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Biomarkers in fish from dioxin-contaminated fjords

K. HYLLAND[1,2], O. Ø. ASPHOLM[3], J. A. KNUTSEN[4], & A. RUUS[1]

[1]*Norwegian Institute for Water Research, Oslo, Norway,* [2]*Department of Biology, University of Oslo, Oslo, Norway,* [3]*DNV, Høvik, Norway,* [4]*Institute for Marine Research, Research Station Flødevigen, Hisøy, Norway*

Abstract

The Grenland fjords, southern Norway, have been heavily contaminated by dibenzo-*p*-dioxins and dibenzofurans (dioxins) over decades through inputs from a magnesium smelter. Despite radically decreased inputs since 1990, there are still high levels of dioxins in both biotic and abiotic components of the fjords. The aim of the study was to establish whether biomarkers' responses in three fish species, Atlantic cod (*Gadus morhua* L.), sea-trout (anadromous brown trout, *Salmo trutta* L.) and flounder (*Platichthys flesus* L.), could be used to discern the effects in the most contaminated ecosystem, Frierfjord, from the effects in the adjacent, less-contaminated ecosystem, Eidangerfjord. Biomarker responses clearly indicated that the three fish species were affected by dioxin exposure. Phase I responses in cod and trout could be used to differentiate exposure in the two fjord ecosystems. Phase II responses (glutathione *S*-transferase) in cod and trout similarly indicated a higher dioxin exposure in Frierfjord compared with Eidangerfjord. Results for glutathione *S*-transferase and glutathione reductase indicated different exposure levels in the two fjords, but also showed seasonal variability, and the results highlighted the need for baseline data for these biomarkers.

Keywords: *Dioxins, Atlantic cod (*Gadus morhua *L.), sea-trout (*Salmo trutta *L.), flounder (*Platichthys flesus *L.), biomarkers*

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Introduction

Sublethal responses in fish to environmental contaminant stress have been studied for decades and a broad range of methods have been identified and applied in environmental assessment (reviewed by Van der Oost et al. 2003). Even for the most widely used methods that clearly and unambiguously identify contaminant-related effects under controlled conditions, there is still a general uncertainty about whether and how they should be used to assess contaminant impact in natural ecosystems. The reservations have predominantly been due to a lack of knowledge about how contaminant-related responses may be modulated by non-contaminant factors such as gender, season, the maturation general health status of the organism, adaptation (will responses prevail following long-term exposure), the dose-dependency of responses, environmental factors such as food availability, temperature and salinity, and behaviour (migration, feeding strategies). It has also become increasingly

Correspondence: K. Hylland, Norwegian Institute for Water Research (NIVA), PO Box 173, Kjelsas, N-0411 Oslo, Norway. E-mail: ketil.hylland@niva.no

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clear that there are obvious differences between species in their response to contaminants even though they may inhabit the same stretch of coast and feed on similar organisms (e.g. Collier et al. 1992, Ruus et al. 2003). Some of the reservations have been partly resolved for some species and responses such as hepatic cytochrome P4501A (CYP1A) activity and metallothionein levels, e.g. seasonal variability (Goksøyr & Larsen 1991, Hylland et al. 1998, Rotchell et al. 1999, 2001), gender differences (e.g. Hylland et al. 1998, Winzer et al. 2002), temperature (Sleiderink et al. 1995) and salinity effects (Baer & Thomas 1990, Schlenk et al. 1996).

In the current study, a fjord system with a known history of dioxin contamination was used to consider some of the issues identified above, i.e. differences between species, seasonal variation, dose-dependency and consistency of responses following long-term exposure. The study area, Frierfjord and Eidangerfjord, near the cities Porsgrunn and Skien, has received high inputs of dibenzo-*p*-dioxins and dibenzofurans (hereafter referred to collectively as dioxins) in 1951–90. Earlier monitoring programmes have clearly shown elevated concentrations of contaminants from this input as far south along the coast as Kristiansand (Knutzen et al. 1997). Although there have been other contaminant inputs into this fjord system, e.g. (polycyclic aromatic hydrocarbons) PAHs and decachlorobiphenyl, recent data suggest that dioxins are the main current contaminants in this area. The inputs were dramatically decreased in 1990, following which there were expectations that levels of dioxins in the biota of the fjord system would return to close to background within a decade. This did not happen. Following an initial decrease, concentrations remained high in fish and shellfish from the receiving waters. An adjacent fjord, Eidangerfjord, was chosen as a reference area, although it was realized that biota in that area would also be exposed to concentrations of dioxins above background levels (Knutzen et al. 2001). The main reason for that selection was to ensure that food webs would be similar. Using the Skagerrak proper as a reference would invalidate direct comparisons due to very different trophic structures. The trophic webs of the two fjords have been described elsewhere (Bergstad & Knutsen 2004). A second reason to select a reference area that would not be entirely free from contaminant exposure was to consider the issue of whether biomarkers could be used to discern between two contaminated areas, but with different levels of contamination.

The main aim of the study was to clarify whether biomarker responses in three fish species would reflect known differences in exposure levels in two fjord ecosystems. An additional aim was to indicate seasonal variability in the responses. Biological effects methods (biomarkers) were chosen according to results in previous studies of dioxin-related effects in fish.

The study included hepatic CYP1A, hepatic glutathione *S*-transferase (GST), hepatic glutathione reductase (GR) and plasma vitellogenin (vtg). Data on tissue dioxin levels have been published elsewhere (Berge et al. 2004, Ruus et al. 2005) and were used to assess exposure in the fish populations studied. Metabolism and excretion of organic contaminants is largely mediated through a two-step process, catalysed by phase I and II enzymes. Phase I enzymes catalyse a range of reactions, but the general outcome is an 'activation' of foreign (and endogenous) molecules. This intermediate may then be acted upon by phase II enzymes, which add a water-soluble ligand so that the complex can be excreted, generally through the bile. Dioxins are known to be strong inducers of phase I enzymes, i.e. P4501A, in fish (Van der Weiden et al. 1990, 1993, 1994, Förlin et al. 1992, Grinwis et al. 2000). P4501A activity has

been widely used as a biomarker in fish for decades (Goksøyr & Förlin 1992) and is presumably the best documented biomarker in ecotoxicology today. In addition to enzyme activity, the concentration of P4501A was also quantified to assess modulation of CYP1A activity by other contaminants or environmental factors. Earlier studies have shown both phase I and II enzymes to be affected in fish collected in this area (Hylland et al. 1997). Phase II enzymes such as GSTs have also been shown to be affected by dioxins in other studies (Schrenk et al. 1995, Hou et al. 2001). The effects of dioxins on antioxidant enzymes fish have not been widely documented earlier, but they have been indicated in birds and mammals (Slezak et al. 2002, Hassoun et al. 2003, Hilscherova et al. 2003). In the present study, GR was included as a marker for changes in the intracellular availability of reduced glutathione, i.e. antioxidant stress. There have also been indications that some dioxins may be anti-oestrogenic in vertebrate systems (Smeets et al. 1999, Rankouhi et al. 2004). To investigate such effects in the two chose ecosystems, a marker for environmental oestrogens, vtg, was included. The species studied in the two ecosystems were sea-trout (anadromous brown trout; *Salmo trutta* L.), Atlantic cod (*Gadus morhua* L.) and flounder (*Platichthys flesus* L.). For Atlantic cod, a distinction was made between juvenile and mature fish, whereas only sexually mature individuals were used for sea-trout and flounder. It is well established that biological processes in temperate fish species have seasonal cycles (e.g. Hylland et al. 1998) and samples were thus taken for the three species at three times of the year: January, April and November.

Materials and methods

Areas and sampling

Fish were sampled in two fjords, Frierfjord (closest to the source at Herøya and thus the highest dioxin exposure) and Eidangerfjord (Figure 1). The two sampling areas are only separated by a few kilometres and a sill at Brevik. Data on contaminants (Berge et al. 2004, Ruus et al. 2005) and tracking experiments (Bergstad et al. 2004) indicate that there is limited movement of Atlantic cod and flounder between the two fjords. Data on sea-trout, however, indicates some exchange between the two ecosystems (Knutsen et al. 2003).

Fish for analyses of biological responses were collected during three sampling campaigns: in April and November 2000 and in January 2001. Fish were kept alive on-board R/V *G. M. Dannevig* until sampling. Before sampling, each individual was stunned by a blow to the head, weighed and measured. Blood was taken from the caudal vein using a heparinized and aprotinized syringe. The sampled blood was then kept on ice until centrifugation and separation of plasma from blood cells. The plasma was frozen in liquid nitrogen and stored at -80°C until analysis. The body cavity was opened, the liver dissected out, and sections transferred to cryovials and frozen in liquid nitrogen. Liver samples were stored at -80°C until analysis.

General status of fish

The general conditions of cod and trout were assessed using the formula (Le Cren 1972):

Weight/(length^3)*100.

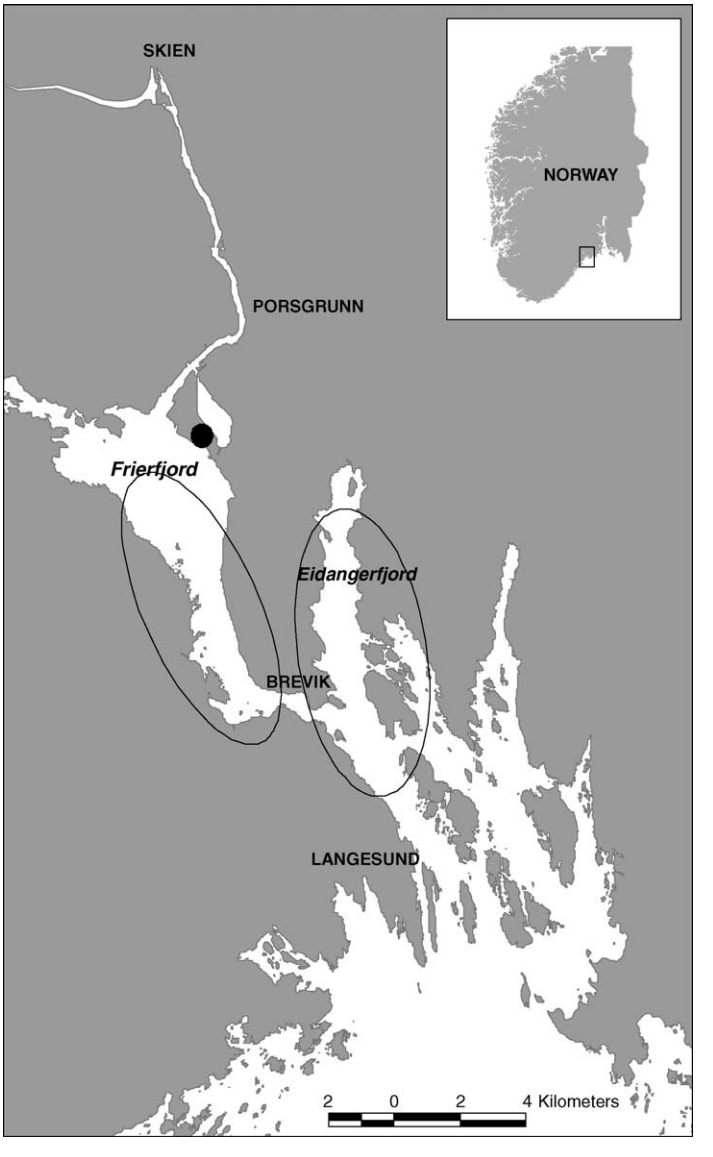

Figure 1. Overview of the areas investigated (Frierfjord and Eidangerfjord); the location of the main dioxin source is indicated by a black marker.

Normal values will be around 1 for both species. There was not a sufficient number of flounder for the method necessary to determine condition in flatfish (Ricker 1979). A liver somatic index (LSI) was calculated for all three species as:

(Liver weight)*100/(body weight – gonad weight).

Sample treatment and biochemical assays

Liver samples were thawed on ice and homogenized in 5 vols ice-cold 0.1 M potassium phosphate buffer, pH 7.8, with 0.15 M KCl, 5% glycerol and 1 mM DTT.

The resulting homogenate was centrifuged at 10 000*g* for 30 min at 4°C and the supernatant (S9 fraction) removed and centrifuged at 50 000*g* for 2 h. The resulting supernatant, the cytosolic fraction, was removed, mixed and divided into three vials before being frozen at −80°C until analysis. The pellet, the microsomal fraction, was resuspended in ice-cold homogenizing buffer with 1 mM EDTA and 20% glycerol, divided into three vials and frozen at −80°C. Protein was analysed in cytosolic and microsomal fractions using Lowry et al.'s (1951) method adapted for a plate-reader. Bovine IgG was used as standard in the assay. The concentration of P4501A in the microsomal fraction was determined using a direct ELISA with antisera purchased from Biosense (Bergen, Norway). Microsomal samples were diluted to exactly 10 μg ml^{-1} in coating buffer. A total of 100 μl was then pipetted in quadruplicate into wells of 96-well microtitre plates. Following incubation overnight in a moist chamber, the plates were washed three times in Tris-buffered saline (TTBS, pH 8.6, with 0.05% Tween-20) and blocked for 30 min with TTBS containing 1% bovine serum albumin (BSA). Following the wash, each well then received primary antisera – NP-7 for cod (dilution 1:800), CP226 for trout and flounder (dilutions 1:1000) – in TTBS with 0.1% BSA. Following overnight incubation, plates were again washed three times in TTBS and secondary antiserum pipetted in to wells (Sigma GAR-HRP in TTBS with 0.1% BSA). The plates were incubated for 8–10 h and then washed five times in TTBS. Colour development was by the substrate TMB, the reaction terminated by adding 2 N sulphuric acid, and the product quantified by reading at 450 nm. P4501A activity in the microsomal fractions was quantified from the amount of resorufin produced in a method adapted to a plate-reader from the original procedure described by Burke and Mayer (1974). Total GST activity was determined in the cytosolic fraction using CDNB as substrate. The method used was that described by Habig et al. (1974) adapted to a plate-reader. The activity was determined kinetically against a blank and presented as nmol min^{-1} mg^{-1} cytosolic protein. GR activity was determined in the cytosolic fraction using an adaptation of the method described by Cribb et al. (1989). Activity was determined kinetically and calculated as nmol min^{-1} mg^{-1} cytosolic protein. Analyses of vtg in mature male cod used a competitive ELISA established at NIVA (described by Scott & Hylland 2002). Plasma from oestradiol-treated cod was used as standard and coating antigen (Hylland & Haux 1997). The concentration of vtg in the standard plasma was determined by HPLC and protein determination. Vtg is stable for years when stored in plasma, but it quickly breaks down when purified. Cod vtg is especially sensitive to breakdown during preparation and storage (Silversand et al. 1993).

Chemical analyses and calculation of TEQ

Chemical analyses were performed as described by Ruus et al. (2005). The analyses of PCDD/PCDFs were performed at the Norwegian Institute for Air Research (NILU) as follows. The samples were homogenized in Na_2SO_4, before extraction by direct elution with cyclohexane and dichloromethane. Aliquots of the lipid extracts were used to determine the lipid content gravimetrically. [^{13}C]-labelled 2,3,7,8-substituted PCDDs/PCDFs (2,3,7,8-tetra-CDD, 1,2,3,7,8-penta-CDD, 1,2,3,4,7,8-hexa-CDD, 1,2,3,6,7,8-hexa-CDD, 1,2,3,4,6,7,8-hepta-CDD, octa-CDD, 2,3, 7,8-tetra-CDF, 2,3,4,7,8-penta-CDF, 1,2,3,4,7,8/1,2,3,4, 7,9-hexa-CDF, 1,2,3,6,7,8-hexa-CDF, 2,3,4,6,7,8-hexa-CDF, 1,2,3,4,6,7,8-hepta-CDF, octa-CDF) were added as internal

standards before the extraction step. The samples were then cleaned with a multi-column system with different types of silica, aluminum oxide and 196-activated carbon. The cleaned samples were concentrated to approximately 10 μl and a [^{13}C]-labelled recovery standard (1,2,3,4-tetra-CDD) was added. Determination of all 2,3,7,8-substituted congeners was performed by the use of gas chromatography with high-resolution mass spectrometry (GC/MS; Micromass Autospec Ultima, Micromass, Manchester, UK) equipped with a Restec RTX 2330 (Restec; now Eclipse Scientific Group, Cambridgeshire, UK) or J&W DB-5 MS column (Agilent; Matriks, Norway), dependent on the type of sample). Acceptable recovery of the internal standards was set within 40–120% of the added standard amount. Analytical quality was certified by participation in international intercalibration tests, and the laboratory is accredited to the requirements of the International Standardization Organization/International Electrotechnical Commission (ISO/IEC)-17025. A toxicity equivalency quotient (TEQ) was calculated using toxicity equivalency factors (TEFs) as suggested by Van den Berg et al. (1998). Chemical analyses were performed on pooled samples of fish from the two areas.

Statistical treatment of results

Variables were checked for normality and homogeneity of variances before tests (Levene's test). Appropriate transforms were used where required. If homogeneity of variances could not be achieved, non-parametric Kruskal–Wallis analyses were used to compare groups (Sokal & Rohlf 1981). In the cases where variances were homogeneous, two- and three-way ANOVAs were used to test differences between groups including fjord, season and sex as factors where appropriate (Sokal & Rohlf 1981). The level of significance for rejection of H_0: 'No difference between groups' was set to 0.05.

Results

The individual size of the fish sampled was reasonably homogeneous for all three species (Tables I and IV), although the median size of the sampled fish did vary somewhat on sampling occasions for sea-trout (Table II) and juvenile cod (Table III). There were no significant differences in the condition index between the two ecosystems Frierfjord and Eidangerfjord for either Atlantic cod or sea-trout (Tables I and II; statistical analyses not shown). There were similarly no differences in condition for juvenile cod collected in the two areas (Table III).

There were no significant differences in LSI in mature cod of either sex collected during any of the three sampling periods. Juvenile cod from Frierfjord had a significantly higher LSI than juvenile cod from Eidangerfjord in April, whereas the situation was the opposite in January (statistical analyses not shown). LSI in juvenile cod was generally at the same level as for mature cod, indicating that there is good food availability for juvenile cod in both areas. It can be speculated that the availability of food is lower in Frierfjord than in Eidangerfjord during the winter and that food availability increased for juvenile cod in spring in Frierfjord. The LSI did not change much during different seasons for either trout or flounder collected in the two areas (Tables II and IV).

There was higher median activity of hepatic CYP1A (ethoxyresorufin O-deethylase; EROD) in mature cod from Frierfjord compared with cod from Eidangerfjord on all

Table I. Adult Atlantic cod (*Gadus morhua* L.) sampled in April and November 2000 and in January 2001: values are the median (minimum–maximum).

Area	Gender	Month	Count	Length (cm)	Weight (g)	CI	LSI
Eidangerfjord	female	January	8	45 (36–52)	806 (457–1690)	0.89 (0.83–1.5)	1.62 (1.15–2.75)
		April	9	44 (36–68)	816 (36–68)	0.97 (0.77–1.2)	2.25 (1.77–5.36)
		November	11	37 (34–49)	543 (433–1150)	0.97 (0.91–1.1)	2.28 (1.14–3.23)
	male	January	12	41 (36–55)	593 (426–1770)	0.93 (0.73–1.1)	1.90 (0.90–2.98)
		April	11	42 (38–53)	646 (461–1170)	0.85 (0.79–0.93)	1.95 (0.83–2.88)
		November	9	42 (36–59)	765 (470–1740)	0.96 (0.84–1.1)	2.01 (1.38–3.33)
Frierfjord	female	January	12	50 (36–73)	1120 (475–3360)	0.96 (0.76–1.2)	1.24 (0.76–1.2)
		April	14	46 (34–83)	915 (350–5510)	0.95 (0.80–1.2)	1.79 (1.13–2.85)
		November	10	49 (36–79)	1170 (457–4380)	1.0 (0.89–1.1)	2.27 (1.23–5.06)
	male	January	8	40 (32–63)	673 (273–2460)	1.0 (0.83–1.2)	1.55 (1.06–2.62)
		April	8	42 (34–65)	683 (379–2510)	0.89 (0.86–.096)	1.58 (1.28–2.85)
		November	10	42 (38–75)	771 (543–3980)	0.99 (0.92–1.1)	1.90 (0.982–2.72)

CI, condition index; LSI, liver somatic index.

Table II. Juvenile Atlantic cod (*Gadus morhua* L.) sampled in April and November 2000 and in January 2001: values are median (minimum–maximum).

Area	Month	Count	Length (cm)	Weight (g)	CI	LSI
Eidangerfjord	January	13	18 (14–30)	52 (21–270)	0.90 (0.62–1.0)	1.82 (0.952–2.61)
	April	23	26 (15–34)	160 (28–370)	0.95 (0.78–1.1)	1.90 (1.09–2.70)
	November	18	21 (15–32)	84 (29–380)	0.97 (0.85–1.2)	1.95 (1.14–3.88)
Frierfjord	January	9	28 (22–30)	200 (99–270)	0.90 (0.81–1.0)	1.38 (0.843–1.05)
	April	6	23 (12–30)	125 (20–220)	0.98 (0.82–1.2)	2.48 (2.00–3.30)
	November	8	27 (22–31)	190 (110–250)	1.0 (0.83–1.1)	2.00 (1.53–3.03)

CI, condition index; LSI, liver somatic index.

sampling occasions and for both sexes (Figure 2). In a three-way ANOVA model with fjord, season and sex as explanatory variables, fjord ($p < 0.001$), season (0.004) and an interaction between season and sex contributed significantly to explaining variability (Table V). Surprisingly, there were no differences in hepatic EROD between male and female cod, even though fish were sampled immediately before and after spawning (January and April) for this species (they spawn in February–March in this area). There were only minor differences for mature cod between fjords and sampling periods for the phase II enzyme GST (Figure 2), but there was a significant interaction between fjord and season in a three-way ANOVA model (Table V). Dioxin exposure, as determined from tissue residues, was higher in both male and female cod from Frierfjord compared with Eidangerfjord in April, the only sampling period for which data were available for replicate analyses of pooled samples (Figure 2).

Juvenile cod also appeared to have higher hepatic P4501A activity in fish from Frierfjord compared with fish from Eidangerfjord (Figure 3), but there were no significant differences in a two-way ANOVA (with fjord and season as factors).

Seasonal variability in hepatic EROD was more obvious in male than in female sea-trout (Figure 4). A three-way ANOVA model (with fjord, season and sex as factors) for hepatic EROD in trout indicated significant differences, but an interaction between season and sex was the only single component that came out as significant (Table V). There were higher median activities of hepatic GST in Frierfjord sea-trout compared with sea-trout from Eidangerfjord during the two periods for which material was analysed, April and November (Figure 4). There was a weak interaction between fjord and sex ($p = 0.02$), and both fjord on its own and season came out as significant factors ($p < 0.001$) in a three-way ANOVA (Table V). Although a very limited dataset, hepatic dioxin (TEQ) levels did not appear to differ greatly between the two areas for trout (data from Ruus et al. 2005).

There were clear seasonal differences in hepatic EROD activity in female flounder from the two areas (Figure 5). There was a trend towards elevated EROD activity in flounder collected in Frierfjord compared with flounder collected in Eidangerfjord in January for both sexes, but this was not statistically significant in a two-way ANOVA for January data with fjord and sex as factors. Due to a limited material, a two-way ANOVA with fjord and season as factors was possible only for female flounder. The results indicated that there is a strong seasonal component, but did not support differences between the areas for EROD activity (Table V). The variation in hepatic GST in flounder could not be explained by any of the factors available in this study (fjord, season). A two-way ANOVA for the concentration of CYP1A protein in female

Table III. Sea-trout (*Salmo trutta* L.) sampled in April and November 2000 and in January 2001: values are median (minimum–maximum).

Area	Gender	Month	Count	Length (cm)	Weight (g)	CI	LSI
		January	18	31 (23–54)	279 (126–1330)	0.93 (0.81–1.0)	0.89 (0.65–1.3)
	female	April	8	34 (27–51)	479 (158–1670)	1.0 (0.80–1.9)	0.94 (0.73–1.8)
Eidangerfjord		November	9	35 (27–51)	317 (163–960)	1.0 (0.88–1.4)	0.94 (0.88–1.9)
		January	2	30–48	270–1036	0.94–1.0	0.83–0.78
	male	April	2	28–34	227–359	0.91–1.0	0.89–1.3
		November	2	56–75	2010–3230	0.84–1.1	0.85–1.1
		January	14	30 (24–46)	268 (128–806)	1.0 (0.83–1.1)	0.87 (0.63–1.3)
	female	April	15	32 (25–39)	287 (142–590)	0.99 (0.83–1.1)	1.1 (0.77–2.2)
		November	14	31 (24–63)	309 (151–2530)	0.98 (0.82–1.2)	0.85 (0.68–1.8)
Frierfjord		January	5	35 (29–38)	353 (269–505)	0.92 (0.82–1.1)	1.2 (0.78–1.5)
	male	April	9	35 (21–54)	406 (95–1710)	0.99 (0.86–1.1)	1.2 (0.66–2.8)
		November	6	33 (23–63)	372 (121–2200)	1.0 (0.88–1.1)	0.85 (0.69–1.0)

CI, condition index; LSI, liver somatic index.

Table IV. Flounder (*Platichthys flesus* L.) sampled in April and November 2000 and in January 2001: values are median (minimum–maximum).

Area	Gender	Month	Count	Length (cm)	Weight (g)	LSI
Eidangerfjord	female	January	10	34 (32–45)	490 (375–1180)	2.6 (1.6–3.5)
		April	19	34 (27–41)	384 (255–801)	1.8 (1.3–2.8)
		November	10	38 (27–45)	719 (265–1530)	2.6 (1.1–3.8)
	male	January	4	30 (25–32)	344 (183–438)	1.4 (0.93–2.4)
		April	1	30	341	1.6
		November	8	31 (28–32)	351 (302–461)	1.8 (1.3–2.3)
Frierfjord	female	January	4	39 (31–42)	691 (367–1030)	2.1 (1.5–3.0)
		April	19	35 (26–40)	430 (162–656)	1.9 (1.0–3.3)
		November	19	37 (31–44)	638 (373–1260)	2.9 (1.2–4.1)
	male	January	6	32 (27–35)	338 (230–493)	1.6 (1.2–2.5)
		April	0			
		November	1	36	563	2.3

LSI, liver somatic index.

flounder indicated significant differences for season ($p = 0.01$) and fjord (0.006) (data not shown). A comparison between hepatic concentrations of CYP1A and CYP1A activity (EROD) in flounder indicated a correlation with no obvious indications of inhibition in fish from any fjord (Figure 6). Similar relationships were seen for trout and juvenile cod (CYP1A was not determined for mature cod).

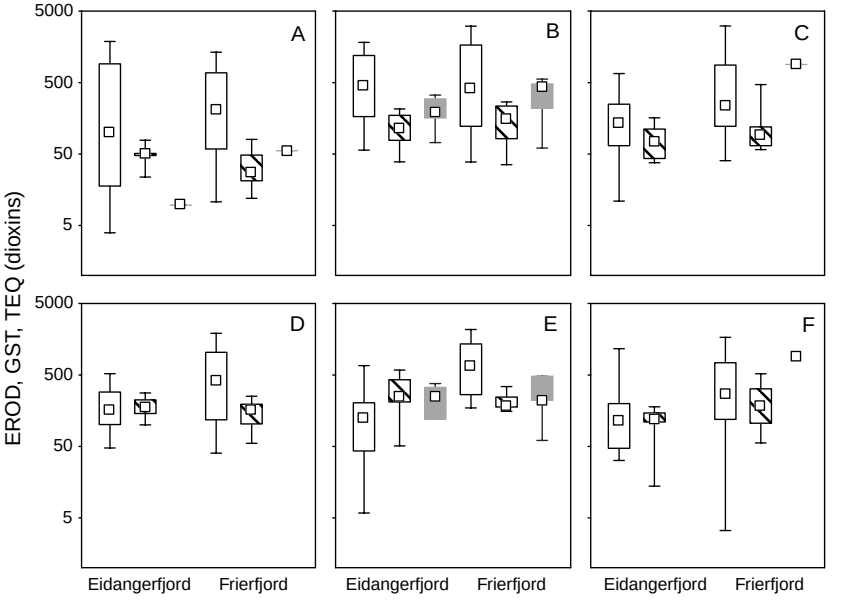

Figure 2. Hepatic CYP1A activity (EROD, pmol min^{-1} mg^{-1} protein; open bars), glutathione *S*-transferase (GST, nmol min^{-1} mg^{-1} protein; hatched bars) and hepatic concentration of dioxins (shown as TEQ; grey bars) in mature cod: (A) females sampled in January, (B) females sampled in April, (C) females sampled in November, (D) males sampled in January, (E) males sampled in April and (F) males sampled in November. Figures show median (squares), quartiles (boxes) and 10/90 percentiles (whiskers). Open spaces indicate that data are lacking. Three-way ANOVAs (fjord, season, sex) indicated significant contributions from fjord, season and season × sex interaction for EROD (log-transformed; $p = 0.0004$) and fjord × season interaction for GST (log-transformed; $p = 0.03$).

Table V. Overview of the results from a three-way ANOVA with two-way interactions using fjord, season and sex as factors; only results for which the variables could be transformed to provide homogenous variances are included.

Species	Endpoint	p of the entire model	Significant factors ($p < 0.05$)
Atlantic cod	EROD	0.0004	fjord, season, season × sex
	GST	0.03	fjord × season
	GR	<0.0001	season
	vtg	0.002	fjord, fjord × sex
Sea-trout	EROD	0.02	season, season × sex
	GST	<0.0001	fjord, season, season × sex
	GR	0.07	fjord
Flounder*	EROD	<0.0001	season

*Only the female.
EROD, ethoxyresorufin O-deethylase; GR, glutathione reductase; GST, glutathione *S*-transferase; vtg, plasma vitellogenin

Although there appeared to be elevated activity of GR in female cod from Frierfjord compared with female cod from Eidangerfjord (Table VI), this difference was not significant in a three-way parametric ANOVA (fjord, season, sex), for which only season came out as a significant factor (Table V). Median hepatic GR was higher in sea-trout from Frierfjord compared with fish from Eidangerfjord for both sexes and at both sampling occasions (January, November), supported by results from a three-way ANOVA in which fjord came out as a significant factor (Tables VI and V). Variations associated with both sex and season characterized hepatic GR in flounder from the two fjords and no pattern was apparent in relation to sampling location (Table VI). There was an unintended bias towards female fish in the analysed material for flounder that precluded a comparison between sexes.

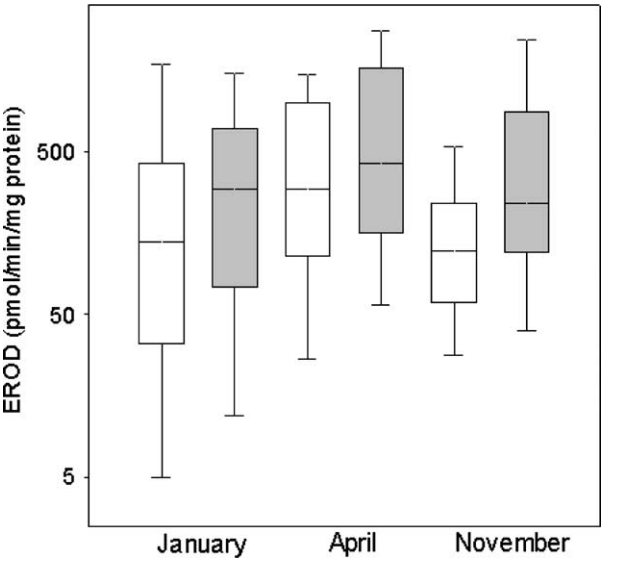

Figure 3. Hepatic CYP1A activity (EROD) in juvenile cod from Eidangerfjord (open bars) and Frierfjord (grey bars) at the indicated points in time. Figures show median (squares), quartiles (boxes) and 10/90 percentiles (whiskers).

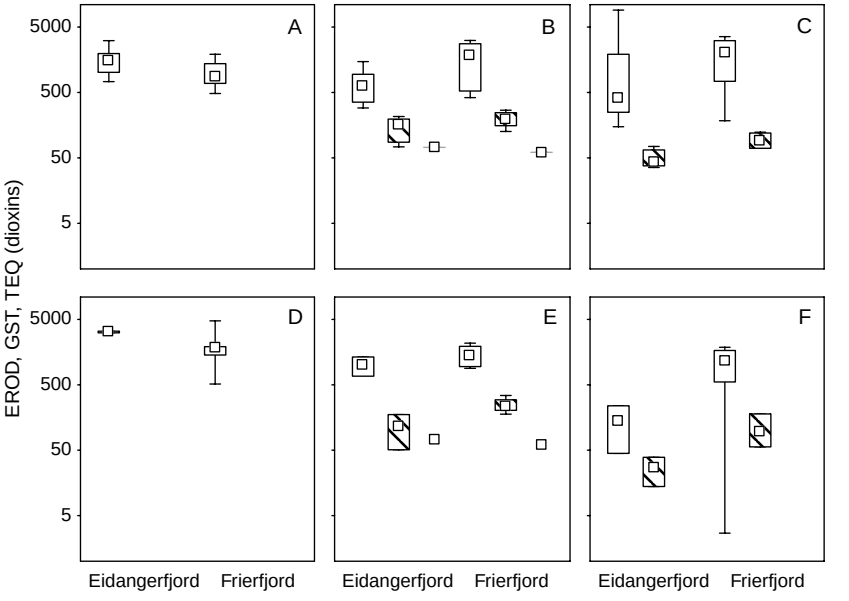

Figure 4. Hepatic CYP1A activity (EROD, pmol min^{-1} mg^{-1} protein; open bars), glutathione *S*-transferase (GST, nmol min^{-1} mg^{-1} protein; hatched bars) and hepatic concentration of dioxins (shown as TEQ; grey bars) in sea-trout: (A) females sampled in January, (B) females sampled in April, (C) females sampled in November, (D) males sampled in January, (E) males sampled in April and (F) males sampled in November. Figures show median (squares), quartiles (boxes) and 10/90 percentiles (whiskers). Open spaces indicate that data are lacking. Three-way ANOVAs (fjord, season, sex) indicated significant contributions from season and season × sex interaction for EROD (log-transformed; $p = 0.02$) and fjord, season, season × sex interaction for GST (log-transformed; $p < 0.0001$).

The results for vtg in male cod indicated a difference between the fjords ($p = 0.002$), but also an interaction between fjord and season ($p = 0.02$) in a two-way ANOVA with fjord and season as factors (Figure 7 and Table V).

Discussion

There is an ongoing discussion on species sensitivity and confounding factors when biomarkers are used for monitoring purposes. Although obviously not the case, species are generally regarded as being equally sensitive to contaminant stress and confounding factors such as different food availability at sampling locations are commonly overlooked. Three species of fish, Atlantic cod, sea-trout and flounder, were sampled during three field campaigns in two areas in the Grenland fjords: Frierfjord, closest to the source of past and, to some extent, present dioxin inputs; and Eidangerfjord, more distant from inputs, but with elevated exposure to dioxins compared with other unpolluted fjords. The choice of the two areas was mediated by a wish to find two systems with a similar trophic food-web structure, but different contaminant (dioxin) exposure. The results of other projects under publication have shown that the two areas did not differ by more than a factor 2–3 in dioxin exposure (Berge et al. 2004, Ruus et al. 2005). The results show that the three fish species appeared to have similar food availability in the two fjord areas through the year, as there were only minor differences in condition-related indices (condition, LSI). Both

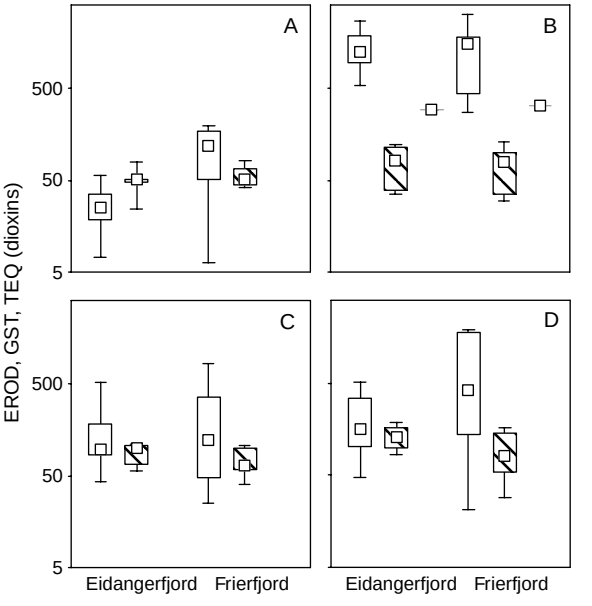

Figure 5. Hepatic CYP1A activity (EROD, pmol min^{-1} mg^{-1} protein; open bars), glutathione *S*-transferase (GST, nmol min^{-1} mg^{-1} protein; hatched bars) and hepatic concentration of dioxins (shown as TEQ; grey bars) in flounder: (A) females sampled in January, (B) females sampled in April, (C) females sampled in November and (D) males sampled in January. Figures show median (squares), quartiles (boxes) and 10/90 percentiles (whiskers). Open spaces indicate that data are lacking or insufficient. A two-way ANOVA (fjord, season) for female flounder indicated a significant contribution from season for EROD (log-transformed; $p < 0.0001$). A similar model was not significant for GST.

areas have shallow bays and mudflats as well as rocky shores. One major difference is the larger freshwater influence in surface waters of Frierfjord compared with Eidangerfjord, which has a negative effect on rocky shore diversity in the upper 5–10 m. There is some indication in the data that juvenile cod in Frierfjord may fare slightly less well than juvenile cod in Eidangerfjord (lower LSI), at least in the winter.

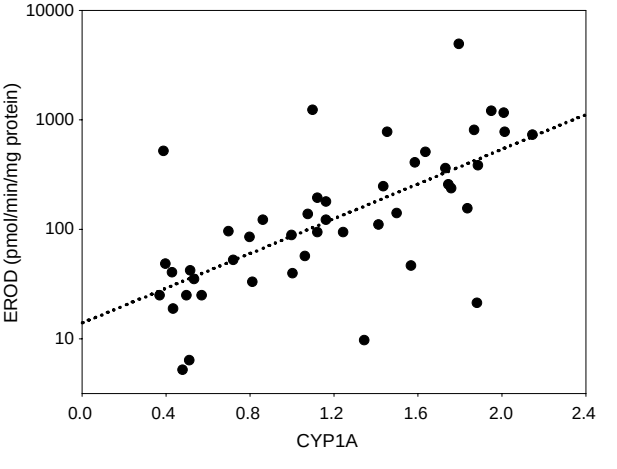

Figure 6. Relationship between cytochrome P4501A concentration (CYP1A) and activity (EROD) in flounder collected in both fjords (linear regression: $r^2 = 0.44$; $p < 0.0001$).

Table VI. Glutathione reductase (nmol min^{-1} mg^{-1} protein) in Atlantic cod, trout and flounder from Eidangerfjord and Frierfjord: values are median (N; minimum–maximum).

Area	Gender	Month	Atlantic cod	Sea-trout	Flounder
Eidangerfjord	female	January			3.1 (5; 2.8–4.6)
		April	9.0 (3; 8.7–14)	3.6 (8; 2.1–9.9)	3.4 (10; 3.1–4.6)
		November	3.8 (6; 2.7–7.5)	2.6 (7; 2.1–2.9)	3.3 (5; 2.4–4.1)
	male	January	13 (0; 5.3–15)		3.6 (4; 3.1–3.8)
		April	12 (7; 10–16)	3.4 (2; 2–9–3.9)	
		November	4.0 (4; 3.1–4.5)	2.2 (2; 1.6–2.8)	3.6 (5; 2.7–4.3)
Frierfjord	female	January			2.9 (4; 2.1–5.4)
		April	11 (7; 6.9–16)	4.1 (6; 1.9–5.5)	4.2 (10; 2.6–6.4)
		November	4.8 (4; 3.2–5.5)	3.0 (7; 2.1–.5.4)	2.0 (10; 1.3–2.8)
	male	January	9.5 (7; 7.5–15)		4.3 (6; 3.5–5.2)
		April	12.8 (3; 10.8–13)	6.4 (4; 2.6–7.0)	
		November	4.1 (6; 2.0–6.4)	2.8 (3; 2.2–3.4)	

Some female trout from Eidangerfjord had a higher condition index than other individuals of that species, possibly reflecting that females are more migratory and move further to sea for feeding as their reproductive success is more size-dependant than that of males (Jonsson & Gravem 1985, Knutsen et al. 2004). The two ecosystems chosen for this study do appear to have relatively similar feeding and habitat conditions for the three species investigated (Bergstad & Knutsen 2004). The major difference between the two ecosystems was different levels of dioxin exposure.

Hepatic P4501A activity in Atlantic cod was high in both areas compared with levels found in other Norwegian coastal areas and offshore (cf. Ruus et al. 2003). In unpolluted areas on the Barents Sea coast, EROD will generally be 10–20 pmol min^{-1} mg^{-1} protein, whereas the hepatic EROD activity in cod at reference areas further south can be up to 90 pmol min^{-1} mg^{-1} protein (all analyses at the same laboratory). Both male and female cod from Frierfjord sampled in November

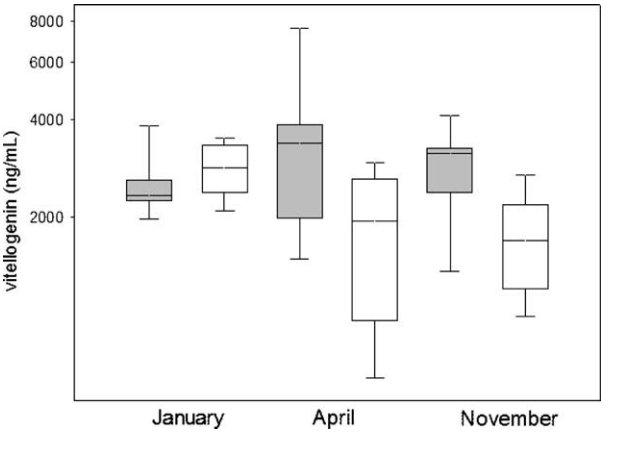

Figure 7. Plasma vitellogenin in male cod at the indicated time points from Eidangerfjord (open bars) and Frierfjord (grey bars). Figures show median (squares), quartiles (boxes) and 10/90 percentiles (whiskers). A two-way ANOVA (fjord, season) indicated a significant contribution from fjord and fjord × season (log-transformed; $p = 0.002$).

(sampling for other monitoring programmes, e.g. the OSPAR JAMP (Oslo and Paris Commissions Joint Assessment and Monitoring Programme)) is generally done in the autumn (September–November) and had medians at two to three times above such background levels, whereas more than half the cod from Eidangerfjord had EROD activities above this level. There was seasonal variation in EROD in female cod, especially in Eidangerfjord (medians varied by a factor four), but there was very little change in median hepatic EROD in male cod for the period investigated (below a factor of two). Variability appeared to be greater in Frierfjord cod, which would indeed be the case if accumulated contaminants were released internally following mobilization of fat for spawning. There are little previous data available on the seasonality of EROD in cod, but seasonality appears smaller than that observed for other species, e.g. Atlantic salmon (Goksøyr & Larsen 1991). For flounder, median hepatic EROD in females from natural populations is known to vary by a factor of six from autumn to spring in unpolluted areas (Christensen 1995). Seasonal variation in CYP1A activity has been addressed for some fish species, e.g. Atlantic salmon (Goksøyr & Larsen 1991), winter flounder (Vandermeulen & Mossman 1996), and flounder and eel (Rotchell et al. 1999). A general observation is the large species variability in gender-dependent seasonal patterns of EROD for those species. The activity of hepatic EROD in juvenile cod was to a large extent similar to that seen for mature individuals; an apparent seasonal variability was somewhere in between male and female, which could mean that even juveniles have sex-specific activity patterns of hepatic EROD through the year. The material available through this study was not sufficient to clarify this issue, although the sex of the juveniles was determined. Other biomarkers in cod were more affected by season than EROD. Hepatic GST varied differently for male and female cod through the year. Common to both sexes was higher activity in individuals from Frierfjord compared with Eidangerfjord in November, a confirmation of earlier observations (Hylland et al. 1997). However, this pattern was reversed in January and it again appeared to be modulated in a sex-dependent manner in April. The data prompt the conclusion that further studies are needed on the seasonality of hepatic GST, and preferably split into GST isoforms (Bhagwat et al. 1998, Hoarau et al. 2004, Perez-Lopez et al. 2002). Other studies have also shown a high degree of individual variability in GST expression (Schrenk et al. 1995), an issue which also merits further study.

There is evidence that migration of Atlantic cod between the two fjord ecosystems is limited. Genetic analysis has documented a differentiation between coastal cod from different fjords, suggesting existence of separate biological populations (Knutsen et al. 2003). The genetic results are supported by earlier tagging experiments conducted with cod in fjords along the Norwegian Skagerrak coast, the results of which indicated that most fish stay close to where they were marked and released (Danielssen & Gjøsæter 1994, Bergstad & Knutsen 2004).

P4501A activity (EROD) was markedly elevated in sea-trout from the two ecosystems compared with control levels found in other studies (e.g. Behrens & Segner 2001) and compared with levels found in Atlantic salmon, *Salmo salar* (Larsen et al. 1992). The high hepatic EROD activities in sea-trout from both areas also indicate that the population is resident in the Grenland fjords, but probably migrate between the two fjords studied here. The limited data available for dioxin levels also suggest that there is exchange of sea-trout between the two areas, in correspondence with the conclusions of Knutsen et al. (2001). Fjord did not come out as a significant

factor in statistical analyses, but there appeared to be trend towards elevated EROD in Frierfjord trout compared with Eidangerfjord trout in two months, April and November, possibly reflecting recent dioxin exposure due to migration (from the open sea or Skien River). The same argument holds for hepatic GST, which was elevated in trout of both sexes and in April and November (no data for January) in Frierfjord sea-trout compared with fish from Eidangerfjord. As for cod, further studies are needed to clarify seasonal variability and the persistence of contaminant induction of hepatic GST in sea-trout. Flounder appeared to be less affected by exposure to dioxins than the other two species, although the median levels for female flounder from both fjords were higher by a factor of two to three compared with data for flounder collected at more or less pristine areas along the Norwegian west coast (Ruus et al. 2003). Hepatic EROD in male flounder was high in both fjords in January (the only month for which there were a sufficient number of samples), but this could be associated with maturation processes. The seasonal variability for female flounder was in correspondence with that observed elsewhere (Rotchell et al. 1999). Hepatic GST appeared to have similar activity in flounder across exposure, seasons and sex, which is somewhat surprizing in view of the large seasonal amplitude in phase I activity and the results reported by Scott et al. (1992), in which environmental contaminants (but not dioxins) were induced or depressed hepatic GST levels in flounder in short-term studies. Leaver et al. (1992) indicated that glucuronidation would be the most important phase II system in flounder, followed by GST, whereas sulphonation would be less important. The results of that study also indicated differential regulation of different GST isoforms, which could at least partly explain the results found here.

There was a clear correlation between hepatic P4501A activity and concentration for juvenile cod, sea-trout and flounder. Although not necessarily evident from correlation plots, there was no evidence for inhibition of activity (which could have been seen as high scatter or subpopulations of samples 'levelling off'). CYP1A concentration generally followed the patterns of CYP1A activity for the three species (results only shown for flounder).

There were indications of increased GR in Atlantic cod (female) and sea-trout (both sexes) in Frierfjord fish compared with fish in Eidangerfjord, but there is an obvious need for a better understanding of sex-dependent, seasonal and adaptational modulation of hepatic GR before drawing conclusions about impacts due to pollution. There is a scarcity of studies concerning seasonal variability of antioxidant parameters and those available concern other species than those studied here. Ronisz et al. (1999) investigated antioxidant responses, including GR, in eelpout (*Zoarces viviparus* L.) sampled twice annually over a number of years, but found little variation.

Seasonal variation of hepatic GR in (female) flounder was different in the two fjords, which may either be a sign of antioxidant stress or of the influence of factors that were not available in this study. Including only a single marker for antioxidant stress may also be insufficient to detect effects since there are well-known species differences in response pathways; as many markers as feasible should be used to elicit such responses (Regoli et al. 2003).

Dioxins are putative endocrine disrupters in fish and the oestrogen-regulated protein vtg was measured in male cod sampled from both ecosystems. The seasonal pattern differed for the two areas and vtg remained elevated in both areas throughout the year, in Frierfjord more so than in Eidangerfjord, compared with data from other coastal areas in Norway (Scott et al. 2005). Inhibition of vitellogenesis following

dioxin exposure has earlier been documented *in vitro* with fish hepatocytes (Rankouhi et al. 2004), but to see such effects there would be a need for oestrogens in the ecosystem. Although there have been some indications of oestrogenic effects in cod from this area earlier (Hylland & Braaten 1996), there are no known sources of substances other than dioxins. Any mechanism for such effect is not known, but further studies would be merited concerning interactions between dioxins and Ah-receptor mediated processes with vitellogenesis in cod.

In addition to the effects reported above, dioxins are known to affect immune responses in fish (Spitsbergen 1986), development (Prasch et al. 2003) and circulation failure (Teraoka et al. 2003). Interestingly, there appeared to be a relationship between CYP1A induction and systemic effects in two studies with zebrafish (*Danio rerio* Walb.) (Prasch et al. 2003, Teraoka et al. 2003). Although there are no such data available for the species used here, such links between CYP1A induction and systemic effects in fish should be considered in a general health assessment of fish in the Grenland fjord system.

The results from the present study indicate that biomarker responses can be used to separate effects in coastal areas with different levels of pollution inputs, but that the following issues need be considered: (1) ideally more than one species should be used, but if not, the selected species should be known to be sensitive to the stressors monitored for; (2) there should be baseline data available for the biomarker in the selected species (in case all stations are impacted); (3) there is a need for a minimum of chemical data to assess exposure, preferably measured in the same material as used for biomarker analyses; and (4) care must be taken in considering the time of year for sampling (which should be outside the periods of gonad maturation and spawning).

Conclusions

In both cod and trout, individuals from Frierfjord generally had a higher activity and level of P4501A than fish from Eidangerfjord, indicating that the two-to-three-times higher dioxin exposure caused increased phase I enzyme activity. Results for phase II enzymes also pointed in the same direction for the same species, but more research is needed to clarify seasonal modulation of the response. The results indicate that fish in the area respond to contaminant exposure (presumably dioxins) and that the differences in exposure between Frierfjord and Eidangerfjord can be seen in the response in some species, i.e. cod and sea-trout, but not in other species, i.e. flounder. Compared with reference levels, hepatic EROD levels were elevated in both fjords in all three species, presumably reflecting the high background of dioxin exposure in the fjord system. Species differences were even more pronounced for antioxidant stress, as determined through GR induction. There were signs of antioxidant stress in sea-trout, whereas results for the other two species were unclear. Salmonids are generally viewed as sensitive compared with other fish. In addition, sea-trout may have probably had a shorter residence time in the Frierfjord and possibly less time to adapt than more stationary species such as cod and flounder. No conclusions could be drawn about the possible impacts on oestrogenic regulation in cod, although there were differences in the seasonal variation of vtg between the two cod populations.

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