reference ('clean') one, it did not differ significantly. But the Pb level in fish liver did differ significantly and was higher at the contaminated sites than at the reference site. Pb in concentrations >1 mg kg^{-1} d.w. was found in two liver samples collected at C1 and C2 in 2002. Studies of surficial sediments from the southern Baltic Sea off the Polish coast indicated much higher concentrations of heavy metal pollutants in the Gulf of Gdańsk compared with the levels found in the vicinity of the Łeba site (Szefer et al. 1995). The Vistula discharge entering the Gulf is a key source of trace metals, and it also affects their subsequent distribution in the water and sediments. Renner et al. (1998) determined that the contribution of the Vistula River is of key importance as regards the supply of Ag, Cu, and Zn into the Gulf of Gdańsk; Cd and Pb are supplied in part by the river and in part by atmospheric transport.

Heavy metal concentrations in the analyzed samples were usually higher at the contaminated sites than at the reference one. The only exception was the Cd level in fish liver, which was significantly higher at the latter site in comparison to the polluted sites. Poland is one of the largest sources of Cd emissions in the HELCOM countries. The annual Cd emissions in Poland in 2002 and 2003 were 52.5 and 48.7 tonnes per year, respectively, which accounts for 30% of the total deposition in the Baltic Sea area (Bartnicki et al. 2005). The concentrations of Cd, Pb and Zn were larger in the southwestern Baltic, where atmospheric emission is more extensive (HELCOM 2003). Metal levels measured by Pempkowiak et al. (2006) in zooplankton samples decreased seawards, with the exception of Cd, the concentrations of which were higher in offshore samples than in near-shore samples. According to those authors, this can be explained by the assimilation of dissolved Cd by algal blooms, which leads to Cd depletion in zooplankton.

Modeled GST activity was significantly higher at the contaminated sites (C1–C3) in comparison to the reference site. The covariates of Pb concentrations in fish liver were significant in the GST model and positively impacted GST activity. Korashy & El-Kadi (2006) showed that some trace metal cations (Hg, Pb, Cu) can modulate the expression of this enzyme. Lenártová et al. (2000) observed a change in the specific isoenzyme pattern of glutathione S-transferases in the livers of chubs (*Leuciscus cephalus*) after their exposure to metal pollutants in industrial areas. Barata et al. (2005) reported significant positive correlations between the biological response in GST activity and Pb concentration levels in caddis-fly larvae.

The GST activity model also indicated that PCB concentrations in fish liver had a significant, positive impact on GST activity. The results of the chemical analysis performed during the present study indicated that PCB concentrations in liver tissues were significantly higher at the contaminated sampling sites than at the clean ones. Pazdro (2004) reported that the sediment samples taken from an open-sea reference site had the lowest concentrations of PCBs compared to the sediment samples collected from the Gulf of Gdańsk. Huuskonen et al. (1996) observed enzymatic induction of GST following the PCB congener treatment of rainbow trout. Similar results were obtained by Martinez-Lara et al. (1996) for gilthead sea bream (*Sparus aurata*) and by Schmidt et al. (2004) for carp (*Cyprinus carpio*).

In the IDH model the area effect was significant and estimated to be the highest at the reference site and at site C2. Concentrations of HCHs and PCBs in muscle tissue had a significant, negative impact on IDH. HCH and PCB levels in a few of the muscle tissue samples from this study were higher than the reference level suggested by Green & Knutzen (2003) for other flatfish species such as dab, plaice, and lemon sole. Concentrations of Cu, Zn, and DDT in fish muscles had a significant, positive impact on IDH. The positive effect of Cu and Zn on IDH activity can be explained by the fact that this enzyme (as well as ME) is activated by metal cations. In the model fitted for ME activity, the area effect was not significant. The results suggest either that flounder has a relatively low sensitivity to ME induction by compounds present in the Gulf of Gdańsk environment, or that the fish are highly sensitive to ME down-regulation by these compounds, or both. Flounder is considered to be a rather stationary species, whereas ME activity seems to play a more crucial role in the metabolism of the muscles of migratory fish (Mommsen 2004).

We cannot rule out the influence of many other compounds present in the Gulf of Gdańsk on measured biomarker activity. Recent studies of the Baltic clam, *Macoma balthica* (L.), from the Gulf of Gdańsk have shown that sediments from nearly all sites exhibited acute toxicity that permitted

classifying the sediments as toxic according to the Recommendations of Helsinki Commission (HELCOM Recommendation 23/11 adopted March 6, 2002) (Sokołowski et al. 2004).

5. Conclusions

The observed differences in enzymatic biomarker levels are of concern, since they suggest that chronic exposure to low level mixtures of contaminants may be taking place in the studied area. According to the literature, all the measured contaminants have been shown to have a potential effect on enzymatic biomarkers in laboratory studies. However, no conclusive evidence was found in this study for a clear link between the presented biomarker responses and contaminant concentrations in selected flounder tissues. The situation prevailing in the natural environment – chronic exposure to such compounds in combination with other contaminants and the influence of environmental conditions – may not provide the clear responses required in environmental monitoring exercises. Further clarification of the role that environmental chemicals play in the 'health status' of fish populations will require more detailed, focused investigations.

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