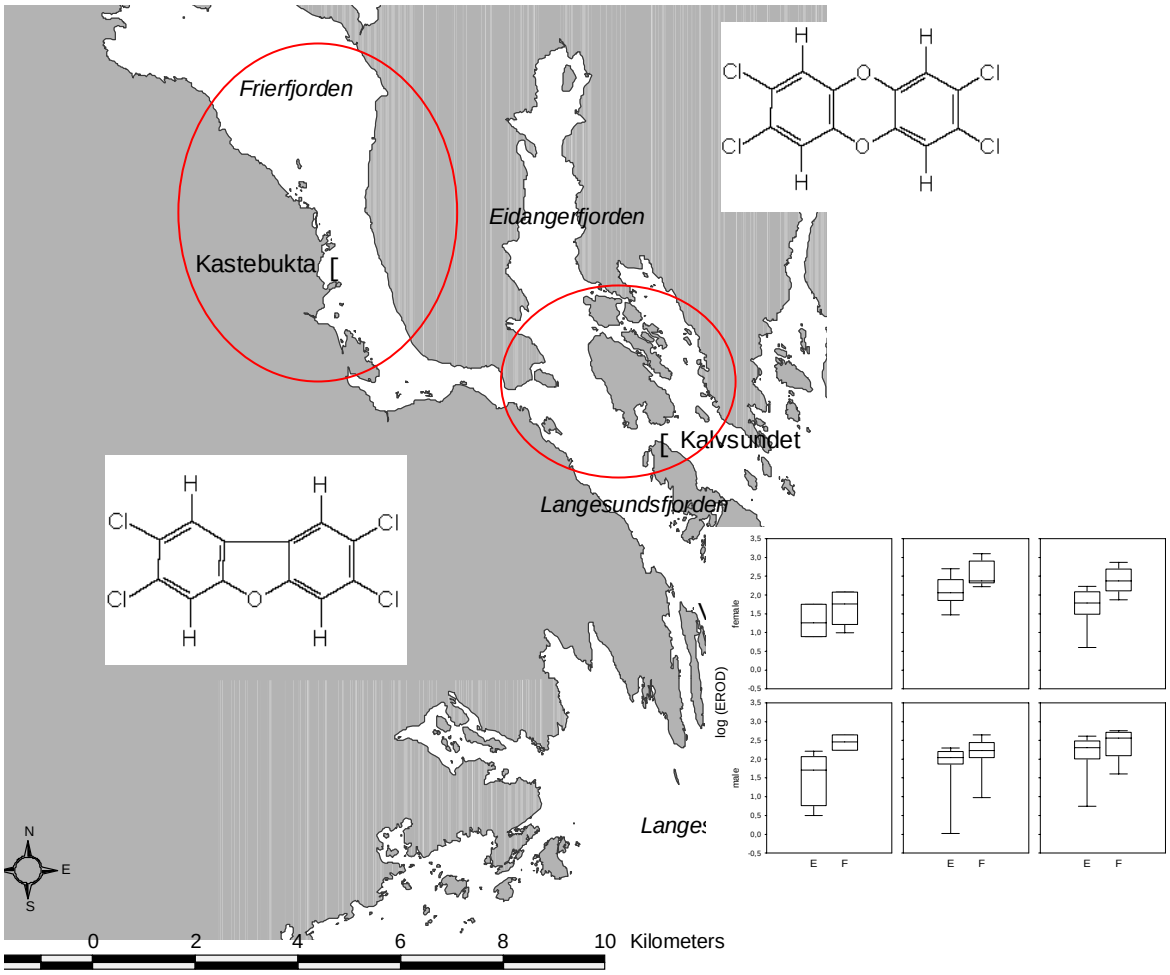
NIVA

REPORT SNO 4857-2004

Biomarker responses in fish from Frierfjord and Eidanger



Norwegian Institute for Water Research
– an institute in the Environmental Research Alliance of Norway

REPORT

Main Office
P.O. Box 173, Kjelsås
N-0411 Oslo, Norway
Phone (47) 22 18 51 00
Telefax (47) 22 18 52 00
Internet: www.niva.no

Regional Office, Sørlandet
Televeien 3
N-4879 Grimstad, Norway
Phone (47) 37 29 50 55
Telefax (47) 37 04 45 13

Regional Office, Østlandet
Sandvikaveien 41
N-2312 Ottestad, Norway
Phone (47) 62 57 64 00
Telefax (47) 62 57 66 53

Regional Office, Vestlandet
Nordnesboder 5
N-5008 Bergen, Norway
Phone (47) 55 30 22 50
Telefax (47) 55 30 22 51

Akvaplan-NIVA A/S
N-9005 Tromsø, Norway
Phone (47) 77 68 52 80
Telefax (47) 77 68 05 09

Title	Serial No.	Date
Biomarker responses in fish from Frierfjord and Eidanger	4857-2004	30.06.2004
	Report No. Sub-No. O-22090 31	Pages Price 43
Author(s) Ketil Hylland Ole Øystein Aspholm Jan Atle Knutsen Anders Ruus	Topic group Ecotoxicology	Distribution Open
	Geographical area Telemark	Printed NIVA

Client(s)	Client ref.
Norwegian Research Council Norsk Hydro	

Abstract

Tthere have been high inputs of dioxins and dibenzofurans into the Frierfjord. The aim of the project reported here was to clarify whether there were observable responses in fish collected from Frierfjord and the adjacent Eidangerfjord that could be associated with exposure to dioxins and dibenzofurans. Three species of fish, Atlantic cod (*Gadus morhua*), trout (*Salmo trutta*) and flounder (*Platichthys flesus*) were sampled during three field campaigns. Other results have shown that the two areas did not differ by more than a factor 2 to 3 in dioxin exposure. In both cod and trout, individuals from Frierfjord generally had higher activity and level of cytochrome P4501A than fish from Eidangerfjord, indicating that the 2-3 times higher dioxin exposure causes increased phase-I enzyme activity. Cod and flounder from both areas had much higher hepatic cytochrome P4501A activity than that seen in reference areas. Differences between the three species were apparent for both phase-II and antioxidant enzyme responses. It can be concluded that dioxin inputs in to the fjord does affect fish in the area and that the effects decreased with decreasing exposure.

4 keywords, Norwegian	4 keywords, English
1. biomarkører	1. biomarkers
2. fisk	2. fish
3. dioksiner	3. dioxins
4. biologiske effekter	4. biological effects

Ketil Hylland
Project manager

Torsten Källqvist
Research manager

Jens Skei
Head of research department

ISBN 82-577-4540-5

Contaminants in the Grenland fjords

Biomarker responses in fish from Frierfjord and Eidanger

Subproject 3 – biological effects

Preface

The current report forms part of a 3-year project on the abiotic transport, bioaccumulation and effects of dioxins and dibenzofurans in Frierfjord and Eidanger. The main project was managed by Kristoffer Næs, NIVA, and was a collaboration between NIVA, IMR (Flødevigen) and NGI. NIVA was in charge of the activity described herein, which was performed in collaboration with IMR (collection of material, herring egg exposure).

Oslo, 5. July, 2004

Ketil Hylland

Contents

Summary

As a result of industrial activities at Herøya, near the cities Porsgrunn and Skien, there have been high inputs of dioxins and dibenzofurans into the Frierfjord. Earlier monitoring programmes have clearly shown elevated concentrations of contaminants from this input as far south along the coast as Kristiansand. The inputs were dramatically decreased in 1990, following which there were expectations that levels would return to normal within a decade. Concentrations remained high in biota in the fjord system. This report addresses results from a sub-project that addressed sublethal effects of dioxins on biota (fish) in the Grenland fjord system. At the conception of this project there were discussions on the selection of a reference area for comparison with Frierfjord, the immediate recipient of the industrial effluents and the area with the highest dioxin/dibenzofuran load. Although it was realised that biota may be affected there as well, the adjacent fjord, Eidanger, was chosen as a reference area. The main reason for that selection was to ensure that food webs would be similar. The aim of the sub-project reported here was thus to clarify whether there were observable responses in fish in the two fjord systems that could be associated with exposure to dioxins and dibenzofurans.

Three species of fish, Atlantic cod (*Gadus morhua*), trout (*Salmo trutta*) and flounder (*Platichthys flesus*) were sampled during three field campaigns in Frierfjord and Eidangerfjord. The results of other projects under publication have shown that the two areas did not differ by more than a factor 2 to 3 in dioxin exposure. The results from the current project have shown that all three fish species have similar condition in the two fjord areas through the year. The results do however indicate that food availability may be lower in Frierfjord in winter for juvenile cod.

In both cod and trout, individuals from Frierfjord generally had higher activity and level of cytochrome P4501A than fish from Eidangerfjord, indicating that the 2-3 times higher dioxin exposure causes increased phase-I enzyme activity. Both cod and flounder had much higher hepatic cytochrome P4501A activity than that observed for fish sampled in reference areas (no reference data was available for trout). The results thus indicate that fish in the area respond to contaminant exposure (presumably dioxins) and that the differences in exposure between Frierfjord and Eidanger can be seen in the response in some species (cod, trout), but not in other (flounder).

Species differences became more obvious in the results for the phase-II enzyme glutathione *S*-transferase (GST) than for cytochrome P4501A. There was clearly elevated activity in the enzyme in Frierfjord trout compared to trout from Eidangerfjord at all times of the year and for both sexes. In cod, the pattern varied somewhat, but there appeared to be some induction in fish sampled in Frierfjord (as found earlier). In flounder there were no differences between fjords, if anything lower activity in Frierfjord individuals compared to Eidangerfjord fish. Despite the result for flounder, the results for the other two species indicates that exposure in the fjord system affects phase-II activity as well as phase-I.

The results found in this project did not give any indication that fish in Frierfjord experienced a higher hepatic free radical exposure than fish in Eidangerfjord. There were also no indications that exposure to Frierfjord water affected herring larvae (early development).

1. Introduction

As a result of industrial activities at Herøya, near the cities Porsgrunn and Skien, there have been high inputs of dioxins and dibenzofurans into the Frierfjord. Earlier monitoring programmes have clearly shown elevated concentrations of contaminants from this input as far south along the coast as Kristiansand (Knutzen et al., 1999). The inputs were dramatically decreased in 1990, following which there were expectations that levels would return to normal within a decade. Concentrations remained high in biota in the fjord system, however, which prompted the initiation of the current research activity. The aim of the main project was to clarify abiotic transport, bioaccumulation and effects of dioxins and dibenzofurans in this fjord system.

At the conception of this project there were discussions on the selection of a reference area for comparison with Frierfjord, the immediate recipient of the industrial effluents and the area with the highest dioxin/dibenzofuran load. Although it was realised that biota may be affected there as well, the adjacent fjord, Eidanger, was chosen as a reference area. 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 comparisions due to very different trophic structures. The trophic webs of the two fjords have been reported elsewhere (Bergstad & Knutsen, 2004a).

The aim of the sub-project reported here was to clarify whether there were observable responses in fish in the two fjord systems that could be associated with exposure to dioxins and dibenzofurans.

Biological effects methods were chosen to clarify possible effects of dioxins or dibenzofurans. Dioxins are known to be strong inducers of phase-1 enzymes, i.e. cytochrome P4501A, in fish (Grinwis et al., 2000; van der Weiden et al., 1990, 1993, 1994). Earlier studies have shown both phase-1 and phase-2 enzymes to be affected in fish from this area (Hylland et al., 1998). Hence, both phase-1 and -2 enzymes were assayed. Both enzymatic activity and concentration were determined for cytochrome P4501A to assess inhibition by other contaminants or environmental factors. Effects of dioxins/dibenzofurans on antioxidant enzymes in fish have not been documented. In the present study, glutathione reductase was included as a marker for changes in the intracellular availability of reduced glutathione. Dioxins are known to be anti-estrogenic in mammalian systems, which prompted the inclusion of the estrogen-marker vitellogenin in the analytical programme. Finally, a small experiment was performed with herring eggs to clarify whether there were sufficient levels of dioxins in the water column to affect embryonal development in fish. Fish embryonal development is known to be highly susceptible to dioxin exposure (Chen & Cooper, 1999; Walker & Peterson, 1992; Walker et al., 1996).

There was a focus on responses in fish. To widen the ecological and comparative scope of the study, three species were included: sea-trout (*Salmo trutta*), Atlantic cod (*Gadus morhua*) and flounder (*Platichthys flesus*). For Atlantic cod, a distinction was made between juvenile and mature fish. It is well established that biological processes in temperate fish species have seasonal cycles (e.g. Hylland et al., 1998). Samples were thus taken for the three species at different times of the year.

2. Interpretation of biological effects

Effects of contaminants may be assessed at different levels of biological organisation – community, population and individual survival, reproduction, growth and health (for an overview see chapters in Lawrence & Hemingway, 2003). There have also been attempts to "convert" tissue levels of contaminants to effects through different types of assessment criteria (OSPAR 1998). The latter approach is scientifically problematic and will not be further treated in this report. The methods used here all belong to the category of methods used to assess the health of individuals, commonly referred to as biomarkers. Biomarkers are more or less contaminant-specific and will generally be found to respond to low levels of contaminant stress and also respond early in a potential detrimental process. On the other hand, the interpretation of biomarker responses is not obvious and has been discussed extensively over the past two decades. A review of various techniques and their possible implementation in risk assessment has been published recently (van der Oost et al., 2003). A description of the methods used here can also be found in that paper.

At this point in time biomarker responses are generally used in holistic assessments including chemistry and environmental data. Such an approach leans heavily on the experience of the scientists making the assessment and is commonly referred to as a "weight of evidence" approach. In addition to the need to combine chemical and biological methods, it is important to be aware that biomarkers should or could only very rarely be used in isolation – sufficient data should be available to enable a holistic assessment of the health of each individual analysed. This assessment needs additional data such as the gender of individuals, their condition and their reproductive status. Such data is needed for a proper analysis of "pure" contaminant-affected biomarkers.

Despite a continuing need for some extent of case-by-case analysis of responses, there is an obvious need for objective assessment criteria. The two main approaches are outlined below.

2.1 Baseline responses

For most biomarkers it is possible to define a range of normal values for any given species. It is however important to be aware that this normal value may differ between sexes, it may differ according to reproductive status, recent food intake and it will probably vary through the year (see example for EROD in flounder, Figure 1). For some methods there are also indications that size and/or age may affect reponses (in addition to the individual being juvenile or mature). The use of baseline or background values therefore requires extensive knowledge of the biomarker in question. In addition, the variability of biomarker responses within an otherwise homogenous group differs due to individual differences and unknown factors.

Within the Norwegian JAMP, preliminary ranges of expected background responses for some biomarkers have been suggested for two of the species studied here, Atlantic cod and flounder (Table 1). As will be apparent, such baseline data can be very important to make a correct assessment of effects, especially in a situation where fish at the reference location are also affected.

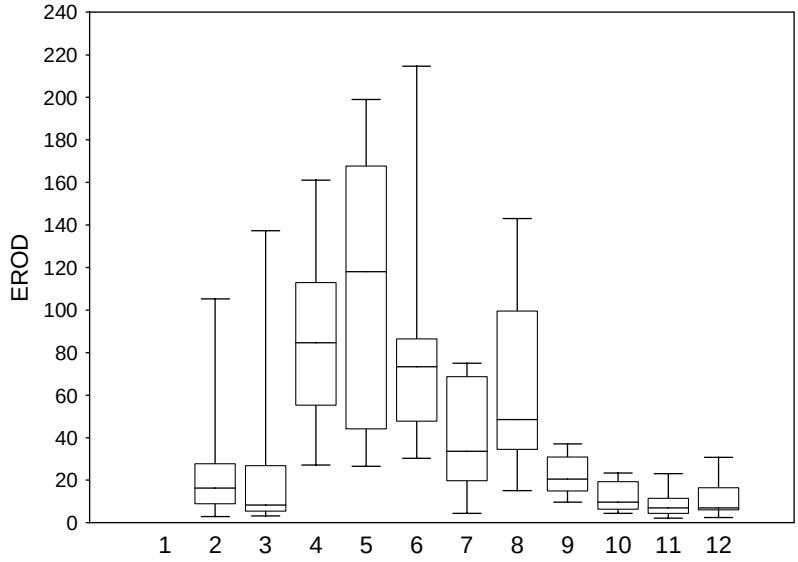


Figure 1. Seasonality in hepatic EROD in female flounder (data from Christensen, 1995).

Table 1. Ranges for selected biomarker responses in Atlantic cod and flounder. Data from the Norwegian JAMP (Ruus et al., 2003).

Species	Method	Count	Background range
Atlantic cod	ALA-D (ng PBG/min/mg protein)	129	15-21
	EROD (pmol/min/mg protein)	139	9-95
	MT (µg/mg protein)	131	6.5-15.8
flounder	ALA-D (ng PBG/min/mg protein)	36	13-21
	EROD (pmol/min/mg protein)	16	10-43
	MT (µg/mg protein)	6	8.4-15.3

2.2 Comparing populations

The "classic" approach to biomarker monitoring is the use of reference populations sampled simultaneously as the exposed population(s). The major requirement is of course that the environment at the different locations is similar except for the contaminant exposure. It is nearly impossible to ensure or test this requirement, but good and appropriate environmental data (hydrography, contaminant levels) are essential.

There are three main problems associated with fish monitoring using exposed locations and a reference location: migration of fish, the length of exposure and adaptation to contaminants. Fish move and information about the exposure history is therefore not immediately available. All available information about a given species in the area(s) in question should be collected and analysed to predict the area integrated over for collection in one or more points (i.e. where would the fish have been expected to move over the relevant period). Exposure may to some extent be estimated through chemical analyses of contaminants in tissues or, better, of metabolites in bile. Unfortunately, migrations or local movement of fish can not be interpreted in the same way for all biomarkers since they have different response times. Generally, two weeks are sufficient to elicit responses for most biomarkers, but some will respond within only one week. Ambient temperature will decrease the time for responses. The third issue concerns adaptation in individuals that remain in contaminated areas for

a long time. Such individuals may then accumulate high levels of persistent contaminants, but may then have biomarker responses at “normal” levels (following an initial increase). There is very limited knowledge about adaptation processes in marine fish species.

The study reported herein included to areas, Frierfjord and Eidanger. It was expected that Frierfjord would have a higher contaminant stress, but it was known before the study was initiated that fish in Eidanger would also be exposed to dioxins/dibenzofurans (Knutzen, 1995). A direct comparison between these two areas would therefore not be between a contaminated and a “clean” area, but between a contaminated (Frierfjord) and a less contaminated area (Eidanger).

2.3 Correcting for other factors

In an ideal world it would be possible to select only one species, of one sex of the same size and condition, at all locations used in a monitoring study. In addition, sampling should be done at the same time and with similar environmental conditions (temperature, salinity). Whereas it is possible to accomplish some, it is never possible to comply with all the above conditions. The most crucial factors are indicated in the OSPAR guidelines for biological effects monitoring (JAMP 1998).

Fixing as many factors as possible prior to sampling is commonly termed “stratified” sampling. Statistical tools, i.e. ANCOVA and multiple regression, should be used to correct for “irrelevant” factors in detecting contaminant-related impacts on organisms.

3. Materials and methods

3.1 Collection and sampling

Fish for analyses of biological responses were collected on three occasions: in April 2000, in November 2000 and in January 2001. Fish were kept alive onboard R/V GM Dannevig until sampling. An overview of fish sampled can be found at Appendix A.

Prior to sampling, each individual was stunned by a blow to the head, weighed and measured.

Blood was sampled from the caudal vein using a heparinised and aprotinised syringe. The sampled blood was then kept on ice until centrifugation and separation of plasma from blood cells. The plasma was then 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.

3.2 Calculation of condition and LSI

Condition of cod and trout was assessed using the formula weight/(length^3)*100 (Le Cren, 1972); 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).

Liver somatic index (LSI) was calculated for all three species as liver wt/body wt*100. Ideally, gonad and stomach weights should be deducted, but these were not available for the fish sampled in this programme.

3.3 Sample treatment

Liver samples were thawed on ice and homogenised in 5 volumes of ice-cold 0.1 M K-phosphate buffer, pH 7.8, with 0.15 M KCl, 5% glycerol and 1 mM DTT. The resulting homogenate was centrifuged at 10 000 x *g* for 30 mins at 4°C, the supernatant (S9 fraction) removed and centrifuged at 50 000 x *g* for 2 hrs. 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 homogenising buffer with 1 mM EDTA and 20% glycerol, divided into three vials and frozen at -80°C.

3.4 Analysis of protein

Protein was analysed in cytosolic and microsomal fractions using Lowry's method (Lowry et al., 1951) adapted for plate-reader. Bovine IgG was used as standard in the assay.

3.5 Analysis of CYP1A protein

The concentration of cytochrome P4501A in the microsomal fraction was determined using a direct ELISA. Microsomal samples were diluted to exactly 10 µg/mL in coating buffer. One hundred µL was then pipetted in quadruplicate into wells of 96-well microtiter plates. Following incubation overnight in a moist chamber, the plates were washed three times in TTBS (Tris-buffered saline, pH 8.6, with 0.05% Tween-20) and blocked for 30 mins with TTBS containing 1% BSA (bovine serum albumin). Following 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 hrs 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 405 nm.

3.6 Analysis of EROD (CYP1A activity)

EROD (7-Ethoxyresorufin-*O*-deethylase) is a specific cytochrome P450 reaction where ethoxyresorufin is used as substrate (Burke & Mayer, 1974). Cytochrome P450 1A catalyse the deethylation of 7-ethoxyresorufin to resorufin. Cytochrome P4501A activity in microsome fractions was quantified from the amount of resorufin produced in a method adapted to platereader. EROD was reported as pmol/min/mg microsomal protein.

3.7 Analysis of GST activity

Total glutathione *S*-transferase (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 plate-reader. Activity was determined kinetically and calculated as mmol/min/mg cytosolic protein.

3.8 Analysis of glutathione reductase

Glutathione reductase 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 µmol/min/mg cytosolic protein.

3.9 Analysis of vitellogenin

Analyses of vitellogenin in mature male cod used a competitive ELISA established at NIVA (described in Scott & Hylland, 2002). Plasma from estradiol-treated cod was used as standard and coating antigen. The concentration of vitellogenin in the standard plasma was determined by HPLC and protein determination. Vitellogenin is stable for years when stored in plasma, but quickly breaks down when purified. Cod vitellogenin is especially sensitive to breakdown during preparation and storage.

3.10 Statistical analyses

Variables were checked for normality and homogeneity of variances prior to tests. Appropriate transforms were used where necessary. One-way and two-way ANOVAs were used to test differences between groups (Sokal & Rohlf, 1981). The level of significance for rejection of H_0: no difference between groups was set at 0.05.

4. General status of fish

4.1 Condition

There were no differences in condition of female cod between the two fjords or between seasons (Figure 2). Male cod from Frierfjord had higher median condition index than cod from Eidanger at all three sampling occasions, but differences were only significant in April (after spawning). It can not be assessed from the available data whether the observed differences simply reflect larger proportions of reproductively active individuals in Frierfjord compared to Eidangerfjord. There were no differences in condition for juvenile cod collected in the two areas (Figure 3).

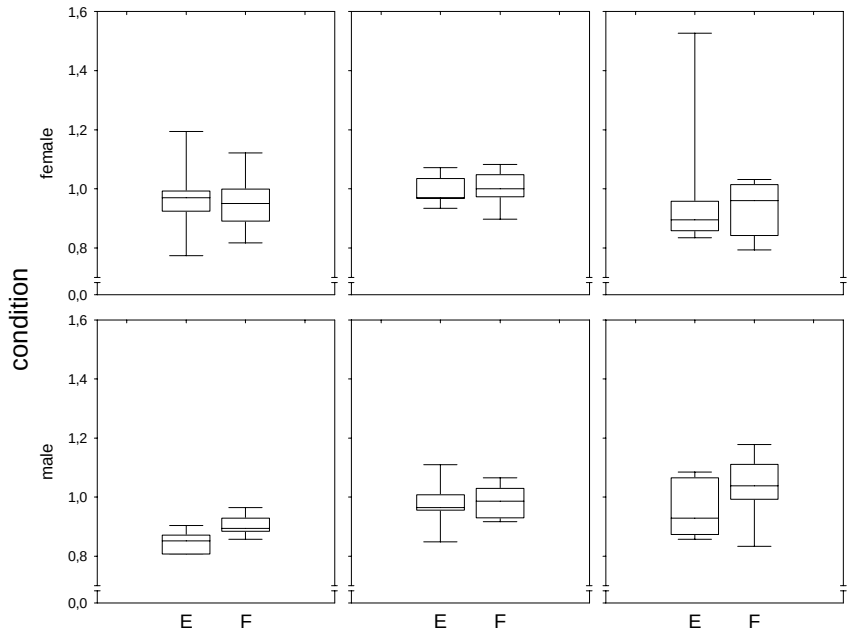


Figure 2. Condition of mature cod collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

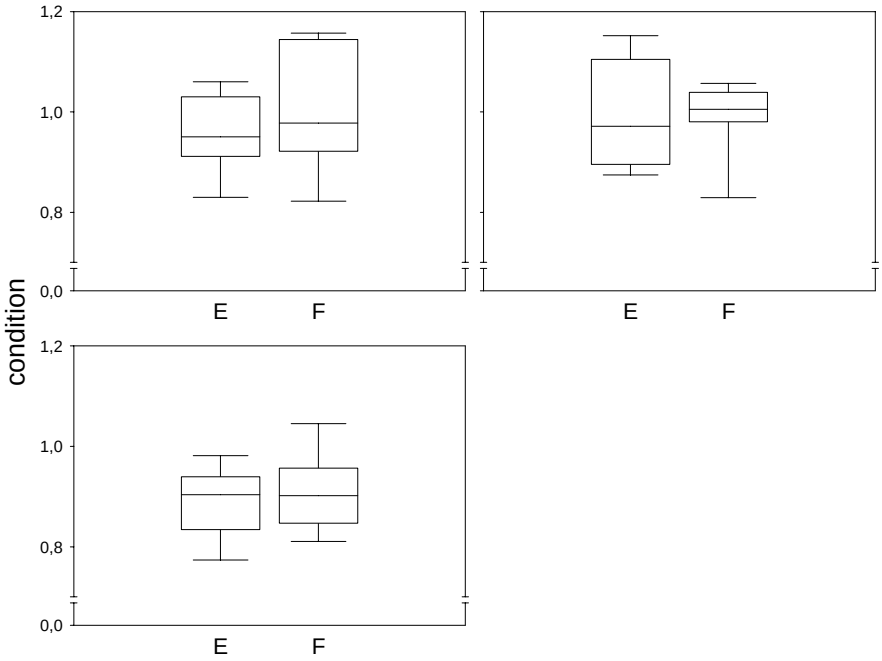


Figure 3. Condition of juvenile cod collected in April 2000 (left panel), November 2000 (right panel) and January 2001 (bottom panel). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

There were no differences in median condition for either sex of trout between the two areas (Figure 4). There is no obvious explanation why some female trout from Eidanger had much higher condition than the other fish, although difference in stomach content (i.e. methodological artefact) is one possibility.

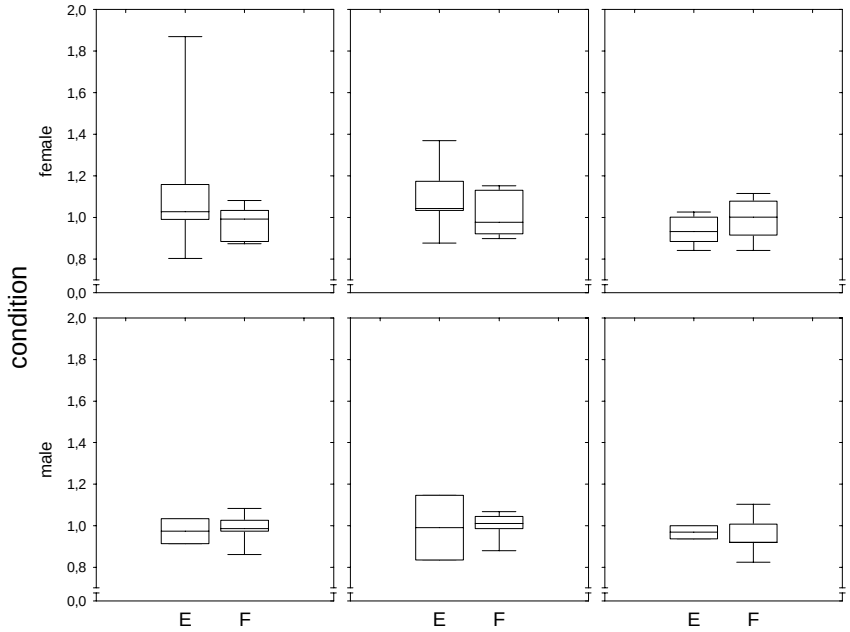


Figure 4. Condition of trout collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

For reasons explained in section 3.2, condition was not calculated for flounder.

4.2 LSI

There were minor differences in liver-somatic index (LSI) in mature cod of either sex (Figure 5). There were indications that cod of both sexes from Frierfjord in January had lower LSI than cod from Eidanger, but the differences were not statistically significant.

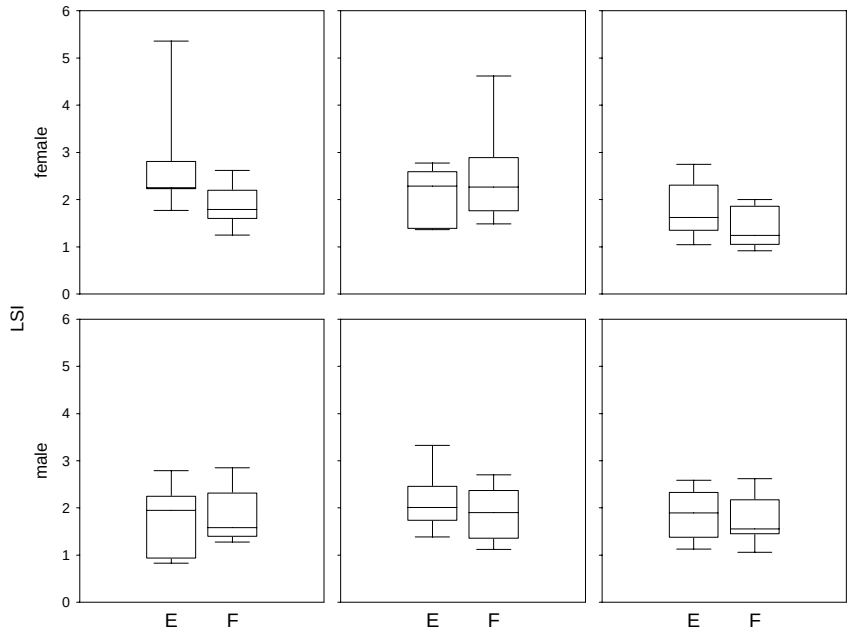


Figure 5. Liver-somatic index (LSI) in mature cod collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

There were obvious differences in LSI for juvenile cod collected in the two areas (Figure 6). Juvenile cod from Frierfjord had higher LSI than juvenile cod from Eidanger in April, whereas the situation was opposite in January. In November there were no differences. LSI 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 food availability is lower in Frierfjord than in Eidanger during winter and than food availability increases for juvenile cod in spring in Frierfjord.

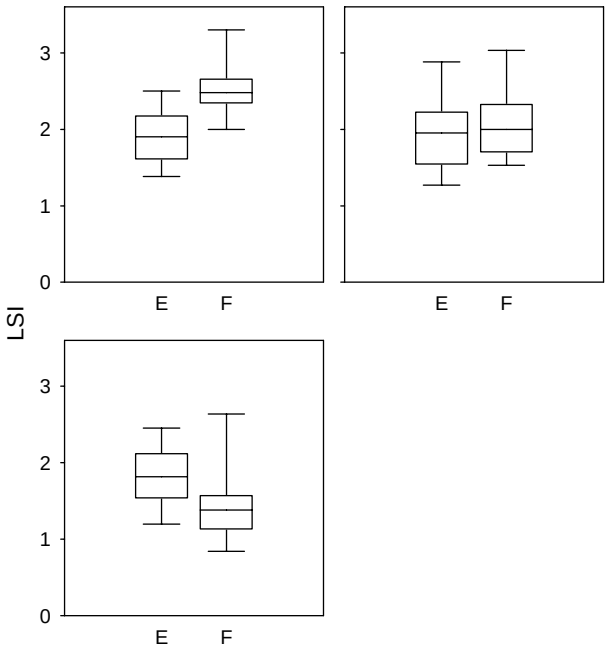


Figure 6. Liver-somatic index (LSI) in juvenile cod collected in April 2000 (left panel), November 2000 (right panel) and January 2001 (bottom panel). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

There were no obvious patterns in LSI for male or female trout collected in the two areas (Figure 7). Median LSI was lower in both sexes of Frierfjord trout compared to Eidanger trout in November (following spawning), but the differences were not statistically significant.

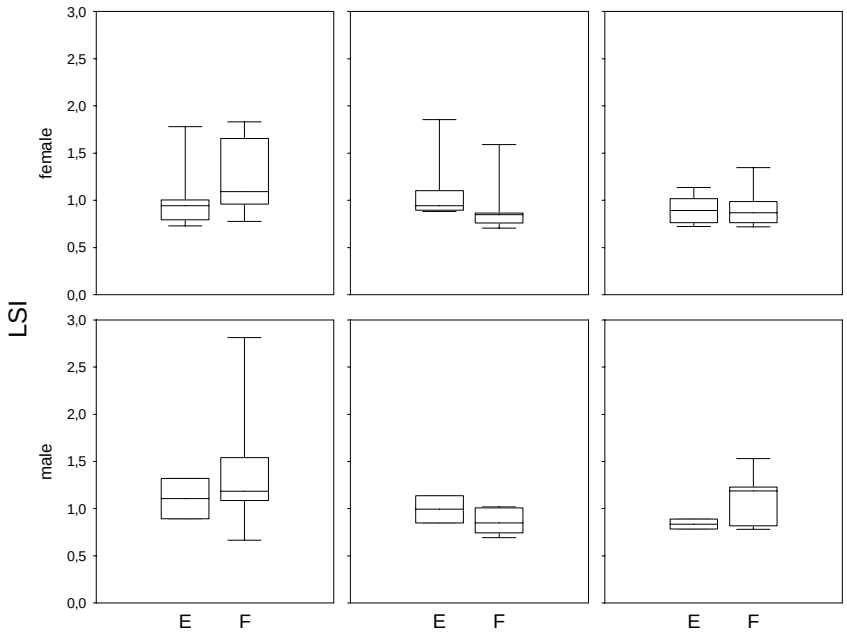


Figure 7. Liver-somatic index (LSI) in trout collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

There were no obvious differences in LSI for flounder collected in the two areas (Figure 8). Due to skewed distributions between the sexes, data was limited for some months and locations. This may be due to spawning migrations (to deeper waters).

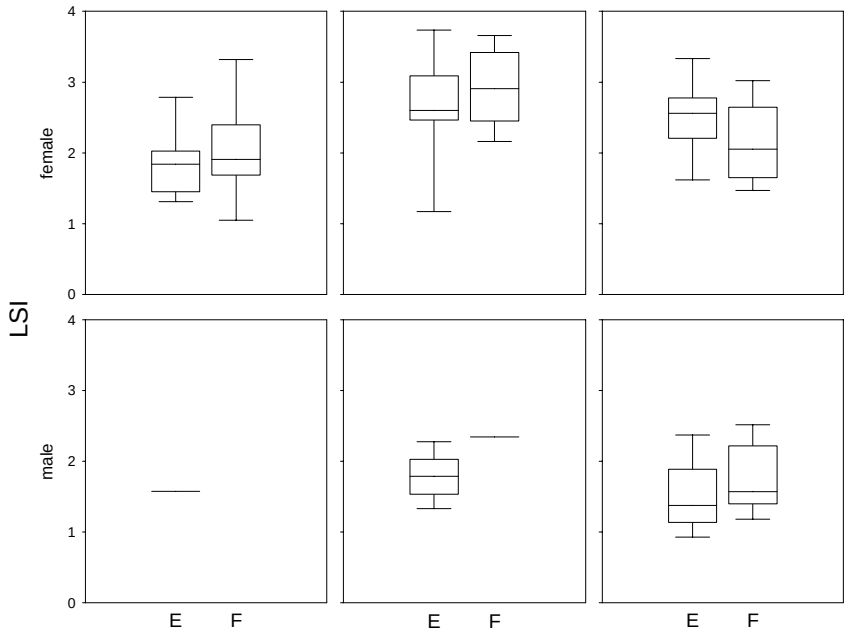


Figure 8. Liver-somatic index (LSI) in flounder collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

5. Phase-I responses

Phase-I activity is the initial metabolisation of hydrophobic substances. The main component of phase-I metabolism is the cytochrome P450 enzyme system. Planar hydrophobic organic contaminants such as some PAHs, planar PCBs, dioxins and dibenzofurans are both metabolised by and induce the synthesis (and hence activity of) one member of the cytochrome P450 family of enzymes, cytochrome P4501A (CYP1A). The enzymatic activity of this enzyme is most commonly determined as deethylation of ethoxyresorufin, referred to as EROD. In addition to activity, the amount of CYP1A protein and mRNA can be determined. CYP1A protein is useful because it can indicate whether the activity of the enzyme is inhibited (the enzyme is present, but does not catalyse the reaction).

5.1 CYP1A protein

CYP1A protein was determined in selected samples and species. The results for small cod indicated consistently higher levels of the protein in fish from Frierfjord compared to fish from Eidanger (Figure 9). This result fits well with results for EROD (see below) and shows that the exposure to contaminants in the two fjord systems to juvenile cod is sufficiently different to elicit different responses.

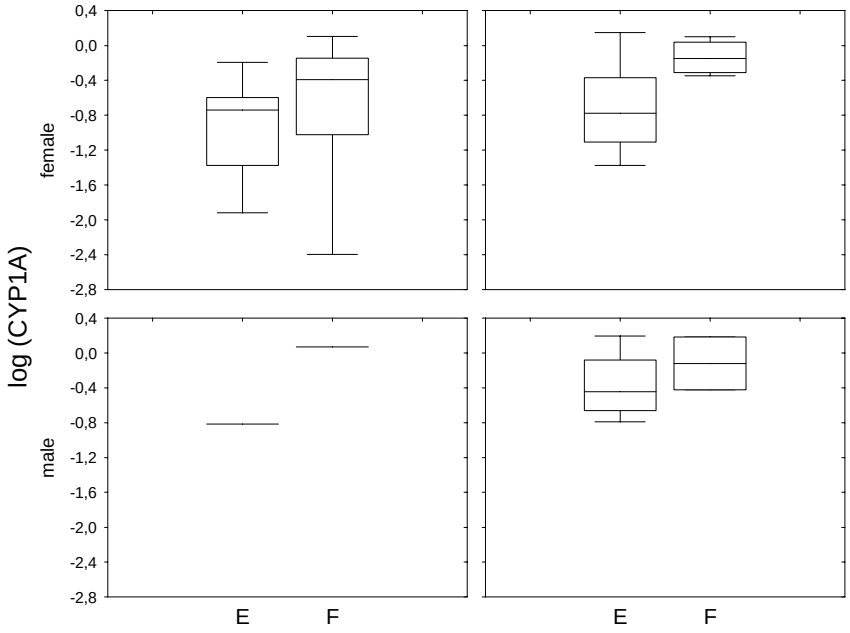


Figure 9. CYP1A protein in small cod collected in November 2000 (left panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

Results for trout indicated higher levels of hepatic CYP1A in trout from Frierfjord compared to trout from Eidanger in November, whereas there were no obvious differences for trout collected in January (Figure 10). The differences were very obvious for males and less so for females.

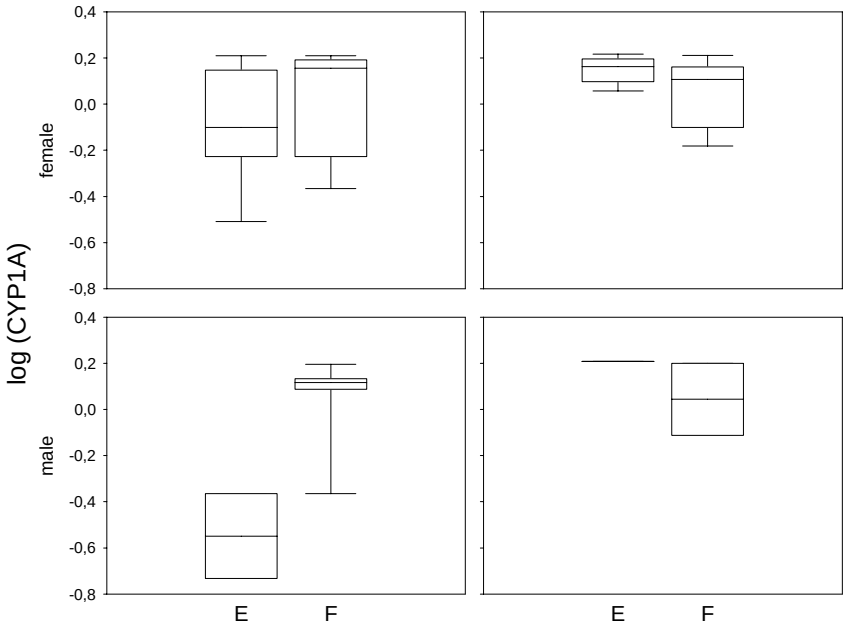


Figure 10. Hepatic CYP1A concentration in trout collected in November 2000 (left panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

Median hepatic concentrations of CYP1A were consistently higher in flounder collected in Frierfjord compared to flounder collected in Eidanger, for both sex and time of year (November, January) (Figure 11). An ANCOVA indicated that there were also differences between the two sexes, but the low number of observations for males at some points in time precludes generalisations.

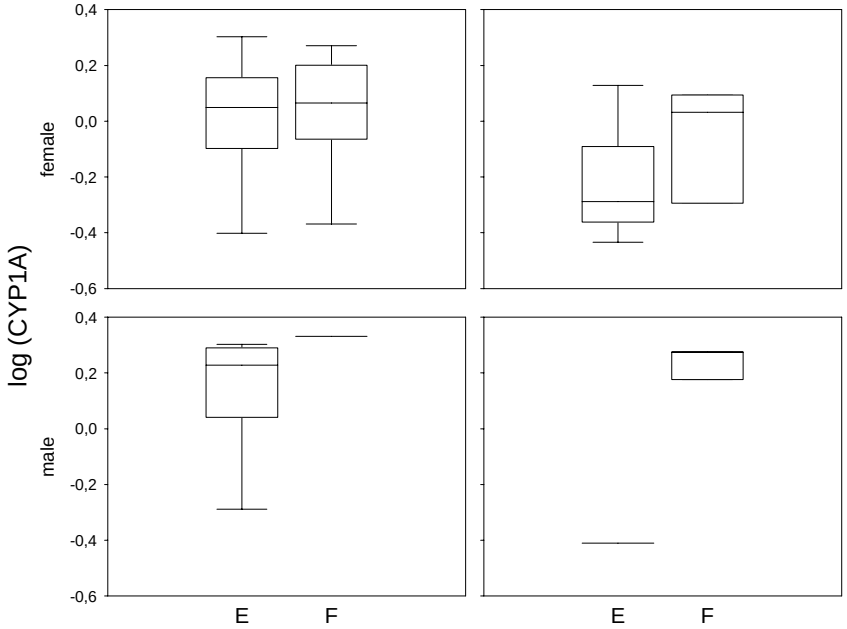


Figure 11. Hepatic CYP1A concentration in flounder collected in November 2000 (left panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

5.2 EROD activity

Cytochrome P4501A activity has been widely used as a biomarker in fish over the past decades (Goksøyr & Förlin, 1992) and there has ben an increasing measurement of the concentration in addition to activity (see e.g. van der Oost et al., 2003). There was consistently higher activity of CYP1A (EROD) in mature cod from Frierfjord compared to Eidanger (Figure 12). In addition to indicating a difference in exposure between the two fjord areas, the results also show that the levels are generally elevated in this region compared to other coastal areas in Norway. There is surprisingly little difference in levels between different periods, which shows that EROD can be used in mature cod sampled both during the autumn and the winter. Activity is somewhat lower in fish sampled in April than during the other two periods, which is to be expected since this is just after their period of spawning.

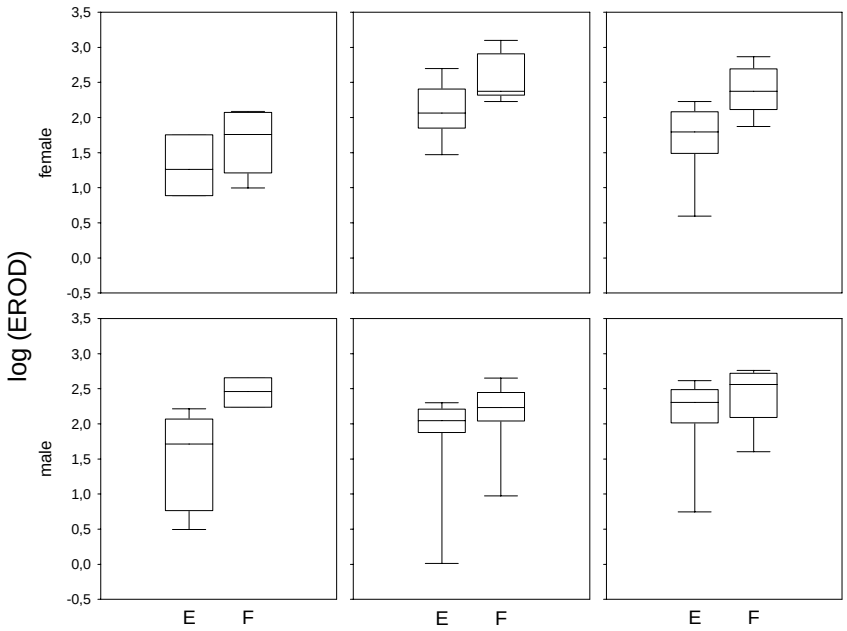


Figure 12. Hepatic CYP1A activity (EROD) in mature cod collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

Results for juvenile cod were similar to those found for mature cod and also harmonised well with results for CYP1A protein reported above (Figure 13). There were few observations at some points in time for male juvenile cod, which precludes further discussion of the apparent differences seen in January data for males. As for mature cod, the results show a consistently higher activity in fish from Frierfjord compared to fish from Eidanger.

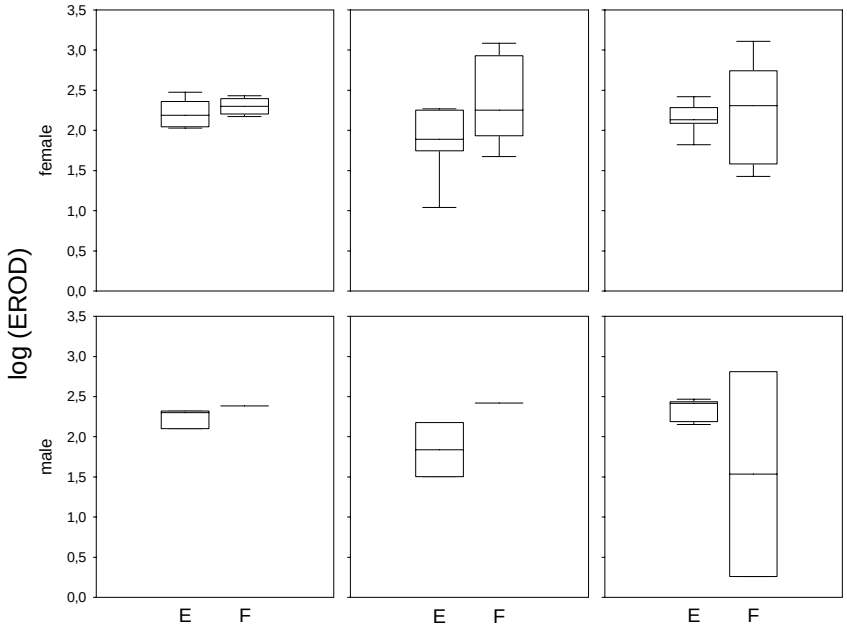


Figure 13. Hepatic CYP1A activity (EROD) in juvenile cod collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

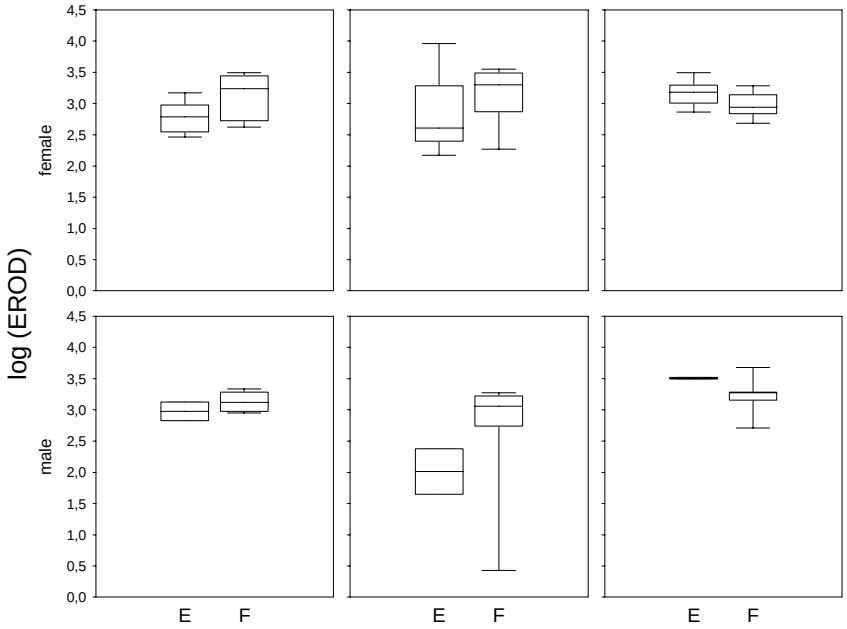


Figure 14. Hepatic CYP1A activity (EROD) in trout collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

Contrary to expectations, seasonal variability was much clearer in male than in female trout (Figure 15). There is no obvious reason for this observation. There were very clear seasonal differences in hepatic EROD activity in flounder (Figure 16). As for other fish, there was a trend towards increasing

EROD activity in flounder collected in Frierfjord compared to flounder collected in Eidanger. This difference was apparent for female flounder collected at all three times of the year.

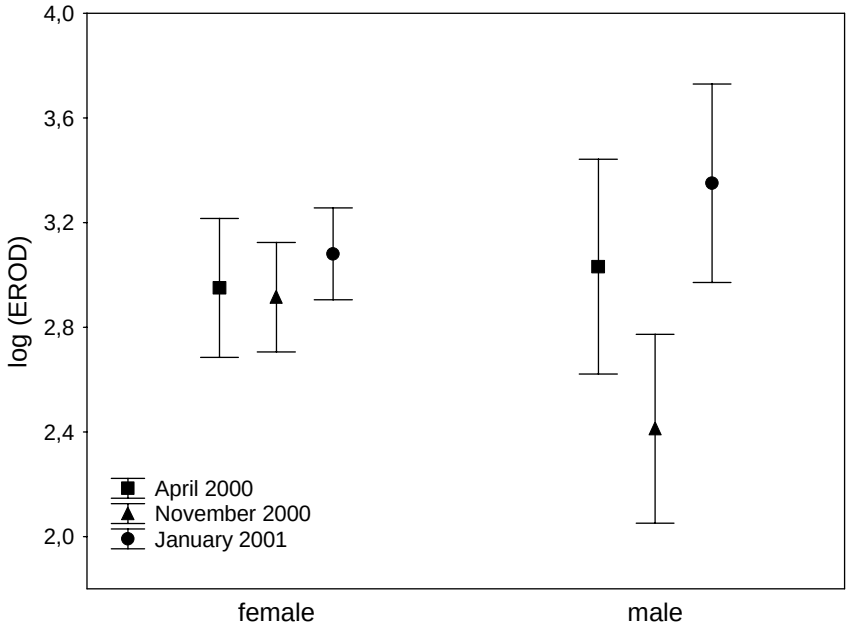


Figure 15. Interaction between sex and time of year in hepatic EROD activity in trout (from the ANOVA analysis). Values are means and 95% confidence intervals.

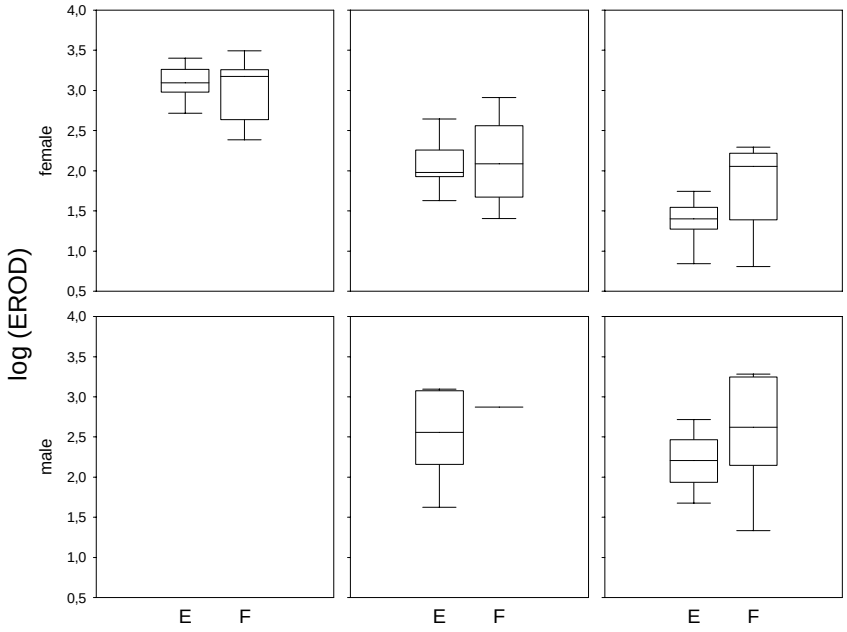


Figure 16. Hepatic CYP1A activity (EROD) in flounder collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

5.3 Relationships between concentration and activity of CYP1A

As mentioned above, cytochrome P4501A is a pivotal enzyme in the metabolism of planar organic contaminants. Under some conditions the activity of the enzyme, generally determined as EROD, may be inhibited, e.g. by organotins. To clarify whether this is the case it is useful to compare the total concentration of the enzyme in addition to determine its activity.

In the current study, such inhibition is presumably more relevant for fish in Frierfjord than for fish in Eidangerfjord due to the more complex exposure in the former area. The results for small cod do not indicate strong inhibition, although activity does appear to level off at high levels of CYP1A (Figure 17). The effect was however more pronounced for cod from Eidanger than for cod from Frierfjord.

There was no evidence for inhibition of cytochrome P4501A activity in trout (Figure 18). Most of the outliers were fish from Frierfjord, but this may not necessarily indicate specific effects (as this was not seen for the other two species).

The relationship between concentration and activity was even clearer for flounder than for the other two species (Figure 19). There was no evidence of inhibition.

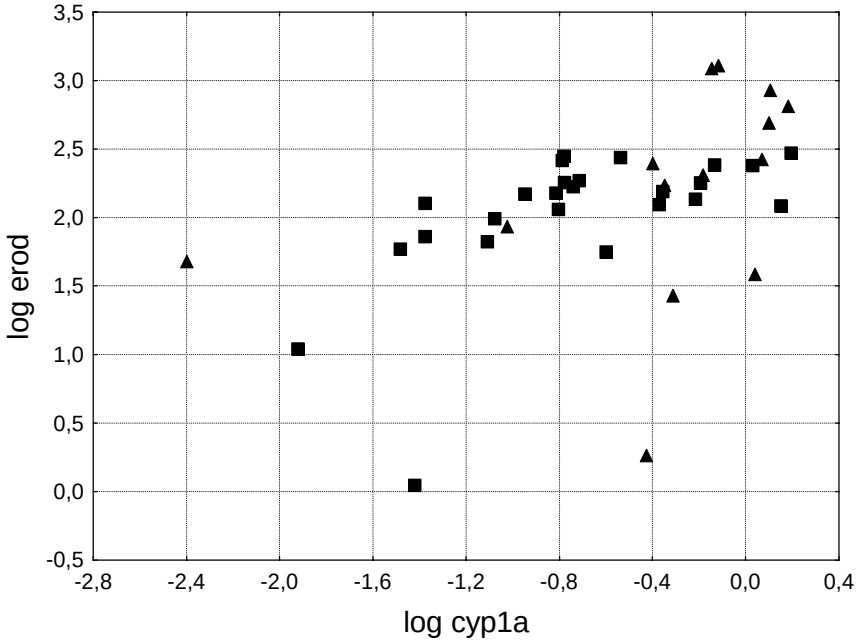


Figure 17. Relationship between cytochrome P4501A concentration (X-axis) and activity (EROD; Y-axis) for small cod. Triangles are from Frierfjord and squares from Eidangerfjord.

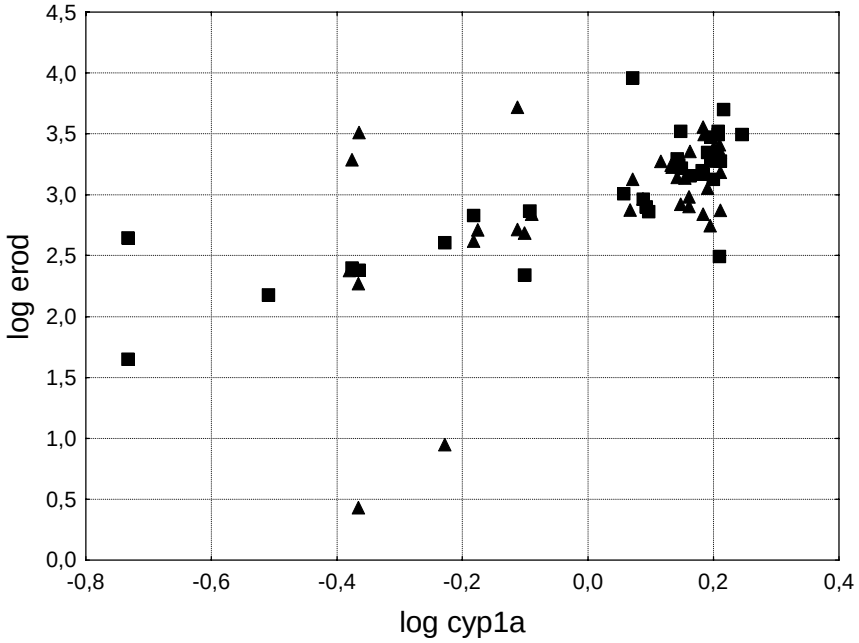


Figure 18. Relationship between cytochrome P4501A concentration (X-axis) and activity (EROD; Y-axis) for trout. Triangles are from Frierfjord and squares from Eidangerfjord.

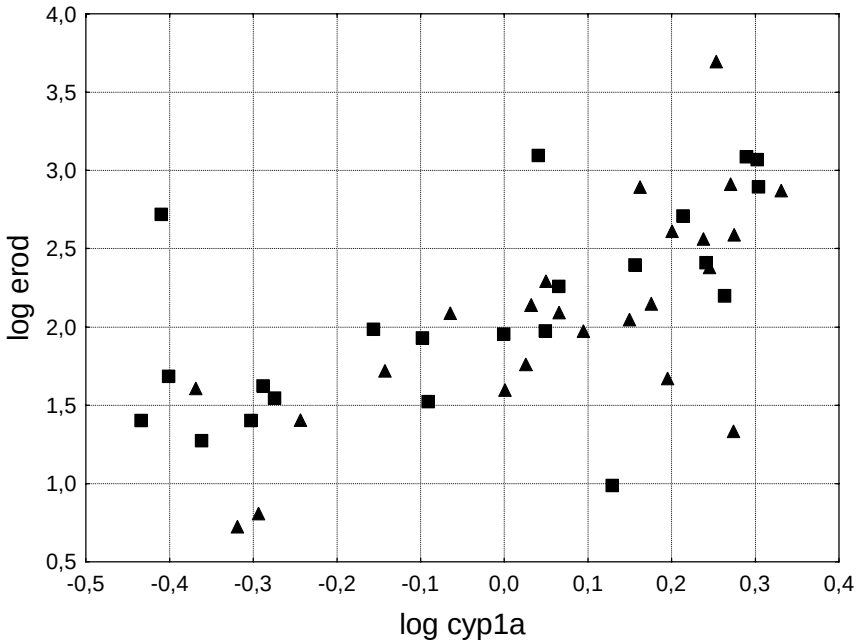


Figure 19. Relationship between cytochrome P4501A concentration (X-axis) and activity (EROD; Y-axis) for flounder. Triangles are from Frierfjord and squares from Eidangerfjord.

6. Phase-II responses

Following activation of hydrophobic contaminants through phase-I metabolism, phase-II enzymes may attach a ligand to the substance to make it more water-soluble and thus more easily excreted through the bile. There are three main phase-II systems in vertebrates: glutathione *S*-transferase (GST), UDP-glucuronyl transferase (UDP-GT) and sulphotransferases (ST). Although all three activities are found in fish, there are indications that of the three, GST is most strongly affected by contaminant stress. GST is a family of enzymes with both distinct and overlapping substrate specificities. In the current study, a substrate was used that will include all main GSTs. There is high constitutive activity of GST in liver cells, but it has been shown to increase under some types of contaminant stress (van der Oost et al., 2003).

Hepatic GST activity in mature cod showed an intriguing relationship. For both males and females there were lower activities in individuals from Frierfjord compared to fish from Eidanger in April, whereas the situation was reversed in November (Figure 20). Earlier studies with cod collected in October also showed significantly elevated GST in cod from Frierfjord compared to fish from other areas.

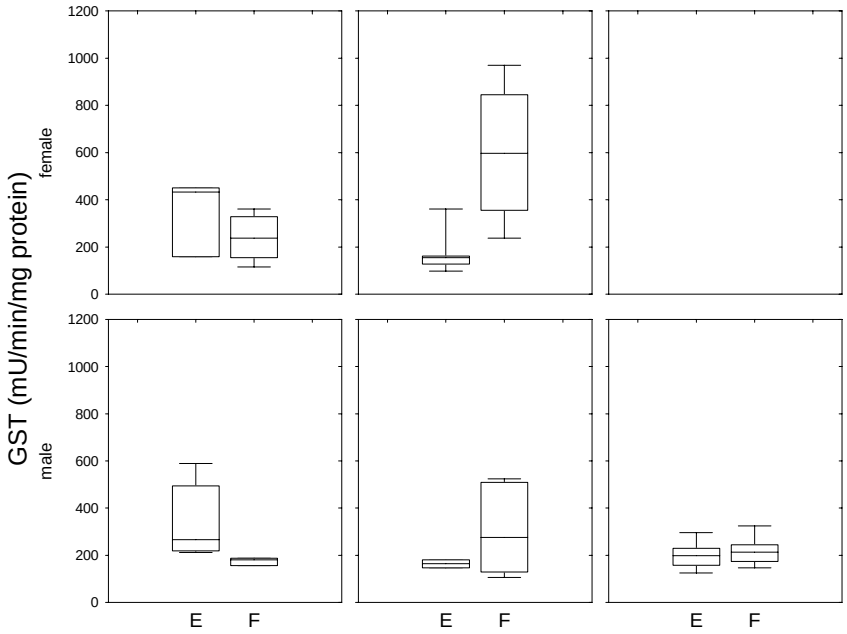


Figure 20. Hepatic glutathione S-transferase activity (GST) in mature cod collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

Hepatic GST in trout from Frierfjord was consistently higher than GST in trout from Eidanger, both in April and in November and for both male and female fish (Figure 21; Figure 22). Although the difference was observed in both sexes, male trout responded most clearly. It is likely that the observed differences are responses to elevated contaminant levels in Frierfjord compared to Eidangerfjord. Similar responses were found for cod in this area in an earlier study (Hylland et al., 1996). There are nevertheless also a range of studies in which hepatic GST in fish does not discriminate between contaminated and clean areas (see van der Oost et al. (2003) for an overview). It is not known if dioxins are particularly efficient inducers of hepatic GST in fish, but the question merits further study.

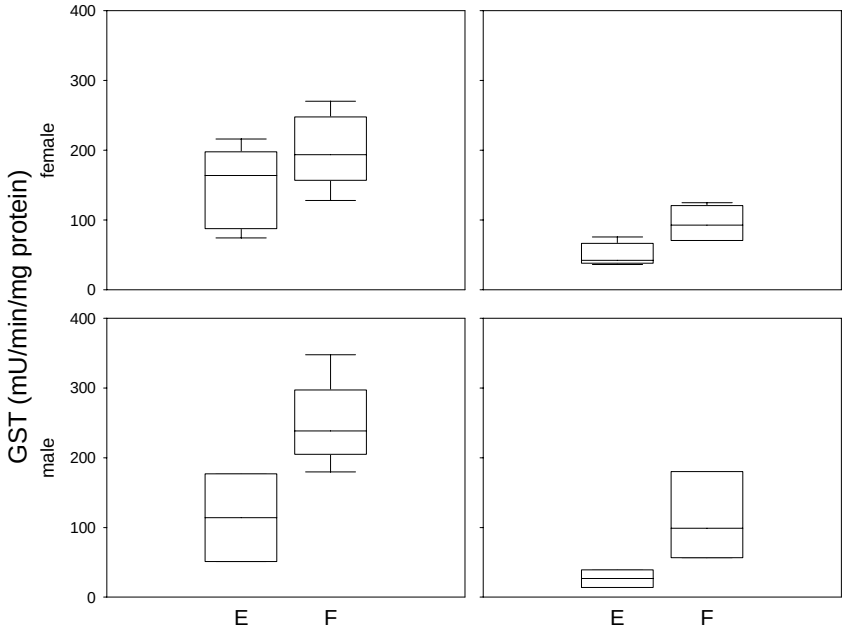


Figure 21. Hepatic glutathione S-transferase activity (GST) in trout collected in April 2000 (left panels) and November 2000 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

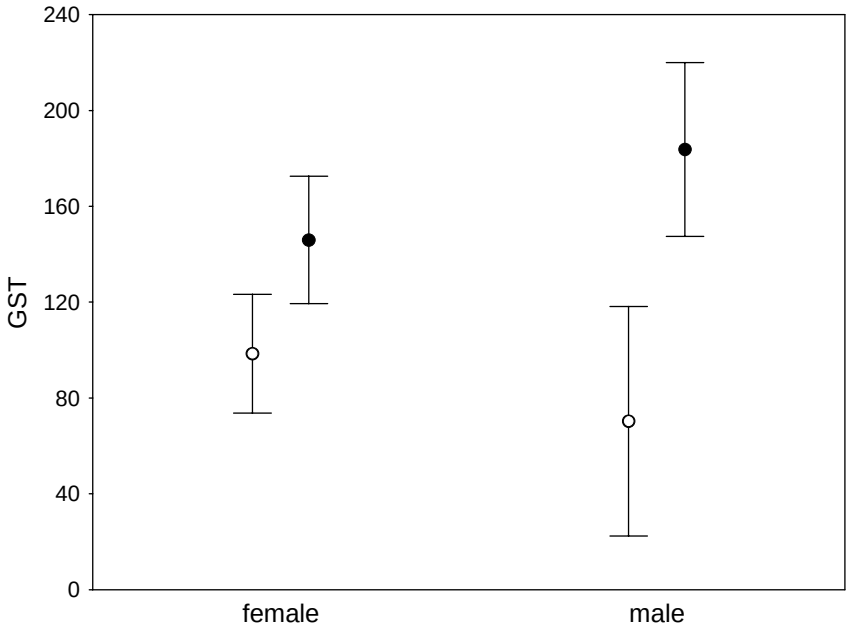


Figure 22. Interactions between sex and fjord in an ANOVA model for hepatic glutathione *S*-transferase in trout; p = 0.065. Closed circles - Frierfjord; open circles - Eidanger. Vertical bars denote 95% confidence intervals.

Contrary to trout, there were no clear patterns in hepatic GST for flounder collected in the two fjords at three times of the year (Figure 23). If anything, GST was lower in flounder from Frierfjord than in flounder from Eidangerfjord (males in January and females in October)

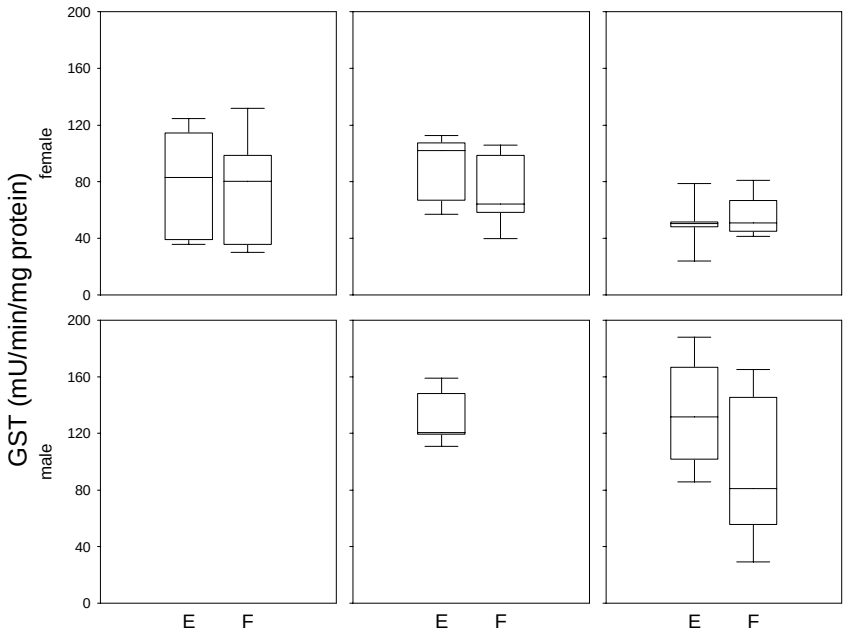


Figure 23. Hepatic glutathione S-transferase activity (GST) in flounder collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

7. Glutathione reductase

Contaminants have different modes of toxic action. The methods described above quantify responses to specific contaminants or contaminant group. In addition some contaminants will cause the generation of free radicals in cells. Cells have mechanisms to protect against damage from free radicals, both enzymes and antioxidants. Glutathione is one of the most important antioxidants in cells. One mechanism for protection is oxidation of glutathione. The main enzyme that catalyses the "regeneration" or reduction of glutatione is glutathione reductase (GR). Glutathione reductase may be induced by free radical stress. It is however important to be aware that this enzyme is only one component in the cellular defense against free radicals. The importance of different components varies for different species.

In mature cod there were no clear seasonal differences, but there was a small significant ($p = 0.02$) difference between fish collected in the two fjords (Figure 24). Cod from Frierfjord had lower hepatic GR activity than cod from Eidangerfjord. With the small differences it is not possible to draw conclusions, although in general an increase in GR would be expected under conditions of elevated cellular levels of free radicals (consumption of reduced glutathione).

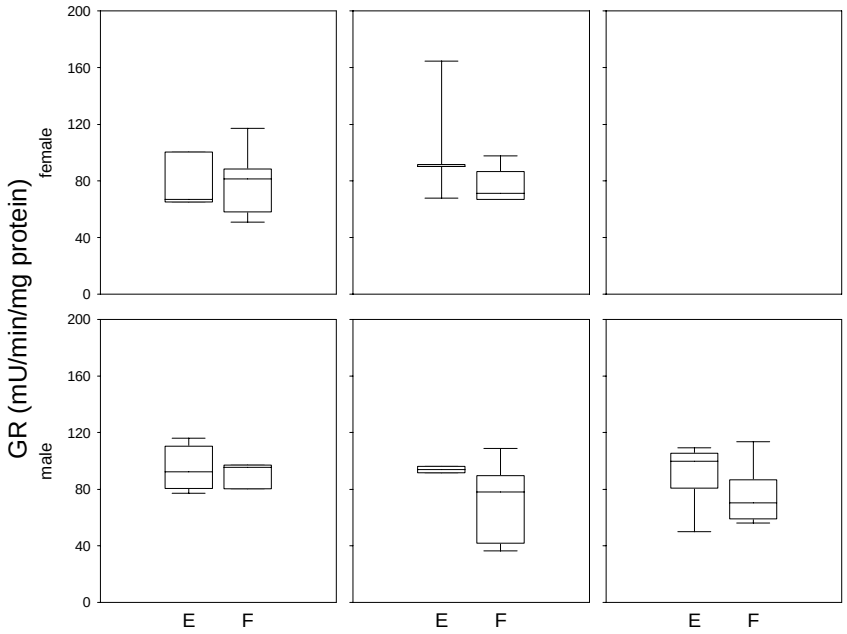


Figure 24. Glutathione reductase (GR) in mature cod collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

The median hepatic GR was higher in trout from Frierfjord than in trout from Eidangerfjord (Figure 25). This applied to both sexes at both sampling points. The apparent differences did not come out as significant in a three-way ANOVA with date and sex as additional explanatory variables. The results for GST and GR point in the same direction, however, in that trout do appear to be affected by conditions in Frierfjord.

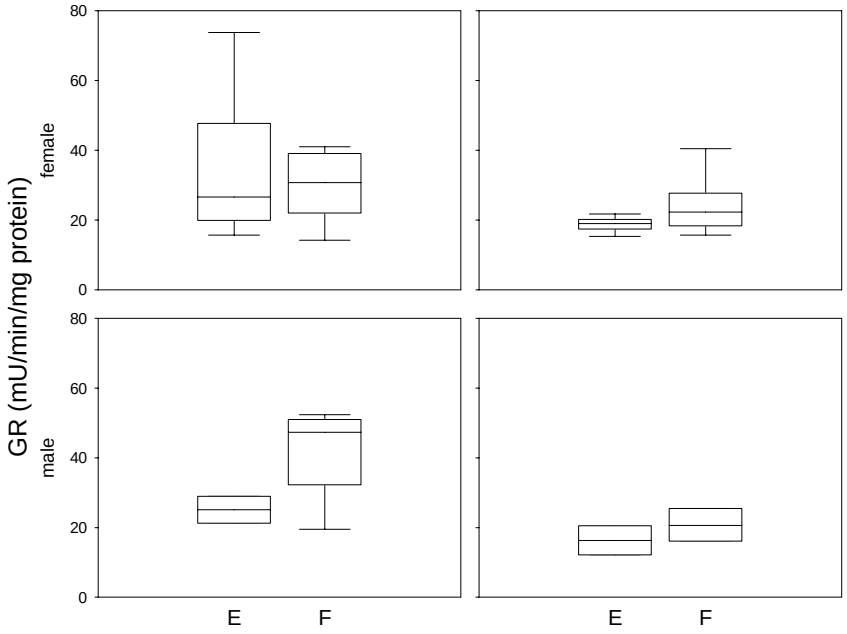


Figure 25. Glutathione reductase (GR) in trout collected in April 2000 (left panels) and November 2000 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

As for GST there was no obvious pattern in hepatic GR activity in flounder, be it male or female (Figure 26). The results indicate that GR activity in this species is affected by both endogenous and exogenous factors.

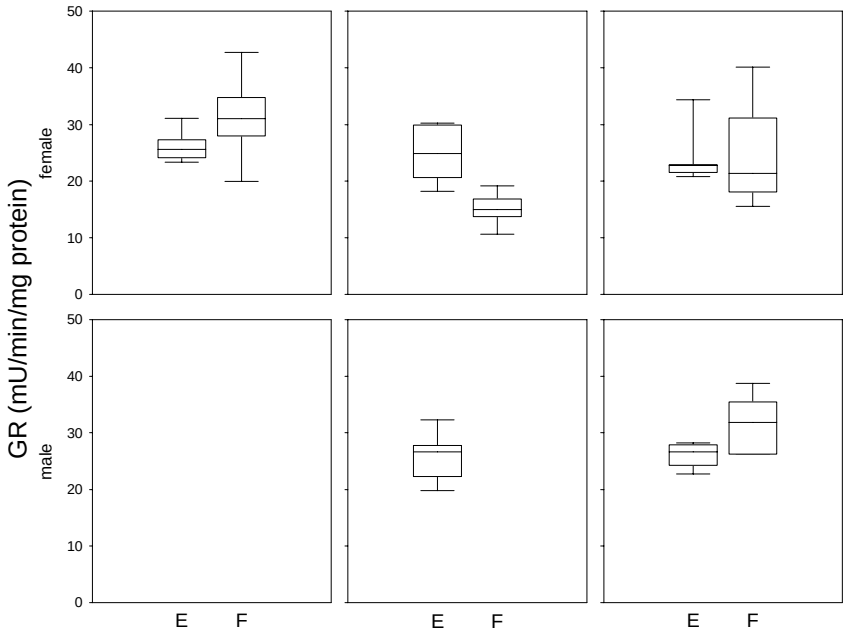


Figure 26. Glutathione reductase (GR) in flounder collected in April 2000 (left panels), November 2000 (middle panels) and January 2001 (right panels). Figures show median, quartiles and 10/90 percentiles. E-Eidanger, F-Frierfjord.

8. Vitellogenin

Selected dioxins have been indicated to be anti-estrogens in some model systems. Plasma samples of mature male cod were therefor analysed for levels of vitellogenin, a yolk-precursor protein, the synthesis of which is regulated by estradiol. Male cod should have low plasma levels of vitellogenin under normal conditions.

The results indicate a difference between the fjords; male cod in Frierfjord appear to have higher levels of plasma vitellogenin most of the year compared to male cod from Eidanger (Figure 27). Exogenous substances can interact with the endocrine system through many mechanisms, including direct binding to receptors, binding to transport proteins and interference with feedback mechanisms. The conclusion to be drawn from the current results is that there appear to be a minor difference in the regulation of vitellogenin synthesis in cod from the two fjords, which may be derived from contaminant exposure.

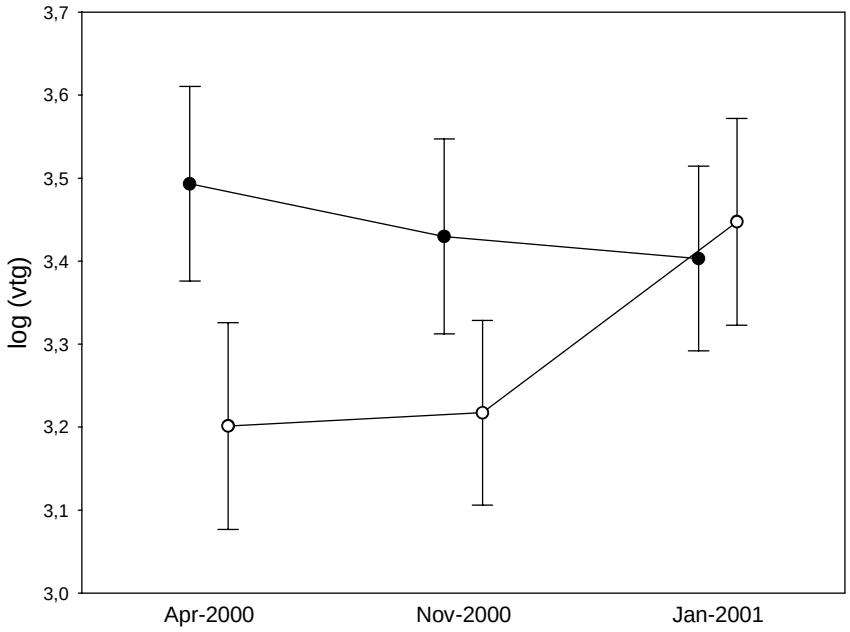


Figure 27. Interactions between date and fjord in an ANOVA model (with date and fjord as explaining variables) for plasma vitellogenin in mature male cod (ng/mL plasma). Closed circles - Frierfjord; open circles - Eidanger. Vertical bars denote 95% confidence intervals; F(2, 48)=4,4262, p=,01721.

9. Herring larvae

Some of the most sensitive organisms or stages in aquatic systems to dioxins are fish larvae (e.g. Walker et al., 1996). Unsuccessful attempts were made to collect wild fish larvae in the two fjord areas as part of the present project. As a substitute, fertilised herring eggs were allowed to adher to glass plates and exposed in both fjord systems. Following exposure, they were transported back to IMR's research station in Flødevigen and allowed to hatch. Hatching was successful, but neither hatching success nor embryonal development appeared to be different between the two fjords. The levels of aberrations were similar to that seen in a control group held at Flødevigen during the exposure period. A more detailed description of this project can be found in Bergstad & Knutsen (2004b).

10. Conclusions

Three species of fish, Atlantic cod (Gadus morhua), trout (Salmo trutta) and flounder (Platichthys flesus) were sampled during three field campaigns in two areas in the Grenland fjords – Frierfjord, closest to the source of previous and possibly present dioxin inputs, and Eidangerfjord, more distant from inputs, but with some contaminant exposure. The choice of the two areas was mediated by a wish to find two systems with similar trophic structures, 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 to 3 in dioxin exposure (Berge et al., 2004).

The current project has shown that all three fish species appear to have similar condition in the two fjord areas through they year. The results do however indicate that food availability may be lower in Frierfjord in winter for juvenile cod.

In both cod and trout, individuals from Frierfjord generally had higher activity and level of cytochrome P4501A than fish from Eidangerfjord, indicating that the 2-3 times higher dioxin exposure causes increased phase-I enzyme activity. Both cod and flounder had much higher hepatic cytochrome P4501A activity than that observed for fish sampled in reference areas (no reference data is available for trout). The seasonal pattern was in concordance with observations elsewhere (for flounder). The results thus indicate that fish in the area respond to contaminant exposure (presumably dioxins) and that the differences in exposure between Frierfjord and Eidanger can be seen in the response in some species (cod, trout), but not in other (flounder). The project has also provided some intriguing data on CYP1A variability in male trout (and lack of variability in female trout) that would merit further study.

Species differences became more obvious in the results for the phase-II enzyme glutathione *S*-transferase (GST) than for cytochrome P4501A. There was clearly elevated activity in the enzyme in Frierfjord trout compared to trout from Eidangerfjord at all times of the year and for both sexes. In cod, the pattern varied somewhat, but there appeared to be some induction in fish sampled in Frierfjord (as found earlier). In flounder there were no differences between fjords, if anything lower activity in Frierfjord individuals compared to Eidangerfjord fish. Despite the result for flounder, the results for the other two species indicate that exposure in the fjord system affects phase-II activity as well as phase-I.

Little is known about seasonal variability in the enzyme glutathione reductase (GR), so the current project also contributed some basic background knowledge about this enzyme. Glutathione reductase is one of the enzymes that will protect cells against free radicals, such as may be found in areas with contaminant exposure (but also in association with variations in oxygen availability). The results found in this project did not give any indication that fish in Frierfjord experienced a higher hepatic free radical exposure than fish in Eidangerfjord.

Dioxins are thought to be endocrine disrupters and the estrogen-regulated protein vitellogenin was measured in male cod in both fjords. The seasonal pattern indicated that there may be conditions that will affect sex steroid regulated processes in cod in Frierfjord, but the available data does not allow further conclusions.

11. References

Berge, J.A., Aspholm, O., Bergstad, O.A., Hylland, K. Knutsen J.A., Ruus, A., Schlabach, M. 2004. Fisk og byttedyr fra Grenlandsfjordene - datarapport for analyse av fett, PCDF/D, n. o. PCB, PCN og stabile isotoper av nitrogen og karbon ($\delta^{13}C$, $\delta^{15}N$). NIVA-report 4795, 235 p. [in Norwegian]

Bergstad, O. A og Knutsen, J.A. 2004a. Marin makro- og megafauna i Grenlandsfjordene og næringsvalg til torsk, skrubbe, sjøørret og pelagisk fisk. Fisken og Havet, in press [in Norwegian]

Bergstad, O.A. og Knutsen, J.A. 2004b. Frekvens av embryonal feilutvikling hos sild (*Clupea harengus*) fra en dioksinforurenset fjord (Frierfjorden, Telemark). Fisken og Havet, in press [in Norwegian]

Burke, M.D., Mayer, R.T. 1974. Ethoxyresorufin: direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug metabol. Disp. 2: 583-588.

Chen, C.-M., Cooper, K.R. 1999. Developmental Toxicity and EROD Induction in the Japanese Medaka (*Oryzias latipes*) Treated with Dioxin Congeners. Bull. Environ. Contam. Toxicol., 63: 423-429.

Christensen, P.G. 1995. Årstidsvariasjon i enzymsystemet cytokrom P4501A i skrubbe (*Platichthys flesus*) fra Hvaleområdet. MSc thesis, University of Oslo, 90 p. [in Norwegian]

Cribb, A.E., Leeder, J.S., Spielberg, S.P., 1989. Use of microplate reader in an assay of glutathione reductase using 5, 5'-dithiobis(2 nitrobenzoic acid). Anal. Biochem. 183, 195-196.

Goksøyr, A., Förlin, L. 1992. The cytochrome *P*-450 system in fish, aquatic toxicology and environmental monitoring. Aquat. Toxicol. 22:287-312.

Grinwis, GCM; Besselink, HT; van den Brandhof, EJ; Bulder, AS; Engelsma, MY; Kuiper, RV; Wester, PW; Vaal, MA; Vethaak, AD; Vos, JG. 2000.Toxicity of TCDD in European flounder (Platichthys flesus) with emphasis on histopathology and cytochrome P450 1A induction in several organ systems. Aquat. Toxicol. 50: 387-401.

Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J.biol.Chem. 249, 7130-7139.

Hylland, K., Bakke, T., Förlin, L. 1998. Overvåking av effekter av miljøgifter på blåskjell og torsk fra Grenlandsfjordene 1996. SFT Report no. 714/97, 28 p. [in Norwegian]

Hylland, K., Nissen-Lie, T., Christensen, P.G., Sandvik, M. 1998. Natural modulation of hepatic metallothionein and cytochrome P4501A in flounder, *Platichthys flesus* L., Mar. Environ. Res, 46: 51-55.

JAMP (Joint Assessment and Monitoring Programme) 1998a. JAMP guidelines for general biological effects monitoring. Oslo and Paris Commissions, 15 p.

JAMP (Joint Assessment and Monitoring Programme) 1998b. JAMP guidelines for contaminant-specific biological effects monitoring. Oslo and Paris Commissions, 38 p.

Knutzen, J. 1995. Miljøgifter i spiselige organismer fra Grenlandsfjordene - senere års utvikling og fremtidsutsikter. Vann, 30: 507-528. [in Norwegian]

Knutzen, J., Bjerkeng, B., Green, N., Kringstad, A., Schlabach, M., Skåre, J.U. 2001. Overvåking av miljøgifter i fisk og skalldyr fra Grenlandsfjordene 2000. SFT Report 835/01. TA-1832/2001, 230 p. [in Norwegian]

Knutzen, J., Becher, G., Biseth, Aa., Bjerkeng, B. Brevik, E.M. Green, N. Schlabach, M., Skåre, J.U. 1999. Overvåking av miljøgifter i fisk og skalldyr fra Grenlandsfjordene 1997. SFT Report 772/99. TA-1661/1999, 195 p. [in Norwegian]

Lawrence, A., Hemingway, K. (eds) 2003. Effects of pollutants on fish. Blackwell Publishers, Oxford.

Le Cren, E.D. 1972. Fish production in freshwaters. Symp. Zool. Soc. Lond. 29: 115-133.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. J. biol. Chem. 193:265-275.

OSPAR. 1998. Report of the Third OSPAR Workshop on Ecotoxicological Assessment Criteria. Report 81, Oslo and Paris Commissions, ISBN 0 946955 79 4, http://www.ospar.org.

Ricker, W.E. 1979. Growth rates and models. In: Fish physiology. Volume 8. Bioenergetics and growth. Hoar,W.S.; Randall,D.J.; Brett,J.R. (eds.), pp. 738-743.

Ruus, A., Hylland, K., Green, N. 2002. Joint Assessment and Monitoring Programme (JAMP). Biological Effects Methods, Norwegian Monitoring 1997-2001. SFT Report no. 869/03. TA-1948/2003, 139 p.

Scott, A.P., Hylland, K. 2001. Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques for the measurement of marine fish vitellogenins. ICES Tech. mar. environ. Sci. 31, 21 p.

Sokal, R.R. & Rohlf, F.J. (1981). *Biometry*, W.H. Freeman & Co, New York, pp. 1-859.

van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003, Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol., 13: 57-149.

van der Weiden, MEJ; Bleumink, R; Seinen, W; Van den Berg, M. 1994. Concurrence of P450 1A induction and toxic effects in the mirror carp (*Cyprinus carpio*), after administration of a low dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Aquat. Toxicol, 29: 147-162.

van der Weiden, M.E.J., Celander, M., Seinen, W., Van den Berg, M., Goksoyr, A., Förlin, L. 1993. Induction of cytochrome P450 IA in fish treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin or chemically contaminated sediment. Environ. Toxicol. Chem., 12: 989-999.

van der Weiden, M.E.J., van der Kolk, J., Penninks, A.H., Seinen, W., van der Berg, M. 1990. A dose/response study with 2,3,7,8-TCDD in the rainbow trout (*Onchorhynchus mykiss*). Chemosphere, 20: 1053-1058.

Walker, M.K., Cook, P.M., Butterworth, B.C., Zabel, E.W., Peterson, R.E. 1996. Potency of a complex mixture of polychlorinated dibenzo-p-dioxin, dibenzofuran, and biphenyl congeners compared to 2,3,7,8-tetrachlorodibenzo-p-dioxin in causing fish early life stage mortality. Fundam. Appl. Toxicol., 30: 178-186.

Walker, M.K., Peterson, R.E. 1992. Toxicity of Polychlorinated Dibenzo-*p*-Dioxins, Dibenzofurans, and Biphenyls During Early Development in Fish. In: Chemically-induced alterations in sexual and functional development: the wildlife/human connection. Colborn, T., Clement, C. (eds). Advances in modern environmental toxicology. Ed. M. A. Mehlmann. Princeton, New Jersey: Princeton Scientific Publishing Co. pp. 195-202.

Appendix A. Morphometric data

sampling	fjord	sex	data	mature cod	flounder	herring	juv cod	trout
Apr 2000	Eidanger	female	fish sampled	9	19		11	8
			mean length-cm	50,3	33,9		25,5	36,5
			mean weight-g	1432,0	423,8		181,1	620,8
			mean GSI-%	4,3	2,9		0,2	0,4
		male	fish sampled	11	1		11	2
			mean length-cm	43,0	31,0		25,7	31,0
			mean weight-g	688,7	248,0		172,5	293,0
			mean GSI-%	0,7	1,5		0,0	0,1
	Frierfjord	female	fish sampled	14	19		4	15
			mean length-cm	51,4	34,1		25,6	31,3
			mean weight-g	1660,9	435,2		162,8	327,6
			mean GSI-%	3,6	6,2		0,4	0,3
		male	fish sampled	8,0			1,0	9,0
			mean length-cm	42,9			21,0	34,9
			mean weight-g	821,0			92,0	515,0
			mean GSI-%	0,2			0,0	0,1
Nov 2000	Eidanger	female	fish sampled	11	10	9	11	9
			mean length-cm	40,0	36,9	27,2	24,5	32,4
			mean weight-g	659,7	780,0	153,6	173,5	408,7
			mean GSI-%	0,7	7,1	0,0	0,3	0,2
		male	fish sampled	9	8	7	2	2
			mean length-cm	43,3	30,5	23,9	30,5	65,5
			mean weight-g	825,2	362,1	99,4	330,5	2769,0
			mean GSI-%	0,3	5,0	0,0	0,5	1,5
	Frierfjord	female	fish sampled	10	19		7	14
			mean length-cm	50,5	37,5		25,3	34,2
			mean weight-g	1494,5	730,2		168,4	526,4
			mean GSI-%	0,8	6,9		0,3	1,9
		male	fish sampled	10	1		1	6
			mean length-cm	47,4	36,0		31,0	36,2
			mean weight-g	1233,0	563,0		247,0	671,0
			mean GSI-%	0,2	6,6		0,0	0,9
Jan 2001	Eidanger	female	fish sampled	8	10	15	8	18
			mean length-cm	44,5	35,3	22,6	20,6	33,9
			mean weight-g	902,9	612,5	122,3	101,1	450,2
			mean GSI-%	1,4	16,8	8,4	2,0	0,5
		male	fish sampled	12	4	5	5	2
			mean length-cm	41,7	29,3	22,8	18,0	39,0
			mean weight-g	725,1	327,0	124,4	53,2	653,0
			mean GSI-%	4,2	3,2	11,7	0,0	0,1
	Frierfjord	female	fish sampled	12	4	16	7	14
			mean length-cm	50,4	37,8	25,3	26,9	31,6
			mean weight-g	1311,8	694,0	164,9	178,0	351,2
			mean GSI-%	0,8	41,6	12,5	0,1	0,6
		male	fish sampled	8	6	3	2	5

			mean length-cm	43,1	31,7	22,7	29,0	34,4
			mean weight-g	998,9	356,0	122,0	239,5	392,4
			mean GSI-%	3,7	2,8	13,5	0,0	0,2

Appendix B. Protein

All data in µg/mL homogenising buffer; analysed – number analysed; mic – microsomal fraction.

sampling	fjord	sex	fraction	data	cod	flounder	herring	small cod	trout
Apr-2000	E	female	cytosol	analysed	9	19			8
				min	6475	6205			4185
				max	9372,5	11290			11140
			mic	analysed	9	19		11	8
				min	1948	874,4		962,8	2368
				max	3378	4743		4887	6226
		male	cytosol	analysed	11				2
				min	5837,5				5255
				max	11755				5510
			mic	analysed	11			11	2
				min	1781			2067	2204
				max	4152			2840	2482
	F	female	cytosol	analysed	14	19			15
				min	8480	3872,5			2950
				max	17157,5	11535			11187
			mic	analysed	14	19		4	15
				min	2921	1143		1664	1257
				max	5242	5457		2470	3536
		male	cytosol	analysed	8				9
				min	9862,5				4835
				max	11242,5				10955
			mic	analysed	8			1	9
				min	2591			1198	1837
				max	4645			1198	4512

Protein continued

sampling	fjord	sex	fraction	data	cod	flounder	herring	small cod	trout
Nov-2000	E	female	cytosol	analysed	11	10	9		9
				min	6117	8675	12432		9160
				max	9543	13065	17322		19022
			mic	analysed	11	10	9	11	9
				min	1645	3419	2343	1764	3509
				max	5180	6014	5838	5473	6211
		male	cytosol	analysed	9	8	7		2
				min	7377	9665	11604		13875
				max	8232	14010	17640		16067
			mic	analysed	9	8	7	2	2
				min	2098	3160	1510	2994	3669
				max	5455	6380	3114	2994	4576
	F	female	cytosol	analysed	10	19			14
				min	8070	8202,5			5367
				max	13689	14852,5			12625
			mic	analysed	10	19		7	14
				min	1862	3381		2049	2406
				max	5740	5912		3511	7193
		male	cytosol	analysed	10				6
				min	6759				7955
				max	11067				12020
			mic	analysed	10	1		1	6
				min	2336	3141		2743	3789
				max	5131	3141		2743	6371
Jan-2001	E	female	cytosol	analysed		10	15		
				min		7644	18820		
				max		12873	18820		
			mic	analysed	8	10	15	8	18
				min	3287	3230	1623	1563	3624
				max	6107	7357	8044	4666	7034
		male	cytosol	analysed	12	4			
				min	5910	8457			
				max	17928	13251			
			mic	analysed	12	4	5	5	2
				min	2256	2980	2033	2506	5968
				max	6390	5719	6572	4487	7321
	F	female	cytosol	analysed		4	16	7	
				min		10650	8604	8430	
				max		11790	14661	8430	
			mic	analysed	12	4	16	7	14
				min	2997	4572	1731	3182	2872
				max	6406	6959	4350	6976	7628
		male	cytosol	analysed	8	6	3	2	
				min	6492	5127	5127	12600	
				max	15582	12765	5127	15903	
			mic	analysed	8	6	3	2	5
				min	2543	2411	1425	4309	4679
				max	5145	5923	3286	5896	6973

Appendix C. CYP1A protein and activity (EROD)

7-ethoxyresorufin *O*-deethylase (EROD) activity (pmol/min/mg protein) and cytochrome P4501A concentration (absorbance corrected for microsomal protein concentration).

date	fjord	sex	data	cod	flounder	herring	small cod	trout
Apr-2000	E	female	analysed - EROD	9	19		11	8
			min - EROD	7,72	417,50		106,77	290,25
			max - EROD	56,81	3446,12		299,01	1484,26
			analysed - CYP1A					
			min - CYP1A					
			max - CYP1A					
		male	analysed - EROD	11			11	2
			min - EROD	3,14			126,69	676,85
			max - EROD	164,50			209,24	1332,02
			analysed - CYP1A					
			min - CYP1A					
			max - CYP1A					
	F	female	analysed - EROD	14	19		4	15
			min - EROD	9,93	143,77		148	418,19
			max - EROD	122,36	3140,55		270,25	3125,50
			analysed - CYP1A					
			min - CYP1A					
			max - CYP1A					
		male	analysed - EROD	8			1	9
			min - EROD	172,73			242	894,10
			max - EROD	450,99			242	2169,86
			analysed - CYP1A					
			min - CYP1A					
			max - CYP1A					

EROD and CYP1A continued.

date	fjord	sex	data	cod	flounder	herring	small cod	trout
Nov-2000	E	female	analysed - EROD	11	10	9	11	9
			min - EROD	13,26	37,11	2,49	1,11	149,68
			max - EROD	545,90	783,21	280,56	280,02	9103,73
			analysed - CYP1A		10		11	9
			min - CYP1A		0,40		0,01	0,31
			max - CYP1A		2,01		0,64	1,62
		male	analysed - EROD	9	8	7	2	2
			min - EROD	1,04	42,13	2,06	31,78	44,64
			max - EROD	199,37	1243,84	160,70	150,23	239,32
			analysed - CYP1A		8		2	2
			min - CYP1A		0,52		0,15	0,19
			max - CYP1A		2,01		0,15	0,43
	F	female	analysed - EROD	10	19		7	14
			min - EROD	148,73	5,28		-0,33	8,84
			max - EROD	1788,46	4949,99		1217,34	5220,38
			analysed - CYP1A		19		7	14
			min - CYP1A		0,43		0	0,42
			max - CYP1A		1,87		1,28	1,63
		male	analysed - EROD	10	1		1	6
			min - EROD	3,31	741,22		263,81	2,69
			max - EROD	452,07	741,22		263,81	1871,19
			analysed - CYP1A		1		1	6
			min - CYP1A		2,14		1,18	0,43
			max - CYP1A		2,14		1,18	1,57
Jan-2001	E	female	analysed - EROD	8	10	15	8	18
			min - EROD	3,96	5,02	1,13	66,12	724,67
			max - EROD	168,50	65,51	272,38	262,50	4990,28
			analysed - CYP1A		10		8	18
			min - CYP1A		0,37		0,04	0,81
			max - CYP1A		1,35		1,41	1,76
		male	analysed - EROD	12	4	5	5	2
			min - EROD	2,45	47,44	54,89	141,94	3119,40
			max - EROD	427,94	522,33	160,44	294,10	3312,30
			analysed - CYP1A		4		5	2
			min - CYP1A		0,39		0,16	1,61
			max - CYP1A		0,39		1,57	1,61
	F	female	analysed - EROD	12	4	16	7	14
			min - EROD	47,49	6,41	0,32	26,89	415,62
			max - EROD	816,90	197,73	330,70	1283,08	2235,30
			analysed - CYP1A		4		7	14
			min - CYP1A		0,51		<0,02	0,42
			max - CYP1A		1,24		1,26	1,63
		male	analysed - EROD	8	6	3	2	5
			min - EROD	40,26	21,54	46,97	1,83	515,30
			max - EROD	576,31	1915,03	258,30	646,40	4770,24
			analysed - CYP1A		6		2	5
			min - CYP1A		1,50		0,38	0,77
			max - CYP1A		1,88		1,52	1,59

Appendix D. GST and GR activity

GR is given in nmol/min/mg protein; GST is given in µmol/min/mg protein.

date	fjord	sex	data	cod	flounder	herring	trout
Apr-2000	E	female	analysed - GR	3	10		8
			min - GR	17,48	6,15		4,22
			max - GR	27,01	9,23		19,84
			analysed - GST	3	10		8
			min - GST	1,05	0,22		0,49
			max - GST	2,99	0,83		1,43
		male	analysed - GR	7			2
			min - GR	20,71			5,71
			max - GR	31,23			7,76
			analysed - GST	7			2
			min - GST	1,40			0,34
			max - GST	3,91			1,17
	F	female	analysed - GR	7	10		6
			min - GR	13,72	5,19		3,82
			max - GR	31,48	12,72		11,02
			analysed - GST	7	10		6
			min - GST	0,77	0,18		0,85
			max - GST	2,40	1,06		1,79
		male	analysed - GR	3			4
			min - GR	21,58			5,25
			max - GR	26,10			14,09
			analysed - GST	3			4
			min - GST	1,04			1,19
			max - GST	1,24			2,31

GR and GST continued.

date	fjord	sex	data	cod	flounder	herring	trout
Nov-2000	E	female	analysed - GR	6	5	7	7
			min - GR	5,32	4,90	3,60	4,12
			max - GR	14,99	8,13	8,46	5,86
			analysed - GST	5	5		7
			min - GST	0,65	0,38		0,24
			max - GST	2,39	0,75		0,50
		male	analysed - GR	4	5	3	2
			min - GR	6,23	5,32	4,16	3,25
			max - GR	9,02	8,67	6,96	5,52
			analysed - GST	2	5		2
			min - GST	0,98	0,74		0,09
			max - GST	1,20	1,06		0,26
	F	female	analysed - GR	4	10		7
			min - GR	6,46	2,63		4,21
			max - GR	10,94	5,68		10,88
			analysed - GST	4	10		7
			min - GST	1,58	0,24		0,47
			max - GST	6,44	0,72		0,83
		male	analysed - GR	6			3
			min - GR	4,02			4,33
			max - GR	12,77			6,86
			analysed - GST	6			3
			min - GST	0,71			0,38
			max - GST	3,48			1,20
Jan-2001	E	female	analysed - GR		5		
			min - GR		5,59		
			max - GR		9,23		
			analysed - GST		5		
			min - GST		0,16		
			max - GST		0,52		
		male	analysed - GR	10	4		
			min - GR	10,63	6,11		
			max - GR	30,15	7,59		
			analysed - GST	10	4		
			min - GST	0,67	0,57		
			max - GST	2,04	1,25		
	F	female	analysed - GR		4	9	
			min - GR		4,18	4,52	
			max - GR		10,80	8,18	
			analysed - GST		4	5	
			min - GST		0,27	0,08	
			max - GST		0,54	0,18	
		male	analysed - GR	7	6	1	
			min - GR	15,07	7,04	8,33	
			max - GR	30,55	10,42	8,33	
			analysed - GST	7	6		
			min - GST	0,98	0,19		
			max - GST	2,16	1,10		

Appendix E. Vitellogenin

date	fjord	data	cod
Apr-2000	E	analysed	11
		min	1487
		max	7614
	F	analysed	8
		min	642,1
		max	2938
Nov-2000	E	analysed	9
		min	1374
		max	4109
	F	analysed	10
		min	870,6
		max	2840
Jan-2001	E	analysed	12
		min	1888
		max	3931
	F	analysed	8
		min	2101
		max	3505