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Some indications of contaminant effects on Baltic cod (*Gadus morhua* L.)

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Abstract

The Baltic Sea has been exposed to severe human impacts. Apart from eutrophication and overfishing, contamination by known organic pollutants such as PAHs, PCBs and certain organo-chlorine pesticides are of major concern, even though the levels of certain substances belonging to the latter two classes of compounds have decreased during the past two decades. Additionally, novel scarcely known and unknown contaminants will also cause biological effects. In the EU-funded project "Biological Effects of Environmental Pollution in marine coastal ecosystems" (BEEP), we assessed to what extent Baltic cod is affected by contaminants. During a cruise in December 2001, cod were collected in the western and southern Baltic Sea, somatic condition factor was estimated and different indicators (biomarkers) for contaminant exposure and biological effects were analysed. In addition, various PCBs and organo-chlorine pesticides were measured in cod liver.

The results obtained demonstrate that the contaminant levels in the Baltic are likely to have a biological effect on cod: in almost all specimens analysed, hepatic EROD activity in the liver was clearly measurable, and 1-OH-pyrene, a common PAH-metabolite, was detectable in bile. Both features indicate an induction of the CYP1A biotransformation system in response to toxic substances. The occurrence of DNA adducts in some of the specimens provide evidence for the presence of genotoxic substances. Acetylcholinesterase (AChE) was measurably inhibited, an indication of exposure to organo-phosphates and related compounds, particularly in specimens taken at Wismar Bay and off the Lithuanian coast. In general, spatial differences in the biomarker responses as well as contaminant loads were found, suggesting differences in concentration and mixtures of organic contaminants in this ecosystem.

Introduction

The Baltic Sea has been subjected to diverse human impacts. Apart from eutrophication and overfishing, contamination by known organic pollutants such as PAHs, PCBs and organo-chlorine pesticides are still of major concern, even though the levels of certain substances belonging to the latter two classes of compounds have decreased during the past two decades (HELCOM 2002). Additionally, novel scarcely known and unknown contaminants may also have an effect on this ecosystem and its biota.

The direct measurement of organic pollutants, by chemical analysis, can provide detailed information regarding the spatial distribution of contamination, but it provides little information on the biological impact of these compounds.

In recent years suitable tools have been developed allowing to assess the exposure to or the damage incurred by environmental pollutants (review: Van der Oost *et al.* 2003). The integrated use of these biomarkers (cellular and/or physiological parameters) has been suggested as an effective means of determining the impact of various pollutants on biota (McCarthy & Shugart 1990).

The mixed function oxygenase (MFO) system plays an important role in the metabolism of many endogenous (e.g. steroid hormones) as well as exogenous substrates (e.g. xenobiotics) in fish. Cytochrome P4501A (CYP1A) is a terminal component of the MFO system. Ethoxyresorufin-O-deethylase (EROD) activity is CYP1A-dependent and therefore a useful marker of MFO induction. The level of this enzyme is known to be induced by exposure to certain bioavailable contaminants, such as PAHs or PCBs. It is therefore considered as a useful biomarker for exposure (Stagg & McIntosh, 1998). Beside EROD activity, production of PAH metabolites in the bile (Stagg 1998) and the formation of DNA adducts have shown great potential for identifying levels of contaminant exposure particularly to different PAHs in recent studies (Ericson *et al.* 1998, 1999a, 1999b, Aas *et al.* 2000, 2002, Ericson & Balk 2000).

Baltic cod is known to be effected by many contaminants. Although PCB and DDT levels in this species have decreased significantly during the past decades, recent levels are apparently still high enough to induce EROD in adults (Schneider *et al.* 2000) or to cause reproduction impairment (Åkerman *et al.* 1996, Petersen *et al.* 1997, Åkerman & Balk 1998) and therefore effecting Baltic cod recruitment (Norrgren *et al.* 1998).

Levels of organo-chlorine pesticides (DDT, DDE) have been assessed in Baltic cod since many years and decades (e.g. Jensen *et al.* 1969, Schneider & Osterroht 1977) because of their high persistence. Even though organo-chlorine pesticides have been replaced by organo-phosphates, pyrethroids and related compounds, nothing is known about their occurrence in and possible neurotoxic effect on Baltic cod.

Inhibition of acetylcholinesterase (AChE) activity in cholinergic areas of tissue has been proposed as a useful molecular biomarker of an effective exposure to organo-phosphates and carbamates (Bocquené *et al.* 1990, Bocquené & Galgani 1998).

As part of the EU-funded project "Biological Effects of Environmental Pollution in marine coastal ecosystems" (BEEP), we assessed to what extent Baltic cod is still affected by contaminants. During a cruise in December 2001, cod were collected in the western and southern Baltic Sea, condition factor, hepato- and gonado-somatic index were estimated and different indicators (biomarkers) for contaminant exposure and biological effects were analysed. In addition, content of major organo-chlorine substances were measured in cod liver.

Methods

Sampling
Female and male Baltic cod (*Gadus morhua*) were caught at 8 different sites (Fig. 1) in December 2001 during a cruise with the fisheries research vessel Walther Herwig III (Bundesforschungsanstalt für Fischerei). The fish were captured using an EXPO trawl, towed at 3-4 knots for 60 minutes. Onboard the fishes were kept in running seawater and dissected within one hour of capture.
Total length, total weight, as well as liver and gonad weight of the fish were measured. Condition factor (CF) was determined as (total weight (g)/length(cm^3) x 100. Somatic indices for liver (HSI) and gonad (GSI) were determined as (liver weight/fish weight) and (gonad weight/fish weight) x 100, respectively.
Central sections of liver tissue were removed for EROD and DNA adducts analyses. They were directly put into cryovials in liquid nitrogen, and later moved to a -80° C freezer. In addition, a central piece of liver was taken and frozen for contaminant analysis. Furthermore, bile samples were taken by the use of a syringe for the estimation of PAH-metabolites and conjugates and kept in dark glass vials at – 20°C. Moreover, a strip of muscle (approx. 2 cm^3) was removed from the dorsal surface for AChE analysis and stored in cryovials in liquid nitrogen.
Information regarding the hydrography at the different sites was obtained using CTD measurements (conductivity, temperature and depth) from bottom and surface areas (Table 1).

EROD activity
Activity of the 7-ethoxyresorufin O-deethylase (EROD) was measured in liver microsomes, which were obtained as described in Beyer *et al.* (1996). EROD analysis was performed using a modification of the method described in ICES TIMES No. 23 (Stagg & McIntosh 1998). EROD activity was measured with a 96-well microplate reader (excitation: 535 nm; emission: 585 nm), after a 5-min incubation at 30°C in 1 ml of a reaction mixture. Reaction was stopped by the addition of 2 ml of cold acetone (Viarengo *et al.* 2000). EROD activity was normalized to protein content in the microsomes and expressed as pmol resorufin min^{-1} mg^{-1} protein. Protein content was measured using a 96-well microplate reader modification of the method described by Bradford (1976) with a bovine serum albumin standard.

AChE activity
Determination of acetylcholinesterase (AChE) activity was conducted according to ICES TIMES No. 22 (Bocquené & Galgani 1998) using about 200 mg of muscle tissue. After homogenisation and centrifugation (20 min, 10,000 x g and 4°C) an aliquot of the supernatant was used for measuring AChE activity at 412 nm using a 96-well microplate reader.

Bile PAH metabolites
Fish bile samples were hydrolysed according to the method of Ariese *et al.* (1997) by adding 480 µl of water and 10 µl of ß-glucuronidase/arylsulfatase (30U/ml-60U/ml) to 10 µl of bile. After two hours of incubation at 37°C the enzymes were precipitated by adding 500 µl of methanol. Samples were centrifuged at 3000 rpm for five minutes (4°C) after which the supernatant was filtered to an HPLC vial and stored at 4°C until analysis. Samples were analysed using a Water Alliance 2960 separations unit with a W474 fluorescence detector.

A Vydac 201TP54 reverse phase column was used as analytical column. Flow rate was 1ml/min and the column was kept at constant temperature (35°C) during the run. Injection volume was 20 µl. Lag time between injections was 9 minutes.

DNA purification and ^{32}P-postlabeling analysis of adducts

Liver tissue samples were semi-thawed and the DNA extracted and purified according to Dunn *et al*. (1987) and Reichert & French (1994), slightly modified as described in Ericson *et al*. (1998) and Ericson & Balk (2000). DNA adducts were enriched using the Nuclease P1 method, 0.8 µg Nuclease P1/µg DNA, and a 45 min incubation period (Reddy & Randerath 1986; Beach & Gupta 1992). Finally, the DNA adducts were radiolabelled using 5´-[?-^{32}P]triphosphate([?-^{32}P]ATP) and T_4 polynucleotide kinase. Separation and clean up of adducts was performed by multidirectional thin-layer chromatography (TLC) on laboratory produced polyethyleneimine cellulose sheets, described as suitable for adducts formed from large hydrophobic xenobiotics, such as 4- to 6- ring, PAHs (Reichert & French 1994; Ericson *et al*. 1999b). Adducts were located and quantified by storage phosphor imaging technology (PhosphorImagerTMSI and ImageQuant 5.0). In addition, several quality control experiments were performed parallel to the analysis of the cod samples. All these quality assurance experiments strongly suggested a faultless assay for the DNA adduct measurements.

Organo-chlorine analysis

Content of the major organo-chlorine substances (PCB congeners 52, 101, 105, 118, 138, 149, 153, 180 and o,p´- and p,p´-DDT, DDE and DDD) were measured in liver samples of male and female cod. Sample extraction and clean-up was carried out as described in Schneider (1982). The gas-chromatographic analysis of the tissues was performed as described in Petersen *et al*. (1997). The analytical quality was repeatedly checked by reference materials and by participation in QUASIMEME Laboratory Performance Studies.

Statistical analysis

Multiple comparisons were performed with the Scheffé test produced by the analysis of variance (ONE-WAY ANOVA) using SPSS (Version 10.0) software package. Prior to comparisons, the homogeneity of variances was tested with the Levene statistic. For parameters with heterogeneous variance the Welch test was applied in combination with the Dunnett test for multiple comparisons. A p-value of <0.05 was considered as statistically significant.

Results and discussion

The data for mean length, weight, Condition Factor (CF), HSI and GSI are summarised in Table 2. In average ten female and ten male cod were sampled at each site. Fish length varied from 29 to 70 cm with means ranging from 41.1 cm (Rügen) to 48.6 cm (Wismar Bay). According to length-age relationships based on data provided by the Bundesforschungsanstalt für Fischerei, Institut für Ostseefischerei Rostock, the majority of cod were 2-3 years old and only at Rügen and Kap Arkona they were younger. Mean condition factor, calculated for cod from each site, ranged from 0.73 to 1.48. The highest median CF (1.04) was found at site Lithuania (Fig. 2). In cod from Gdansk Bay median CF was lowest (0.87). Differences between the sites were also apparent in the hepato-somatic index (Fig. 2). The lowest median was found in specimens from Kiel Bight (2.43), which was significantly different from all other sites.

As a general trend, HSI was higher and showed a greater variability at the sites in the southern Baltic and Baltic Proper, respectively (Bornholm Basin, Slupska, Gdansk Bay or Lithuanian coast).

Biomarker responses were measurable in almost all cod analysed. However, no significant differences between males and females occurred (Schnell, unpubl. data). Therefore, the following assessment of the various biomarkers is based on data for males and females.

Measurement of acetylcholinesterase (AChE) activity, an enzyme which is important for cellular neurotransmitter functioning, revealed median activities between 15 and 75 nmol^{-1} min^{-1} mg protein (Fig. 3). The significantly highest values were measured in cod from Kiel Bight (KB - median: 52.7 nmol^{-1} min^{-1} mg), whereas at the Lithuanian coast (LT) the activities were lowest (median: 26.3 nmol^{-1} min^{-1} mg). Similar low activities (median: 33.3 nmol^{-1} min^{-1} mg) were measured in cod from Wismar Bay (WB). It can be ruled out that the variations in the activities are due to temperature effects. As shown in Table 1, temperature did not differ significantly between the sampling sites. Therefore, the reduced AChE activities at Wismar Bay or the Lithuanian coast may indicate contamination with organo-phosphates and related compounds, substances which are known to inhibit acetylcholinesterase.

As mentioned before, the mixed function oxygenase (MFO) system is an important pathway for metabolism of many different xenobiotics. The degree of induction of cytochrome P4501A (CYP1A) can be assessed through the measurement of ethoxyresorufin O-deethylase (EROD) activity. EROD activity was analysed at all sites, but some locations show clearly higher levels than others, and thereby indicate induction (Fig.3). In Kiel Bight (KB), EROD activity was lowest (median: 12.9 pmol^{-1} min^{-1} mg), whereas in fish from Slupska (SP), it was ten times higher (median: 119.7 pmol^{-1} min^{-1} mg) and in those from the Lithuanian coast, about five times higher (median: 65.7 pmol^{-1} min^{-1} mg). In Gdansk Bay, EROD activity was comparable low (median: 39.9 nmol^{-1} min^{-1} mg).

In order to estimate the present exposure to PAHs, analysis of bile for the present of PAH-metabolites has been proven to be a suitable method. It has also been demonstrated that 1-hydroxy pyrene is a major metabolite in fish exposed to PAHs (Ariese *et al.* 1993). In the present study, this metabolite was analysed in cod from 6 of the 8 sampling sites (Fig. 3). Hydroxy-pyrene was measurable in almost all specimens. The lowest median values (58.3 µg kg^{-1} bile) were found in cod from Kap Arkona (KA) and Kiel Bight (KB - 67.4 µg kg^{-1} bile), and the highest ones in Gdansk Bay (DB - median: 179.3 µg kg^{-1} bile).

Biotransformed PAHs can bind to DNA, thus forming DNA adducts which is widely believed to be an initiating step in chemical carcinogenesis (Shugart *et al*. 1992). The induction of EROD as well as the PAH- metabolite measured in cod at the different sites strongly suggests an exposure to contaminants. The analysis of DNA adducts give evidence for genotoxic effects (Fig. 3). The DNA adduct analysis , ^{32}P-postlabelling methodology, is a sophisticated but also a relatively expensive method. Therefore, this biomarker was only applied on cod specimens that showed a clear EROD or PAH signal.

The highest amount of DNA adducts were found in cod from Gdansk Bay (2.5 nmol per mol normal nucleotides) with one extreme (9.4 nmol per mol normal nucleotides). Cod from Kiel and Wismar Bay showed clearly lower median values (0.3 nmol per mol normal nucleotides), i.e., background levels as observed in non-exposed fish in pristine areas. In general, the amount of DNA adducts was lower than measured in cod from experimental studies performed with juvenile Atlantic cod (Aas *et al.* 2000) or as in Atlantic cod caught in the vicinity of an aluminium works (Aas *et al.* 2001). Beside possible genetic and/or metabolic differences, this is likely due to the fact that cod in the Baltic Sea is in general chronically exposed, although to lower levels of PAH compared to the above cited exposure studies.

The observation of individual cod with DNA adduct levels of around 3 to 9.5 nmol adducts per mol normal nucleotides suggests an exposure situation comparative to a highly contaminated area. Furthermore, it should be mentioned that cod might belong to the group of teleost fish species that are not especially sensitive to DNA adduct formation as a result of PAH exposure, due to their extremely high lipid content in the livers that might withdraw the PAHs from metabolic activation to reactive intermediates that could form the DNA adducts. The differences in the DNA adduct level between the sampling sites, which were also evident in the responses of the other biomarkers analysed (EROD, PAH- metabolites and AChE), suggest spatial differences concerning the severeness of the biological effects. This could partly be caused by the differences in the degree of contamination.

In order to gain an overview regarding the general contaminant level in Baltic cod, content of the major organo-chlorine substances (PCB congeners 52, 101, 105, 118, 138, 149, 153,180, and o,p´- and p,p´-DDT, DDE and DDD) were measured at 4 of the 8 sites, which represent different regions in the Baltic Sea. As shown in Figure 4, PCB content varied between the different locations, though not significantly. The highest PCB levels were measured in cod from Kiel Bight (KB – median: 872 ng g^{-1} lipid), whereas PCB content in Bornholm cod was lowest (504 ng g^{-1} lipid). Content of organo-pesticides (DDT/DDE/DDD), on the other hand, was lowest at Kiel Bight (KB – 489 ng g^{-1} lipid) and somewhat higher in cod from Wismar Bay (WB – median: 599 ng g^{-1} lipid). In cod from Gdansk Bay (median: 1013 ng g^{-1} lipid) and Bornholm Basin (965 ng g^{-1} lipid) organo-chlorine pesticide levels were significantly higher ($p < 0.05$). It should also be pointed out that cod from the Bornholm Basin showed the highest absolute values and greatest variability from all 4 sites.

Conclusions

The results obtained with the different biomarkers document that contaminant levels in the Baltic are likely to influence the physiology of cod, even though the content of the commonly monitored organo-chlorines have, in general, significantly decreased during the past two decades in the Baltic Sea (HELCOM 2002). Other contaminants, not being monitored that regularly and novel scarcely known and unknown contaminants are probably also of importance for the biomarker signals observed.

The spatial variations in the biomarker responses suggest differences in the concentration and mixtures of the various organic contaminants in this ecosystem. This was confirmed to some extent by the chemical analysis of the major PCBs and organo-chlorine pesticides as model contaminants. The greater individual variability in the biomarker response at some sites might be caused by cod migration activities.

The more pronounced biomarker responses particularly in cod from the central and eastern Baltic Sea may also indicate that other factors, such as low salinity or reduced oxygen concentrations, have an effect on the biological response to organic contaminants in Baltic cod.

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Table 1: Water depth, temperature (T), salinity (S) and oxygen saturation at the different sampling sites. KB = Kiel Bight; WB = Wismar Bay; KA = Kap Arkona; RÜ = Rügen; BB = Bornholm Basin; SK= Slupska; DB= Gdansk Bay; LT = Lithuania

Date	Area	Depth (m)	T (°C)	S (PSU)	O_2-Saturation (%)
12.12.01	KB	3.5	5.6	16.3	101.2
		17.5	8.2	20.9	86.0
11.12.01	WB	3.0	5.5	9.9	100.0
		22.0	7.4	18.0	95.1
10.12.01	KA	3.0	5.7	7.7	100.8
		40.0	10.0	14.0	84.0
02.12.01	RÜ	4.0	6.8	7.7	99.9
		25.0	6.9	7.7	99.6
09.12.01	BB	3.5	6.0	7.2	100.9
		55.0	8.2	11.6	34.0
03.12.01	SP	3.0	6.3	7.0	102.6
		66.5	7.1	12.1	31.9
08.12.01	DB	3.0	6.6	7.1	101.1
		67.	5.4	8.4	52.6
		81.0	5.4	7.1	13.3
07.12.01	LT	2.5	7.4	7.2	101.3
		47.0	7.4	7.1	100.9

Table 2: Morphometric data, body and organ indices (CF= condition factor, HSI= hepato-somatic index; GSI= gonado-somatic index) as well as maturity level of *Gadus morhua* from different sites in the Baltic Sea. The results are expressed as mean ± SD
*[1]estimated using lenth-age relationships based on data provided by Bundesforschungsanstalt für Fischerei / Institut für Ostseefischerei Marienehe
*[2]according to the index scale in Bleil & Oberst (2000)

Sampling site	N	total length [cm]	total body weight [g]	liver weight [g]	gonad weight [g]	CF	HSI	GSI	Age [y] *[1]	Gonadal maturity*[2]
Kiel Bight	18	42.4 ± 6.8	838 ± 386	21.94 ± 12.50	18.76 ± 27.73	0.98 ± 0.09	2.70 ± 1.12	2.22 ± 2.62	1-3	1-2
Wismar Bay	20	48.6± 5.4	1211 ± 462	57.75 ± 39.62	51.83 ± 51.99	1.01 ± 0.11	4.43 ± 1.38	4.09 ± 3.17	2-3	1-3
Kap Arkona	21	43.5 ± 4.3	828 ± 261	43.33 ± 16.30	22.70 ± 26.16	0.98 ± 0.09	5.25 ± 1.41	2.51 ± 2.56	1-2	1-3
Rügen	20	41.1± 6.0	710 ± 308	31.50 ± 15.99	14.38 ± 20.81	0.99 ± 0.14	4.46 ± 1.27	1.67 ± 1.81	1-2	< 3
Bornholm Basin	22	42.8 ± 5.1	743 ± 743	42.05 ± 21.31	10.91 ± 12,47	0.93 ± 0.12	5.61 ± 2.17	1.67 ± 2.14	2-3	1-2
Slupska	20	43.7 ± 4.9	786 ± 304	54.75 ± 27.22	10.14 ± 5.29	0.91 ± 0.08	6.90 ± 1.50	1.41 ± 0.78	2-3	< 3
Gdansk Bay	20	48.5 ± 7.8	1083 ± 551	70.5 ± 46.17	26.75 ± 21.42	0.89 ± 0.11	6.19 ± 2.07	2.41 ± 1.32	2-3	< 3
Lithuania	22	42.5 ± 11.1	902 ± 635	58.59 ± 44.87	14.85 ± 15.15	1.03 ± 0.12	6.52 ± 2.15	1.43 ± 0.92	1-3	< 3

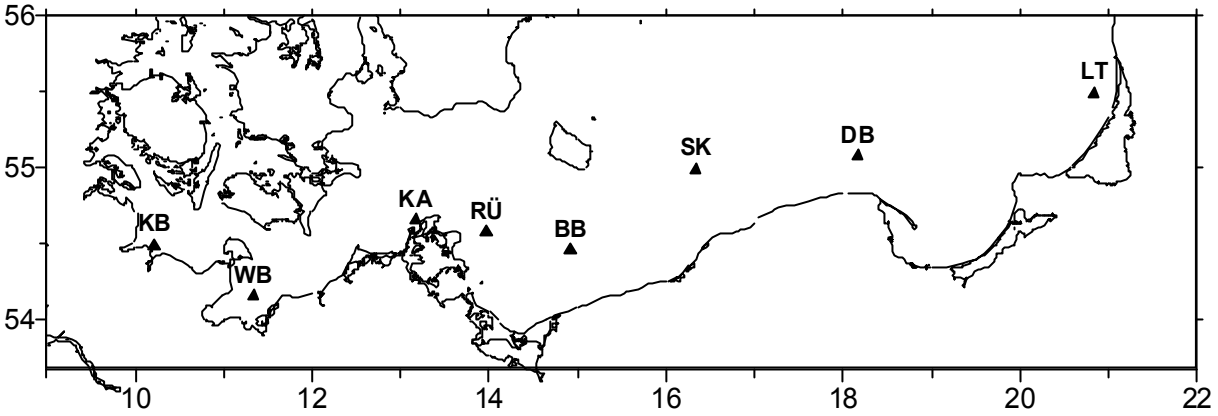

Fig. 1: Sampling locations in the Baltic Sea (KB = Kiel Bight; WB = Wismar Bay; KA = Kap Arkona; RÜ = Rügen; BB = Bornholm Basin; SK= Slupska; DB= Gdansk Bay; LT = Lithuania)

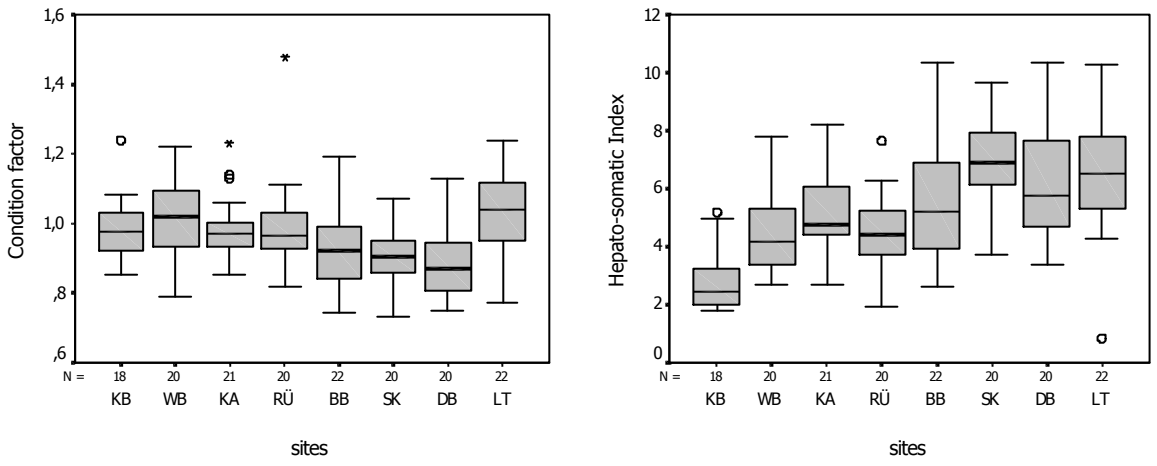

Fig. 2: **Condition factor** and **Hepato-Somatic Index** in *Gadus morhua* at different sites in the Baltic Sea. (KB= Kiel Bight, WB= Wismar Bay, KA= Kap Arkona, RÜ= Rügen, BB= Bornholm Basin, SK= Slupska, DB= Gdansk Bay, LT= Lithuania).

Box-Whiskers plot: median (line), 25% and 75% quantiles (boxes), 10% and 90% quantiles (bars), outliners (dots) and extremes (*).

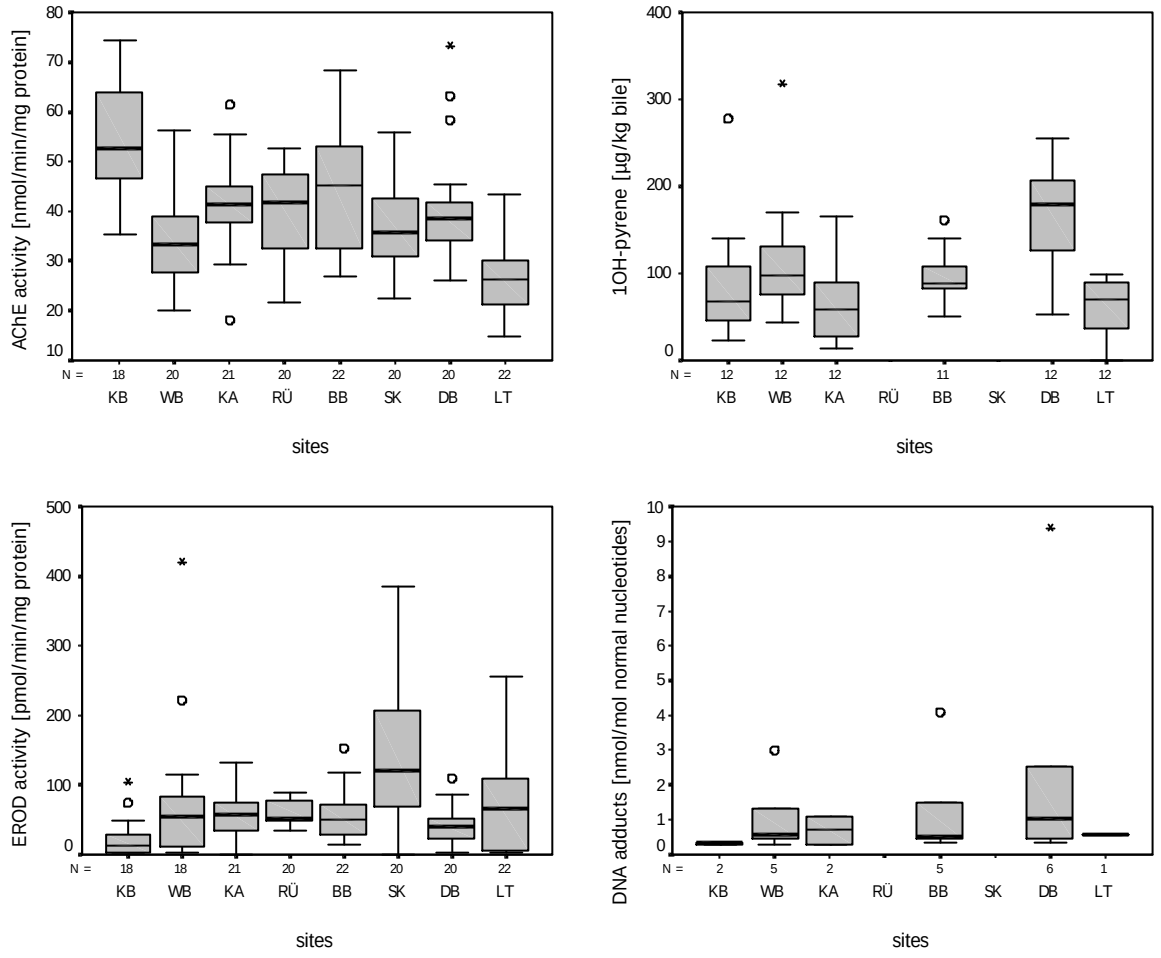

Fig. 3: AChE activity (muscle), **EROD activity** (liver), **concentration of 1-OH-pyrene** (bile) and **levels of hepatic DNA adducts** in *Gadus morhua* at different sites in the Baltic Sea. (KB= Kiel Bight, WB= Wismar Bay, KA= Kap Arkona, RÜ= Rügen, BB= Bornholm Basin, SK= Slupska, DB= Gdansk Bay, LT= Lithuania). Box-Whiskers plot, for key see Fig. 2.

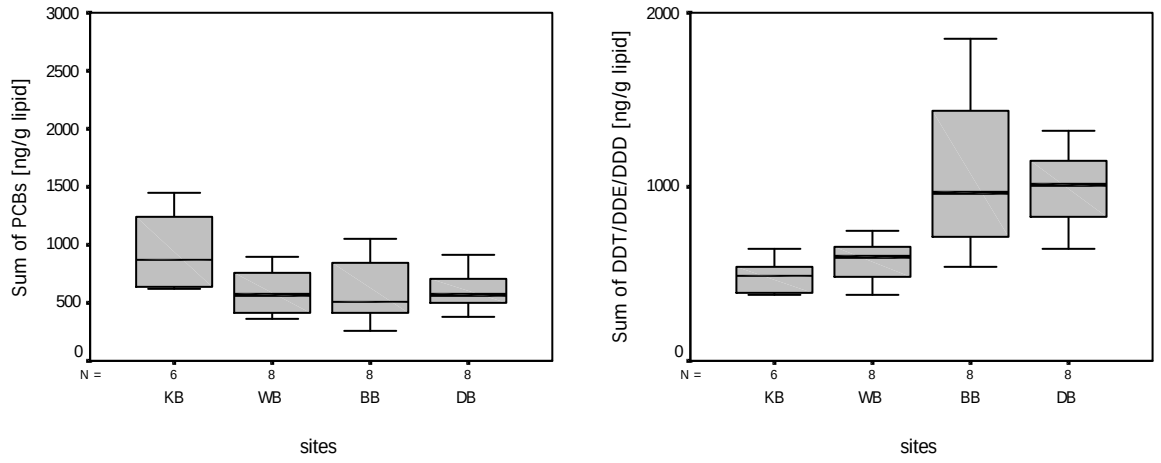

Fig. 4: **Content of PCBs** (sum of 8 congeners) and sum **DDT/DDE/DDD** in *Gadus morhua* (liver) at different sites in the Baltic Sea. (KB= Kiel Bight, WB= Wismar Bay, BB= Bornholm Basin, DB= Gdansk Bay). Box-Whiskers plot, for key see Fig. 2.