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Temporal and Spatial Trends in the Distribution of Contaminants and their Biological Effects in the ICES Area (S)

Baltic cod reproductive impairment: ovarian organo-chlorine levels, hepatic EROD activity, muscular AChE activity, developmental success of eggs and larvae, challenge tests

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Abstract

During the past two decades, different types of reproductive disturbances, including inadequate ovary maturation, low fecundity and early life stage mortality, have been demonstrated for a number of fish species in the Baltic. Suggestions have been made that larval deformities and increased mortality in pelagic eggs of plaice, flounder and whiting and demersal eggs of herring are caused by chloro-organics like DDTs, PCBs and other persistent bioaccumulative compounds. The present study aimed at assessing links between the viability of eggs and larvae from Baltic cod and the concentration of organo-chlorines analysed in the ovaries of the females. Eggs from 32 running ripe female cod from the Bornholm Basin were stripped, artificially inseminated and incubated in Baltic seawater. Enzymatic activity (EROD and AChE) in female tissues as well as viable hatching, larvae survival, larvae growth, and challenge testing with pyrene were the effect parameters applied in the evaluation of potential effects of toxicants on cod reproductive impairment. The results obtained show no recent decline of DDT and PCB burdens in the ovaries. EROD activity levels and an apparent inhibition of AChE indicate that cod in the Bornholm Basin are affected by contaminants. However, with regard to egg and early larval development no significant correlations were found. An apparent relation between elevated EROD activities in mother fish and low lethal body burdens of pyrene in the challenge tests may indicate prenatal damage or a mother-to-young transfer of toxicants.

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Introduction:

During the past two decades, different types of reproductive disturbances, including inadequate ovary maturation, low fecundity and early life stage mortality, have been demonstrated for a number of fish species in the Baltic Sea, including cod (Westernhagen et al. 1988, Åkerman et al. 1996, Åkerman and Balk, 1998). From various studies in the North Sea it is suggested that larval deformities and increased mortality in pelagic eggs of plaice, flounder and whiting and demersal eggs of herring are caused by chloro-organics like DDTs, PCBs, and other persistent bioaccumulative compounds (Hansen et al. 1985, Cameron et al. 1986). Investigations performed on the Baltic cod *Gadus morhua* L. have shown that the reproductive success was seriously impaired as a result of high mortality of the eggs and embryos. Elevated levels of DNA adducts were found in the cod offspring before feeding, indicating a maternal transfer of xenobiotics (Ericson et al., 1996).

Studies performed on early life stages of fish (cod, herring, turbot and zebra fish) have shown that Lethal Body Burdens (LBB) for lipophilic narcotic chemicals were reached at 0.9-4.35 mmol/kg wet weight (Petersen, 1997). If, however fish are already exposed to toxicants, only a low additional accumulation is likely to be needed before a toxic effect is expressed (van Wezel et al. 1996). This approach, known as a challenge test, was used in the present study as a tool for examining whether cod larvae obtained from individual females caught in the Bornholm Basin have different intrinsic sensitivities towards a single known toxicant. A higher intrinsic sensitivity in the different larvae batches could indicate either a prenatal damage or a mother-to-young transfer of toxicants that causes initially higher body burdens of toxicants in the eggs/larvae when they are spawned. As it is impossible to determine all potentially harmful environmental toxicants by chemical measurements, the approach opens the possibility indirectly to compare the toxicant loading in the larvae from the different females.

The present study aimed at assessing links between the viability of eggs and larvae from Baltic cod and the concentrations of some major organo-chlorines analysed in the ovaries of females. In order to evaluate potential effects of toxicants in the ovaries of parental fish, eggs from 32 running ripe female cod from the Bornholm Basin were stripped, artificially inseminated and incubated in Baltic seawater. Hepatic enzymatic activity of 7-ethoxyresorufin O-deethylase (EROD) and acetylcholinesterase (AChE) activity in muscle tissue were used as biomarkers of a biochemical effect of contaminants in the mother fish. Viable hatching, survival after hatch, growth within the first ten days after hatch and challenge testing with pyrene were the effect parameters applied on yolk sac larvae.

Additionally, the enzyme activities of EROD and AChE were measured in spawning male cod in order to estimate the influence of gender on these two biomarkers.

Materials and methods

Sampling

Mature Baltic Cod (*Gadus morhua*) were caught by use of an EXPO trawl in the Bornholm Basin between July 4 and July 13, 1999, on a cruise with the Danish research vessel DANA. Ripe females and males were stripped of eggs and sperm, respectively, and the eggs were fertilised with sperm from 2-4 males as described by Westin and Nissling (1991). All females used were age-determined, and total length, total weight, gutted weight as well as liver and ovary weight were measured. Samples of ovary, liver and muscle tissues were taken from females and males for organo-chlorine analysis, EROD and AChE activity measurements, respectively, and stored in liquid nitrogen.

Organo-chlorine analysis

Content of the major organo-chlorine substances (PCB congeners 52, 101, 105, 118, 138, 149, 153 and 180, o,p´- and p,p´-DDT, DDE and DDD, dieldrin) were measured in the ovaries of the running-ripe females. 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.

Enzyme activity measurements

Activity of the 7-ethoxyresorufin O-deethylase (EROD) was measured in liver microsomes, which were obtained as described in Beyer et al. (1996). The enzymatic activity was measured according to ICES TIMES No. 23 (Stagg and McIntosh 1998) after 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.

Determination of acetylcholinesterase (AChE) activity was conducted according to ICES TIMES No. 22 (Bocquené and Galgani, 1998).

Incubation experiments

After fertilisation, eggs from individual females were incubated in Baltic seawater at 7°C and a salinity of 15 PSU (which allows live fertilised eggs to float while dead and unfertilised eggs sink). The incubation water was prepared from filtered seawater taken from the Bornholm Basin at a depth of approx. 65 m. The incubation water was treated with antibiotics (Nystatin 2 ml/l; Streptomycin 2 ml/l; Doltacillin 1 ml/l). Two replicates of each 150 eggs from each female were incubated in 500 ml of water at 7°C until hatching (up to 15 days). After hatching, viable larvae from each female were transferred to two replicates each containing 20 larvae. The water was renewed daily throughout the incubation period. Newly fertilised eggs were incubated in climate rooms on board the DANA. After the end of the cruise, eggs and hatched larvae were transported to climate rooms at VKI where survival and growth were recorded until day 10 post hatching. Dry weight determinations were performed on 5 samplings of each 10 larvae on approx. 5 days old post hatch larvae. A lipid concentration of 13.3% (Petersen, 1997, Rainuzzo et al., 1992) of the dry weight was used for estimating the lipid contents of the larvae.

A total of 32 egg batches obtained from 32 different females were used for the incubation experiments.

Challenge experiments

Challenge tests were performed on all 32 egg batches. The tests were started with 5 to 6 days post hatch larvae. The experiments were run for 2 to 4 days, dependent on the survival time of the larvae. Radiolabelled [4,5,9,10-^{14}C] pyrene was obtained from Sigma Radiochemical, St. Louis (USA) and analytical grade pyrene (>96%) obtained from Sigma.

Stock solutions of radio labelled (2μCi/ml) and non-labelled chemicals were prepared in acetone. The test solutions were achieved by mixing radiolabelled and non-labelled substance to obtain a ^{14}C activity of about $1.1 \cdot 10^5$ DPM/l of test solution. Exposures were performed with one lethal concentration (135 μg/l). The acetone concentration in the test solutions did not exceed 119 μg/l. Concurrent control experiments with acetone (119 μg/l) were performed together with controls without any toxicant added.

Thirty larvae from each batch were placed in glass beakers (1000 ml) and exposed until death of the larvae occurred. Each beaker was covered with a glass lid in order to minimise volatilisation. A semi-static test system was used, e.g., the test solutions were renewed every 24h. Temperature, O_2 concentration and pH were measured daily in parallel test beakers prior to the observations of mortality and malformations. Every 24h, samples of old and freshly prepared test solutions, respectively, were withdrawn from each beaker. The actual

concentrations of the radiolabelled chemicals were determined by mixing a 10 ml sample with 10 ml of scintillation cocktail (Insta-gel Plus, Packard) in 20 ml vials. The ^{14}C-activity was determined by liquid scintillation counting.

The test beakers were checked for dead larvae several times daily. Dead larvae, identified by failure to react to the stimulus of being touched with the end of a Pasteur pipette, were removed from the beakers and analysed for content of test substance (Lethal Body Burden). The larvae were rinsed by successive transfer to beakers containing water free of test substance in order to remove radioactivity adhering to the surface of the larvae.

After rinsing, the larvae were transferred to glass vials with 100 µl tissue solubilizer (Packard). After 30 min at room temperature, 10 ml Ultima Gold LSC cocktail were added and the radioactivity in the larvae was determined by liquid scintillation counting.

Data treatment

Correlations between ovary burden and the different effect parameters were tested by use of Spearman Rank correlation test.

Results and discussion

Age, condition index (CI), hepato-somatic index (HSI), gonado-somatic index (GSI), EROD and AChE activity for the individual females are summarised in Table 1 as well as the effect parameters determined on fertilized eggs and larvae from the respective females.

Contaminant content in the ovary (Table 2,Fig. 1)

The mean concentrations (and ranges) of organo-chlorine compounds estimated in the ovaries are listed in Table 2, together with the corresponding data from 1996 in the Bornholm Basin (Petersen et al. 1997). Bearing in mind the general trend of declining DDT and PCB concentrations in biota in the Baltic (Bignert et al., 1996), surprisingly, the mean concentrations in this limited number of cod were markedly higher in 1999 than in 1996, whereas the standard deviations and ranges were somewhat lower, with the exception of dieldrin. Except for dieldrin, the standard deviations exceeded the mean values in 1996, but not in 1999. Hence, contaminant concentrations were much more homogeneous in 1999 than in 1996, rendering any detection of biological effects more unlikely.

A strong correlation ($r^2 = 0.856$) between total amount of PCB and pesticides was found (Fig. 1), which was similar to our previous findings (Petersen et al., 1997). It can be assumed that this close correlation between PCBs and organo-chlorine pesticides indicates similar correlations with many other contaminants of comparable physicochemical properties.

EROD activity (Fig. 2)

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 vertebrates. Cytochrome P4501A1 (CYP1A1) is the terminal component of the MFO system. Ethoxyresorufin-O-deethylase (EROD) activity is CYP1A1 dependent and therefore a useful marker of MFO induction. The level of this enzyme is known to be induced by exposure to certain contaminants, such as PAHs or PCBs. It is therefore considered a useful biomarker for exposure to certain bioavailable contaminants (Stagg and McIntosh, 1998). However, studies on several fish species have shown that EROD activities vary seasonally and are generally lowest in gonadally maturing females (Goksøyr and Larsen, 1991; Lange et al., 1998).

Activity of EROD was visible in most individuals, but as expected was clearly lower in the females compared to the males. In average an activity of about 130 pmol/min/mg protein was found in the males. In the females it ranged from 1.3 to 57 pmol/min/mg protein (Fig. 2).

For comparison, EROD activities in female and male Atlantic cod, caged and exposed to polluted sediments in a Norwegian fjord for a period of three month (Beyer et al., 1996) are also included. As seen from Figure 2, the activities found in the males in the Bornholm Basin are elevated as compared to the Norwegian cod (Beyer et al., 1996), indicating an induction of this biomarker due to contaminant exposure.

An experimental treatment of juvenile, cultivated cod (110-170 g) with TCDD (total dose of 8 µg/kg body weight) at Bergen, Norway resulted in EROD activities between 34-98 (9 days after intragastrical administration) and 51-119 pmol/min/mg protein (17 days after TCDD treatment), respectively (Hektoen et al., 1994). Hence, the average EROD activity in the males from the Bornholm Basin exceeds the maximum values obtained in those exposure experiments. Bearing in mind that all females analysed here were running-ripe and that female hepatic EROD should be at minimum in the spawning time, it can be assumed that EROD was also induced at least in some of the female specimens. This induction need not be caused by the organo-chlorines measured or their closest relatives, such as dioxins or dibenzofurans, but could as well be due to other compounds not measured in this study, such as, e.g., PAHs.

AChE activity (Figures 3,4)

Inhibition of acetylcholinesterase (AChE) activity in cholinergic areas of tissue has been proposed as a useful molecular biomarker of an effective exposure to organophosphates and carbamates (Bocquené et al., 1990, Bocquené and Galgani, 1998). Since the Baltic Sea catchment area is heavily subjected to industrial as well as intensive agricultural effluents, the presence of such AChE inhibiting xenobiotics in the Bornholm Sea can well be expected. Furthermore, World War ammunition, partially containing organophosphorous nerve gases, dumped in the deeper areas of the Bornholm Basin in the late 1940ies and corroding successively, is very likely to act as point source of such substances, particularly for the cod inhabiting the deeper water layers.

All individuals measured in the present study had AChE activities ranging between 10 to 80 pmol/min/mg protein (Fig. 3). The means vary between 37.9 ± 16.2 (females) and 47.3 ± 11.9 pmol/min/mg protein (males). Activity of this enzyme is significantly lower (t-test. = $p < 0.02$) in the females than in the males. Furthermore, AChE activities in the females tend to be lower in animals with higher loads of pesticides, particularly dieldrin (Fig. 4). This negative relation is not significant, but in spite of the wide spread of data points, high AChE activities were not found at high dieldrin concentrations. However, to our knowledge there is neither any hint on AChE inhibition caused by dieldrin, nor can we assume that dieldin concentrations are indicative of a concurrent exposure to well-known AChE inhibitors, such as organo-phosphates or carbamates.

Viable hatching, larval survival and larval growth rates (Table 1,3)

Hatching rates varied between 63 and 96%. When hatching was achieved, 80 to 100% of the larvae survived. Growth rate within the first ten days ranged from 2.1 to 26.9 % (Table 1).

No statistically significant correlations were found when the contaminant concentrations as well as the EROD and AChE activities were calculated against the effect parameters measured. The correlations performed and the r^2 values obtained are given in Table 3. This is in contrast to our previous findings in 1996 where such correlations existed (Petersen et al., 1997). One possible explanation might be that in 1999 the contaminant concentrations were much more uniform than in 1996 (see above). Considering the wide variability in natural hatching success and larval performance, a more pronounced gradient of contaminant burdens in the ovaries would probably be necessary to obtain significant correlations.

Challenge experiments (Fig. 5)

Our hypothesis in the present project was that if the Baltic female cod already suffered from their exposure to toxicants, a lower LBB would be obtained compared to the LBB determined in 'clean' larvae. The range of LBBs found in this study was between 55.6 - 185 mmol pyrene /kg lipid, indicating a difference in the intrinsic sensitivity between the different larvae batches. There was no correlation between the contaminants measured in the parental fish and the LBBs measured in 5-6 days post hatch larvae (Fig. 5). One explanation for not finding any negative correlation between contaminant content and LBB could be that the contaminant loading of the hatched larvae was too low for giving any significant response on a respectively rough effect parameter as death. However, a more probably explanation could be that the contaminants measured were not responsible for the variability in the LBBs obtained. This explanation is more likely, when taking into account EROD as a biomarker of exposure. When EROD activity was particularly high in the females, the LBB in the larvae was always low (Fig. 5). As already pointed out, the activity levels found clearly indicate an induction of this enzyme, which appears to be independent of the contaminants measured.

For AChE such a clear indication was not found (Fig. 5).

Conclusions

- The mean concentrations of DDTs and PCBs found in ovaries of Baltic cod (Bornholm Basin) were higher in 1999 compared to 1996, whereas the standard deviations and ranges were lower. Hence, differences in contaminant burdens in the fish were much less pronounced in 1999 than in 1996, rendering any significant correlation with biological effects more unlikely.
- Ovary-contents of DDTs and PCBs were highly related, indicating that burdens of other contaminants not measured with similar physicochemical behaviour (e.g. planar PCBs or dioxins) were distributed in a similar manner.
- Elevated activities of hepatic EROD particularly in males (as compared to literature data on Norwegian cod) indicate that this biomarker of contaminant exposure was in fact induced in the Bornholm Basin cod. This was less visible in the females, probably due to the known counteracting activity on estrogens during ovary maturation. There were no significant correlations between hepatic EROD and ovary burdens of the contaminants measured.

- Larval hatching rates were relatively high (63 - 96%), and so was survival after hatch (80-100%). Growth rates varied between 4.6-26.9%. None of these rates was correlated with contaminant burdens measured in the ovaries or with hepatic EROD or muscular AChE activities.
- Challenge tests with pyrene showed differences in the intrinsic sensitivity between the larvae batches, as indicated by a relatively high variability in the lethal body burdens (LBBs) determined. A correlation between burdens of contaminants measured in the ovaries and LBBs of the larvae was not found, indicating that the contaminants measured are probably not responsible for the variability in the LBBs obtained. However, the apparent relation between elevated EROD activities in the mother fish and low LBB of pyrene in the larvae may indicate prenatal damage or a mother-to-young transfer of toxicants.

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Successful fertilizations: Female no.	Age	CI g.w/l3	% Hatch	% surv. after hatch	Growth d 0-10 %	egg size mm	larval size day 0
1	6	0,93	65,00	100,00	13,11	1,69	56,47
5	3	0,77	88,00	90,00	9,46	1,72	60,60
6	4	0,75	79,50	100,00	10,70	1,71	60,73
4	4	0,80	63,00	98,00	13,82	1,62	59,07
7	3	0,84	92,50	98,00	9,27	1,84	60,40
10	5	0,71	95,50	92,00	6,67	1,84	59,93
12	3	0,72	92,50	94,00	9,85	1,70	55,47
13	3	0,63	88,00	90,00	11,01	1,73	56,33
17	3	0,73	85,50	99,00	14,71	1,72	53,93
18	3	0,70	77,50	94,00	7,52	1,73	56,27
19	2	0,89	89,50	98,00	8,97	1,67	60,20
20	2	0,75	65,50	98,00	5,23	1,69	58,33
22	3	0,77	75,50	94,00	4,59	1,75	58,13
28	3	0,78	92,50	84,00	9,54	1,75	56,42
31	9	0,65	77,00	96,00	10,84	1,85	57,20
32	3	0,77	90,50	98,00	2,14	1,61	59,20
33	3	0,72	88,50	92,00	4,68	1,67	59,07
41	3	0,80	95,00	92,00	10,62	1,67	55,27
42	3	0,69	95,50	80,00	7,97	1,62	55,00
46	4	0,70	94,00	80,00	16,39	1,69	52,29
47	3	0,80	79,50	88,00	9,27	1,68	56,80
48	4	0,74	96,00	88,00	8,11	1,72	58,33
49	6	1,02	96,00	98,00	13,22	1,78	57,60
51	3	0,79	91,50	92,00	21,38	1,69	50,75
53	6	0,61	79,50	92,00	21,79	1,63	49,20
54	2	0,70	94,00	98,00	10,19	1,77	57,73
55	3	0,80	91,00	100,00	23,14	1,73	52,73
56	3	0,76	88,50	90,00	16,46	1,80	54,67
57	3	0,80	91,00	96,00	26,87	1,74	50,38
58	3	0,75	85,00	98,00	15,01	1,81	59,53
60	5	0,83	85,00	92,00	24,24	1,81	53,93
61	4	0,68	96,50	90,00	9,19	1,72	58,73

Table 1: Parameters measured in running ripe females used in the experiments (T = 7 °, S = 15‰)

Table 2: Arithmetric means, standard deviations and ranges of organochlorine concentations in the ovaries of cod caught in the Bornholm Basin in ´99 and in ´96 (Petersen et al., 1997)

(concentrations are expressed as mg / kg extractable lipid)

	1999		1996	
	Mean +/- S.D.	Range	Mean +/- S.D.	Range
S-PCBs	1.32 +/- 0.80	(0.40 - 3,98)	0.77 +/- 1.02	(0.13 - 5.29)
S-DDTs	1.34 +/- 0.94	(0.31 - 3.95)	0.92 +/- 1,11	(0.14 - 5.43)
p,p´-DDE	1.06 +/- 0.79	(0.26 - 3.28)	0.75 +/- 0.92	(0.12 - 4.42)
Dieldrin	0.039 +/- 0.023	(0.011 - 0.12)	0.021 +/- 0.013	(0.044 - 0.060)

Table 3: Correlations between different xenobiotics monitored in the females, other effect parameters and larval development
(%hatch, larval size day 0, % survival after hatch, growth within ten days)
all females (n=32) July 99

	total contamin. µmol/kg lipid)	Sum PCB µmol/kg lipid)	CI	AChE	EROD	LBB
Egg size (mm)						
%hatch	-0,205	-0,238		0,21		
larval size day 0 (mg)						
%survival after hatch			0,373			0,365
growth 0-10d (%)						0,564
LBB			0,311		-0,258	
Age				-0,483		

Table 4: Correlations between different xenobiotics monitored in the females, other effect parameters and larval development (%hatch, larval size day 0, % survival after hatch, growth within ten days)
3-year-cod (n=18)

	total contamin. µmol/kg lipid	Sum PCB µmol/kg lipid	DDE (DDT??) µmol/kg lipid	CI	AChE	EROD	LBB
Egg size (mm)	0,405	0,478	0,339	0,258		-0,305	
%hatch	-0,229	-0,231	-0,217			-0,351	
larval size day 0 (mg)							
%survival after hatch				0,31			0,28
growth 0-10d (%)				0,312		-0,447	0,439
LBB				0,349		-0,428	
Age							
EROD				0,492			

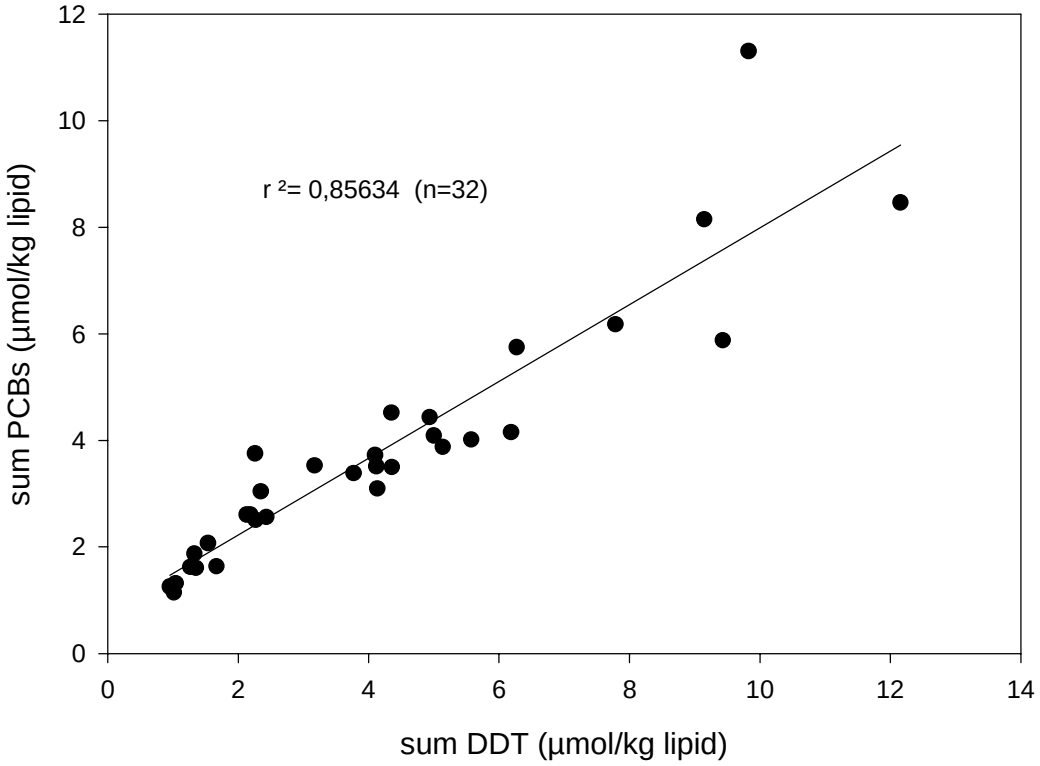


Figure 1. Relation between PCB ∑ PCBs (congeners 52, 101, 105, 118, 138, 149, 153 and 180) and DDT (∑ DDTs,DDD and DDE) content (µmol/kg lipid) in ovaries of female cod from Bornholm Basin (n=32).

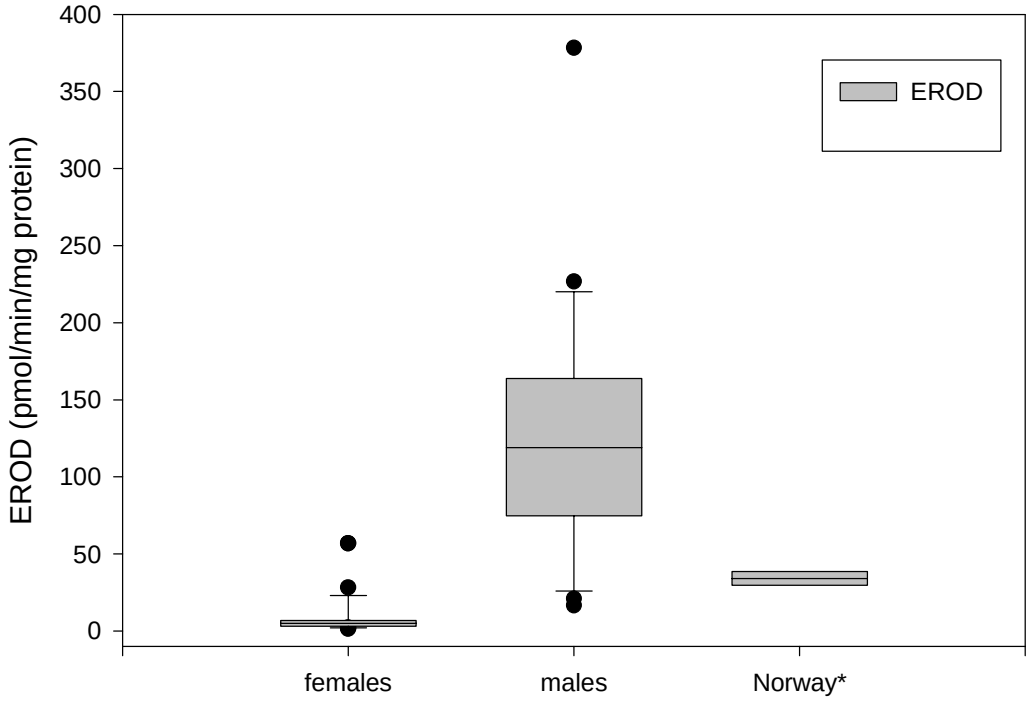


Figure 2. EROD-activity (pmol/min/mg protein) in liver tissue of running ripe female (n= 32) and male (n=24) cod, respectively, caught in the Bornholm Basin (July 1999) in comparison to data from Atlantic cod (females and males; 6:2) subjected to caging at polluted sediments in a Norwegian fjord (Sørfjorden) for a period of 3 months (Beyer et al. 1996).

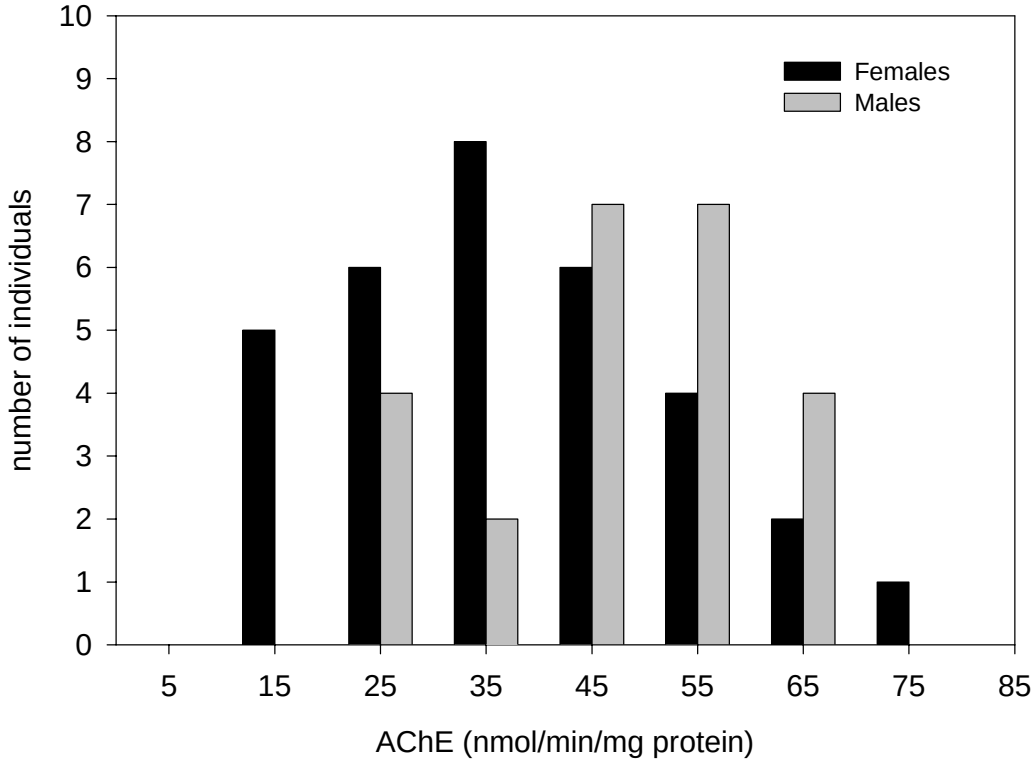


Figure 3. Frequency distribution of AChE activity (nmol/min/mg protein) in muscle tissue of female (n=32) and male (n=24) cod from Bornholm Basin (July 1999)

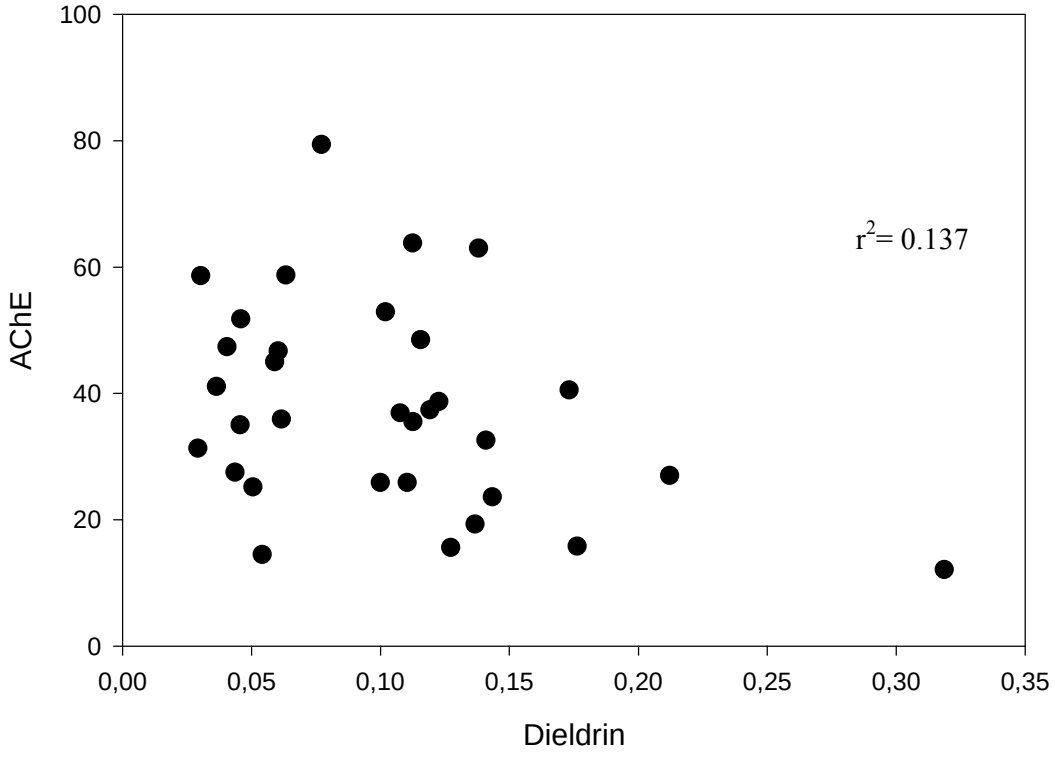
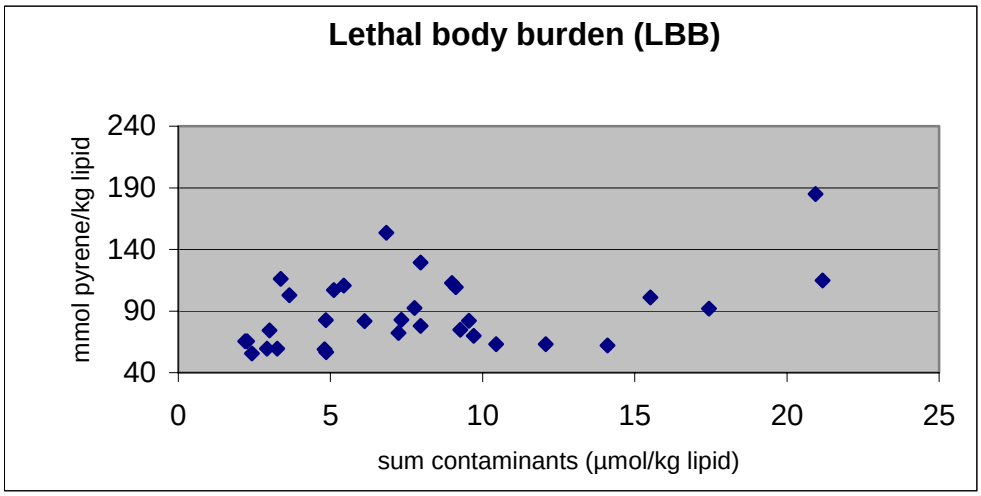
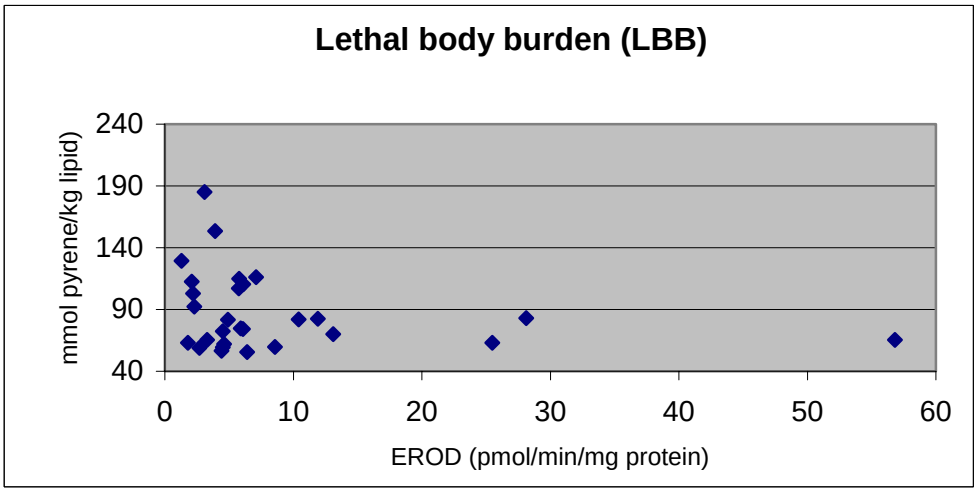


Figure 4. Activity of AChE (nmol/min/mg protein) in muscle tissue versus dieldrin content (µmol/kg lipid) in the ovaries of running-ripe female cod from Bornholm Basin (July 1999).





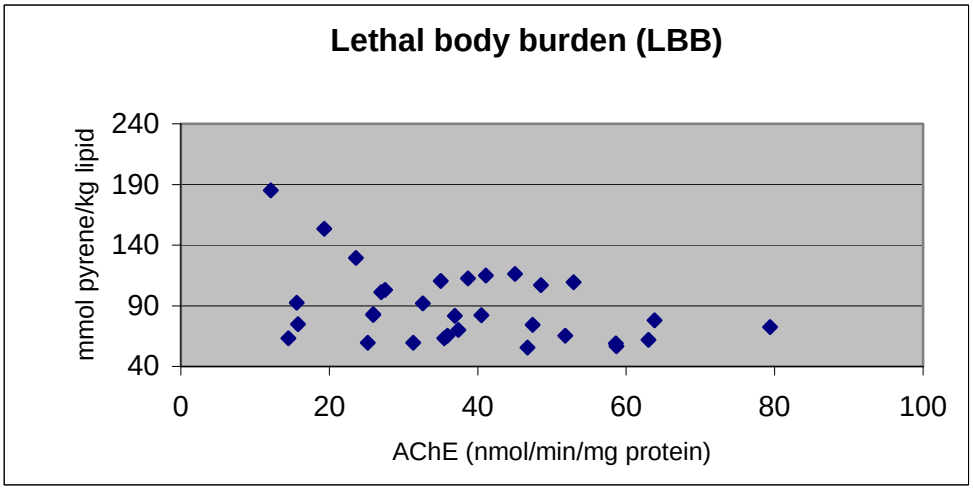


Figure 5. Relation between contaminant content or enzyme activity (EROD, AchE) in female cod (Bornholm Basin, July 1999) and lethal body burdens (LBB) in the larvae.