Coastal baseline

- **Exposure and effects of PAH on fish in the coastal zone around the Faroe Islands**

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Summary

In this study the level of the biomarkers: EROD activity and CYP1A protein in liver, PAH metabolites in bile and vitellogenin in blood, have been analysed in four fish species living in the coastal zone of the Faroe Islands. The fish species analysed were sculpins (*Myoxocephalus scorpius*), dab (*Limanda limanda*, cod (*Gadus morhua*) and flounder (*Plathichthys flesus*). Sculpin from three seasons were analysed (late winter, spring and summer) whereas dab and cod only were analysed two of the seasons (spring and summer) and flounder only one (late winter). The fish were sampled at two locations (Kaldbak and Kirkjubø), but the same species were not sampled the same stations except in July when dab was sampled both in Kaldbak and Kirkjubø.

The results generally show low levels of PAH exposure, although large variations are found within the groups, especially in the EROD results, but also in the concentration of pyrene metabolites in bile and in the the vitellogenin results from July. Dab are found to be most sensitive to the biomarkers of PAH exposure. Sculpins show very low levels of CYP1A activity, probably because the catalytic activity has been destroyed.

The levels of CYP1A induction was influenced by gonadal development in flounder, dab and sculpin, showing negative correlation between CYP1A induction and gonadal developement. The cod sampled were immature and thus not influenced by spawning.

Vitellogenin was analysed in cod and the results show high levels compared to other studies from reference areas.

There was not found correlation between PAH metabolite content in bile and induction of CYP1A.

The most suitable species to use as an indicator organism for the analysed biomarkers is dab or cod. Since the results of the biomarkers show large variation, probably due to small sample sizes, it is suggested that either cod or dab is subjected to further investigation, by analysing a larger number of individuals for the biomarkers, and minimizing the influence of other parameters by sampling only in one season, not influenced by spawning.

Title:

Coastal baseline – Exposure and effects of PAH in the coastal zone around The Faroe Islands., January 2004.

The report was written by Katrin Hoydal who has been the responsible for the sampling, sample preparation and who has taken part in the analyses work at the laboratories. Tórvild Østerø and Jóhannis Danielsen both from Heilsufrøðiliga Starvsstovan (the Food and Environmental Agency) have been assisting in the field, as have also staff from "Hav-, botn- og streym kanningar" and Carlo Petersen. Project leader was Maria Dam.

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The report is also a part of K. Hoydal´s MsSc thesis, whis is done under the supervision of associate professor Ian Lambert, at the University of Copenhagen and professor Ketil Hylland at the Norwegian Institute for Water Research.

Contents

1 Introduction

With the opening of the Faroese offshore area for hydrocarbon exploration activities, large parts of the Faroese society, including the authorities, need to adapt to new challenges and opportunities. In particular there is a need to upgrade knowledge of current pollution status with regard to oil-derived pollutants has arisen and also to establish skills in evaluating and monitoring possible pollution from these sources.

Oil is primarily saturated hydrocarbons, but contains variable amounts of PAHs (polycyclic aromatic hydrocarbons). PAHs are the main toxic component of oils and the heavy PAHs are neither easily evaporated, nor quickly degraded by bacteria or weathering processes. They have, therefore, been used as indicators of oil-derived pollution. The relative composition of the various components in a PAH mixture is often referred to as its profile, or signature, and can be used to trace the source of the PAH.

The aim of the present project is to increase our base of knowledge of oil-derived pollutants as such and the effect of these on the fish in the coastal zone. More specifically, the study is concentrated on PAHs, or rather the biomarkers of PAHs. In addition, vitellogenin has been analysed as another biological effect marker. The level of PAH metabolites, as well as vitellogenin, have been investigated for season-dependent variations.

The results of the study will contribute to establish the ambient level of oil-derived pollution in coastal marine organisms in non-polluted areas of the Faroese coast. This background or "baseline level" will serve as reference, when assessing future environmental status with respect to oil-derived pollution.

1.1 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are compounds consisting of two or more fused benzen rings lying in a single plane. PAHs can be formed by incomplete combustion of organic material at high temperatures (pyrogenic PAH) or by slow transformation of organic matter under pressure (petrogenic PAH). In addition, to a minor extent, PAHs can be formed naturally by biosynthesis in microbes and plants (Neff, 1979; Meador et al.,1995; Aas, 2000). PAH contamination originating from a petrogenic source show dominance of 2- and 3- ring compounds while pyrogenic PAH is dominated by 4- and 5- ringcompounds (Neff, 1979; Meador et al., 1995; Aas et al., 2000b). Pyrogenic PAH can be released to the environment naturally from forest fires and volcanic eruptions or from antropogenic sources such as combustion of fossil fuels and industrial waste. Sources of PAH to the marine environment may both be from deposition of airborne particulates from burning of fossil fuels and direct discharge of oil to the sea by natural seepage, accidental releases or operational discharges of produced water (Aas, 2000). Produced water is a by product from oil production and contains residues of oil. PAH from industrial waste generally has a local distribution near the discharge point, while PAH in oil discharged with produced water can be distributed widespread in the ocean (Aas, 2000). The main source of total PAH to the aquatic environment is by petrolium spillage.

1.1.1 Uptake of PAH

Fish can take up PAH from the water through the gills, and to a minor degree through the skin, or from ingestion through the intestine (Neff, 1979). The uptake routes depend on in which form PAH is found in the water, whether in dissolved state or bound to particulates; the partitioning of PAHs into either of these forms is due to their specific water solubility. Thus the distribution of these compounds in adsorbed and in water phase differs for each PAH (Piccardo et al., 2001). The lighter PAHs seem to remain in the dissolved state to a larger extent than the heavier PAHs, which almost always are bound to particles.

The uptake from water depends mainly on the diffusion over the body surface, of which the gill constitutes most surface area (Randall et al., 1998). The rate of toxicant transfer between the water and organism will depend on the lipofilicity (K_{ow}) of the compound and will vary with oxygen uptake (Randall et al., 1998). Factors that affect the rate of water movement over the gill will also affect the rate of lipophilic chemical uptake, since they are brought into contact with the gills by water flow (Connel, 1988). Hence, factors that increase oxygen requirement (such as temperature rise or exercise) also enhance transfer of toxicant (Randall et al., 1998; Connel, 1988).

Uptake via the food depends on the feeding rate, the concentration of toxicant in the food, the rate at which food is processed by the gut, and the amount of toxicant absorbed versus the amount excreted. The uptake over the gills is found to be the dominating route in fish, while dietary uptake is limited (Randall et al., 1998; Neff, 1979). Studies of dietary uptake in fish generally indicate low uptake efficiency and that uptake efficiency generally declines with increasing chemical hydrofobicity (Meador et al., 1995). The strong particle affinity of PAH can make them more unavailable for metabolism (Næs et al., 1998). It is suggested that uptake from food can be ignored when estimating toxicant body burden in water breathing animals under natural conditions when the feeding rate is low and thus uptake from food plays a minor role compared to the uptake from water (Randall et al., 1998).

1.1.2 Elimination of PAH

Vertebrates can metabolize PAH efficiently by enzymes of the cytocrome P-450 system. The biotransforming processes have been shown to be inducible in several fish species when they are exposed to PAH, by which the excretion capacity for these compounds is enhanced (Aas, 2000). When taken up in fish, PAH undergo a metabolic transformation which creates a polar and more watersoluble metabolite and thereby enhances excretion. The biotransformation process is occuring in two steps. Phase I is an oxidativ step catalysed by the cytochrome P-450 enzym system, by which an oxygen atom is introduced into the molecule. In phase II a larger endogenous molecule (such as glucoronic acid, sulfate, glutathione etc.) is conjugated to the oxidized compound by the aid of different transeferase enzymes. The created metabolite is generally less toxic and can readily be excreted (Goksøyr & Förlin, 1992; Andersson & Förlin, 1992).

However the biotransformation process can also create metabolites that are more toxic than the parental compound. Certain PAHs can by the phase I metabolization be converted into highly reactive metabolites (epoxides, free radicals, etc) which are able to bind covalently to macromolecules such as DNA, creating adducts which eventually can cause mutagenic or carcinogenic effects (Buhler & Williams, 1989; Ericson et al., 1998).

In vertebrates the biotransformational processes are mostly taking place in the liver, which is the major organ involved in metabolization of organic xenobiotics, but is also found to occur to some degree in other extrahepatic tissues such as kidney, heart and gills (Husøy et al., 1994; Stegeman et al., 1990).

1.2 The Cytochrome P-450 system

The cytochromes P-450 comprise a superfamily of enzymes which function as mixed function oxygenases (MFOs) involved in metabolisation of both endogenous compounds, such as steroid hormones, steroids and fatty acids, and exogenous xenobiotics, such as PAH, PCB[1] and various drugs (Parke, 1990). Many of them have shown to exhibit induction, which is substrate induced genomal regulation of their enzyme activity, following exposure of the animal to a specific substrate or chemical agent (Parke, 1990).

The gene family inducable by planar molecules such as PAHs, PCBs and PCDDs[2] is the P4501 family (Parke, 1990; Stegeman et al., 1992). In mammals, which is the species group most intensively studied, the P4501 family is found to comprise only one subfamily (P4501A) with two genes, CYP1A1 and CYP1A2 (Nebert & Gonzalez, 1990). Until recently, evidence for only one subfamily has been found in teleost fish species and analyses of DNA sequences have lead to the assumption that this subfamily corresponds to the CYP1A1 family in mammals (Stegeman, 1989) and that the CYP1A2 gene has evolved by a gene duplication event after the separation of fish and mammalian predecessors (Nebert & Gonzalez, 1990; Goksøyr & Förlin, 1992). Evidence for a second CYP1A gene (CYP1A3) has since been found in fish (Berndtson & Chen, 1994). This gene is however not orthologous to the CYP1A2 gene in mammals, but probably the fish CYP1A1 precursor gene has undergone its own duplication event separate from the mammalian duplication event (Berndtson & Chen, 1994).

[1] polychlorinated biphenyls
[2] polychlorinated dibenzo-*p*-dioxins

1.2.1 Induction of cytochrome P-450

Inductive response is a process by which a chemical stimulates the rate of gene transcription resulting in increased levels of messenger-RNA and synthesis of the P450 protein (Andersson & Förlin, 1992). The induction of cytochrome P450 system in fish can be influenced by various factors such as: Species, strain, sex, reproductive stage, temperature, age, dietary factors, inducing agents, antagonistic agents (Goksøyr & Förlin, 1992).

There can be large variations in the inductive response, even between related species. When dab and flounder are sampled at the same place or under same conditions the MFO activity, measured as 7-ethoxyresorufin-*O*-deethylase (EROD) assay, is found to be three to four times higher in dab than in flounder (Krüner & Westernhagen, 1999). Seasonal- and sex differences have also shown to be less pronounced in flounder than in dab (Westernhagen et al., 1999).

In mammals males generally seem to have higher levels of hepatic P450 than females (Andersson & Förlin, 1992) and this seems also to be the case in winter flounder (Pleuronectes americanus) (Vandermeulen & Mossman, 1996; Edwards et al., 1988) and in dab (*Limanda limanda*) at least during some stages of the reproduction cycle (Krüner & Westernhagen, 1999; Lange et al., 1999; Goksøyr et al., 1992).

Seasonal variations in MFO activity can be linked to hormonal status, (e.g. levels of steroid hormones), and changes in the reproductive state can influence the sensitivity to induction in the organism (Payne, 1984). Therefore, seasonal effects are often reflecting the different reproductive periods (spawning, pre-and post spawning). Spawning is particularly important, as it can be a period of marked biochemical, physiological and histological changes (Payne, 1984). Temperature can have indirect influence by modulating seasonal changes in physiology and abundance of chemical or natural inducers can also vary seasonally and possibly play a role (Payne, 1984).

Seasonal variations in MFO activity are differently expressed in males and females and the EROD activity is found to be low, especially in females in the spawning period (Krüner & Westernhagen, 1999; Lange et al., 1998; 1999). The differences in MFO response between the sexes are most likely due to the interaction of the MFO system with steroid hormones in fish (Lange et al., 1999; Edwards et al, 1988; Lindström-Seppä & Stegeman, 1995). Estradiol is a steroid hormone produced by developing ovaries and has been suggested to suppress CYP1A expression in reproductively active females (Stegeman & Hahn, 1994). This explanation is supported by the findings of an inverse relationship between EROD activity and Gonadosomatic Index (GSI)[3] (Khan & Payne, 2002; Lange et al., 1998). Sex and seasonality of the reproduction cycle have been found to be the most important variables influencing the MFO activity in winter flounder (Vandermeulen & Mossman, 1996; Edwards et al., 1988).

In dab an inverse relationship has been found between temperature and EROD activity in the post-spawning season and temperatures appeared to have stronger influence on regional variability in EROD activity than organochlorine concentration (Sleiderink et al., 1995; Lange et al., 1998). However, in the spawning season temperature and EROD activity showed positive correlation (Lange et al., 1998) and as the temperature differences could not be explained by qualitative changes, such as changes in enzyme affinities and temperature optima, Lange et al. (1998) suggested, that the influence by temperature on EROD activity occurs indirectly via its influence on

[3] GSI: (Gonad weight/total weight)x100

the duration of the gonadal cycle. Temperature thus influences the time of spawning, which is assumed to be coupled to the seasonal variation in EROD activity.

It has been suggested that age and size of the fish could be excluded as factors when selecting specimens for MFO measurements in monitoring (Edwards et al., 1988). However, Khan & Payne (2002) found higher activities in adult male than in adult female and juvenile winter flounder.

1.3 Biomarkers

Biomarkers have been defined as " Biological responses that can be related to an exposure to, or toxic effect of, an environmental chemical or chemicals" (Peakall, 1994 in Beyer, 1996)
The effect of chemical contaminants can occur at different biological levels extending from the molecular or biochemical level to the physiology of the individual organism and ultimately to the level of population and ecosystem (Stegeman et al., 1992). Changes at molecular level will underlie the effects at higher level of organisation and can be used as an "early warning" signal of the effects of chemical pollution. There is an advantage of measuring changes at the biochemical level, because the biochemical and molecular alterations are usually the first detectable quantifiable responses to environmental change, and can serve as a markers of both exposure and effect (Stegeman et al., 1992).

Certain criteria have been proposed to define biological responses that can serve as a biomarkers (Beyer, 1996):

1) The assay to quantify the biomarker should be sensitive, reliable, and relatively easy;
2) baseline data for the concentration/activity of the biomarker should be known in order to be able to distinguish between natural variability (noise) and contaminant-induced stress (signal);
3) The basic biology/physiology of the test organism should be known so that sources of uncontrolled variation (growth and development, reproduction, food sources) can be minimized;
4) All the factors, intrinsic as well as extrinsic, that affect the biomarker should be known;
5) It should be established whether changes in biomarker concentration are due to physiological acclimation or to genetic adaption;
6) changed levels of the biomarker should be correlated with the "health" or "fitness" of the organism.

1.3.1 Biomarkers measuring P-450 (CYP1A1) induction

Each of the steps of induction shown in Figure 1.1 (formation of mRNA, protein and catalytic active enzyme) can be analysed by a suitable assay to measure induction (Goksøyr & Förlin, 1992). The formation of mRNA can be analysed by DNA probes, CYP1A protein content by immunodetection analysis (such as ELISA), and the enzyme activity can be analysed by catalytic assays (such as EROD).

Level		Nomenclature	Marker
DNA	→	*CYP1A1*	
▼			
mRNA	→	CYP1A1	DNA probe
▼			
protein	→	P450 1A1	antibody
▼			
enzyme	→	EROD/AHH	catalytic assay

Figure 1.1 Different levels of induction of the cytochrome P450 system and markers, which can be used to detect it. (Redrawn from Goksøyr & Förlin, 1992)

EROD

The catalytic activity of the CYP1A enzyme can be analysed by the 7-ethoxyresorufin-*O*-deethylase (EROD) assay. CYP1A catalyses the O-deethylation of 7-ethoxyresorufin, giving the product resorufin which can be measured fluorimetrically. The resorufin formation depends on the presence of enzyme, substrate, oxygen and the cofactor NADPH (Nilsen et al., 1998).

The catalytic assays EROD and AHH (aryl hydrocarbon hydroxylase[4]) appear to be the most sensitive catalytic probes when determining induction response in fish, showing low or undetectable levels in untreated fish, but are higly induced by exposure to PAH type inducers (Goksøyr & Förlin, 1992). When the two assays (EROD and AHH) are analysed at the same time, the results are highly correlated, and as the EROD assay is highly sensitive and easy and safe to perform, it is most often preferred by researchers when studying PAH induction in fish and mammals (Buhler & Williams, 1989).

CYP1A protein

The measurement of catalytic activity has some disadvantages, because some conditions can lead to underestimation or lack of detection even though induction has occurred. The catalytic activities are sensitive to protein denaturation when conditions during sampling and storage are not optimal (Förlin & Andersson, 1985; Krüner & Westernhagen, 1999). The presence of some compounds, endogenous or exogenous (especially organochlorines), can inhibit the catalytic activity. In addition the sample or tissue may be too small to give measurable catalytic activity. In such cases measurements of the CYP1A protein content are of crucial importance (Goksøyr & Förlin, 1992). Measurement of CYP1A mRNA can also complement catalytic assays, but as mRNA is also very susceptible to degradation, the information gained by this method in environmental monitoring is limited compared to the immunodetectable analysis of CYP1A protein (Goksøyr & Förlin, 1992).

CYP1A protein content can be measured with an enzyme-linked immunosorbent assay (ELISA), which is a semiquantitative assay giving a relative measure of CYP1A in different samples. In the ELISA procedure the antigen is immobilized on a microtiter plate. A primary antibody specific for the antigen is allowed to bind to the antigen and a secondary antibody, conjugated to the enzyme HRP, is allowed to bind to the primary antibody. The immunocomplex is then detected by addition of a substrate, which is cleaved by the conjugated HRP generating a coloured reaction product, which can be detected spectrofotometrically (Nilsen et al., 1998).

[4] BaP-hydroxylase (Buhler & Williams, 1989)

1.3.2 Other biomarkers

PAH metabolites in bile

As most of the PAH taken up in fish is metabolised, chemical measurements of PAH in fish is not a suitable parameter for analysis of PAH exposition (Aas, 2000). Instead the concentration of metabolites, derived from PAH, can be measured. When biotransformated by the CYP1A1 system the metabolites generally are hydrofilic and will readily be excreted. Excretion to the bile is the dominant excretion route in fish of metabolites of, especially, larger PAH molecules (Meador et al., 1995). Hence, analyses of PAH metabolites in bile is a suitable matrix for PAH exposition.
Since the PAHs and their metabolites display strong and characteristic fluorescent properties, semi-quantitative measures of such compounds can be obtained by simple fluorescence analyses of bile samples. All PAH molecules absorb ultraviolet light followed by an emission of light of longer wavelength. The optimal exitation and emission wavelenghts and signal intensity vary between PAH compounds and are dependent on size, structure and eventual substituents and this variability can be utilized in simple detection methods for PAHs (Aas, 2000.)
The PAH–metabolites in bile are mostly found as conjugated OH-PAH, which means that they are bound to proteins. The proteinbound metabolites can be released by treatening the material with an enzyme, β-glucuronidase/aryl sulfatase at 37ºC. The proteins are precipitated by the addition of alcohol. Selected metabolites can then be measured by HPLC with fluorescence detection.

The concentration of PAH metabolites in bile is however influenced by differences in bile density between individuals. The filling and emptying of the gall bladder depends on the feeding status of the individual fish, and the feeding status, thus, influences the bile density and thereby the concentration of metabolites in the bile (Aas, 2000). As the fish empties the bile into the gastrointestinal tract during feeding, the gall bladder will be almost empty in the hours after feeding. Furthermore, the bile first accumulating in the gall bladder after feeding will be rather diluted. After long periods of fasting the bile fluid will be more concentrated and the fluorescence signal may be influenced by accumulation of other metabolic products in the bile (Beyer, 1996). The variations in bile densities can be minimized by normalizing the result for the bile pigment biliverdin. This can be done by measuring the absorbance in the bile at 380nm, as biliverdin has one of its two major peaks at this wavelength.

Vitellogenin

Vitellogenin (Vtg) is the egg yolk precursor, and is normally produced in the liver of mature female fish in response to estradiol in the blood. Estradiol is produced by developing ovaries. If male or immature female fish are exposed to oestrogenic substances, their livers too will be stimulated to produce Vtg and hence they can be used as a biomarker for environmental oestrogens (Scott & Hylland, 2002).

Vtg can be measured by a competitive ELISA test. The principle in this test is that anti-vitellogenin (antibody) in a polyklonal serum binds to vitellogenin (Vtg) in a solution in competition with a known amount of Vtg, which is bound to the walls of a microtitre plate. An enzyme-conjugated secondary antibody then binds to the primary antibody, which has bound to the Vtg on the walls of the plate. By adding a colour substrate the enzyme conjugate will develop a colour and the the colour end product can be measured by reading the absorbance by a platereader. The concentration of Vtg can then be calculated from a standard curve. The more vitellogenin there is in the sample, the less enzyme-conjugated antibody binds to the walls in the plate and the weaker is the colour development.

1.4 Test organisms

The suitability of species chosen to be monitoring organisms has to be evaluated when performing biomarker analyses. The selected species should be representative for habitat and biota and relative stationary to be sure they are exposed to the actual pollution. Among species the most sensitive ones should be selected (Aas, 2000).

In this project species of fish (teleosts) have been sampled as monitoring organisms. Of the fish species sculpins (*Myoxocephalus scorpius*), dab (*Limanda limanda*), flounder (*Platichthys flesus*) and a coastal stage of cod (*Gadus morhua*) called "reyðfiskur" in Faroese (red fish) have been chosen.

The following text is based upon Joensen & Tåning (1969) where no other reference is given.

Dab:
Dab (*Limanda limanda*) is common in the fjords, coves and sounds in the Faroes and is found from the tidal zone to a depth of more than 150 m (not taken outside 200 m contour line). On the whole it is a non-migratory fish and does not move any great distances. It leaves the shallow water to spawn, though finds spawning grounds in the fjords and bays.
According to Joensen & Tåning (1969) spawning starts April, reaches a peak around (end of) May and continues to later in the summer. However, a study from 1996-97 (Dam, 2000) shows that spawning occured as early as in March in 1996 and probably even earlier (in January-February) in 1997.
Males reach a length of about 30 cm, females longer (maximum males 38 cm, females 41 cm) Maturity is reached at a lenght of 18 cm in females and a little less in males.
The diet consists for a large part of mussels (*Abra, Axinus, Cardium* etc.), smaller crustaceans (gammarids, hermit crabs, smaller crabs), and finally bristle worms, echinoderms and smaller fish. The alga *Ulva* commonly found in the stomach of dab.
Dab is important as foodstuff for predatory fish in the area (Cod, Halibut, Monk-fish).

Flounder:
Flounder (*Platichthys flesus*) is found in probably all Faroese fjords and coves where there are freshwater outfalls. Most of its lifetime it lives in the inner parts of the fjords or coves, where the salinity is low (around fresh water outlets). It can frequently be found at depths as low as 0-3 m but is likely to go to deeper water in the winter. Probably it seeks to more open parts or mouths of the fjords or even the banks during the spawning season. Spawning occurs in winter or early spring (until May).
In the Faroes flounder reaches a length of 38-40 cm (maximum 40-45). It feeds mostly on crustaceans (gammarids, crabs etc.), snails and mussels (young Mytilus and similar forms), larvae of aquatic insects and small fish.

Cod:
Cod (*Gadus morhua*) is one of the most common fishes in and around the Faroes. It is mainly found within the 200-300 m contour line, but ranges from depths less than 1m to perhabs 700 m.
Near the Faroes it spawns to the north of the islands (Norðhavið). Spawning takes place at temperature around 6-7ºC. The main spawning season is from early March to May, April being the most important month.

Until the age of 3 years (or more) the cod is rather stationary. When sexually mature the cod migrates to “Norðhavið” (or other locations) to spawn. After spawning they disperse all over the Faroe area and survivors will seek the spawning grounds again the following year.
Many reach sexual maturity at the age of 3 years, but the majority at the age of 4 years, some even later. Cod feeds on nearly everything digestible it comes across. Sand-eel and herring are probably the most favoured food with the addition of crustaceans, brittle star, mussels etc.

Cod which lives among red algae acquire an intense red color and are called “reyðfiskur” (red fish) (they probably get the red color by eating the red sea-weed). “The red fish” is stationary until approximately 1 – 1½ years of age, then it migrates to spawn and live among the cods on the continental shelf (Steingrund, pers. comm.).

Sculpin:
Sculpin (*Myoxocephalus scorpius*) is very common all around the islands in the stony or sea-weed covered areas of the coastal region. It is often found near piers and similar places. It is rare in deep parts of the fjord because of the soft bottom in most of these places. It is found from the low water mark to a depth of ca.200 m, although the number decreases notably from 20-25 m outwards.
The spawning season is from mid-winter until spring.
Sculpins feeds on crustaceans and will indeed take anything.
Females reach a size of approximately 32 cm, males less. In Iceland they may reach length of 40 cm.

2 Methods

2.1 Sampling

Fish were sampled in Kaldbak and Kirkjubøur. The plan was to collect sculpins in Kaldbak and dab in Kirkjubøur, but as there was a not negligible by-catch of other species, these were taken as samples as well. The additional sampling included a coastal stage of Atlantic cod, flounder and dab in Kaldbak, and flounder in Kirkjubøur. The only flounder samples that were analysed were those from Kirkjubøur in February.

In Kaldbak the samples in the first sampling period were secured by fishing with fishing rods at the quay, but as it turned out to be quite difficult to get enough samples and because of the close vicinity to a fish processing plant, the sampling method was changed to fish-traps placed more distantly from land. The traps normally were soaked for 24 hours, before they were taken up, but sometimes 2-4 days elapsed.

In Kirkjubøur the sampling in the first sampling period was done by diving near the quay. In the second and third sample period the fish were caught in fishing nets, which were set in Brandansvík and were deployed for about two hours. Some of the fish was also caught by fishing rod from a boat.

The fish were transported alive in containers with seawater to the laboratory, where the samples were prepared. Table 2.1 gives a summary of the fish catches.

Table 2.1 Fish samples from Kaldbak and Kirkjubøur

	Sampling period	**Short-horn sculpin** (*Myoxocephalus scorpius*)	**Cod** – coastal stage (*Gadus morhua*)	**Flounder** (*Platichthys flesus*)	**Dab** (*Limanda limanda*)
Kaldbak	Jan-feb ’02	6	-	-	-
	Apr-May ’02	12	12	8	-
	July ’02	10	11	1	9
Kirkjubøur	Jan-feb ’02			6	-
	Apr-May ’02			8	7
	July ’02				13

Grey shadow: Not analysed

Prior to dissection the weight and length of the fish were measured. Then the fish was killed with a blow to the head. A blood sample of 1-5 ml was taken with a syringe and a BD vacutainer containing heparin (LH 143 I.U.). The blood sample was kept on ice until centrifugation (5 min. at 3000 rpm). The plasma was transferred into a cryo tube with a pasteur pipette and frozen in liquid nitrogen.

The liver and gall bladder were dissected. The gall bladder was transferred whole into a cryo tube and frozen in liquid nitrogen. When the gall bladder was too big to fit in to a cryo tube, it was pierced and the content carefully poured into the tube. Three samples of liver, approximately 1 g

each, were taken into cryo tubes from each fish (if there was sufficient liver tissue) and frozen in liquid nitrogen.

Sex was determined and the gonads were weighed. The gonads and the rest of the liver and the stomach, with contents, were stored in -20ºC and registered in the Environmental Specimen Bank for use in later studies.

Water temperature was not measured in connection with sampling, but the Faroese Office of Public Works makes regular measurements of sea-water temperatures (one meter above the sea-floor) at selected stations. The stations which are nearest to Kaldbak and Kirkjubø are "Strendur" and "Sørvágur" (see map in attachment 1). The mean sea-temperature for each month during 2002 is shown in attachment 2. The mean temperature in Strendur and Sørvágur respectively was 5,7-6,5ºC in January, 6,2ºC in April (only measured in Strendur) and 10,7-10,2ºC in July.

2.2 Analysis

The analyses were performed at NIVA (Norwegian Institute for Water Reseach) from the 26 August to the 21 September by Katrin Hoydal, under the supervision of the staff at NIVA, except for additional analysis of EROD activity in sculpin by HPLC method, which were performed by the National Veterinary Institute in Norway and the analysis for CYP1A protein, which were performed by NIVA.

The liver samples from the fish were analysed for EROD activity, CYP1A protein content and total microsomal protein content.
Preparation of the samples was done by homogenisation to attain cell lysis without affecting proteins and enzymes. The cytosol and organelles were separated with centrifugation as described in section 2.2.1 .
The protein content in the microsomal fraction of the liver was measured as described in section 2.2.2
The EROD activity was measured fluorimetrically as described in section 2.2.3.
CYP1A protein was analysed by an enzyme-linked immunosorbent assay (ELISA) as described in section 2.2.4
The bile samples were analysed for PAH-metabolites using HPLC with fluorescence detection. The preparation and analysis of bile samples are described in section 2.2.5 and 2.2.6.
The blood samples were analysed for vitellogenin content using an enzyme-linked immunosorbent assay (ELISA) as described in section 2.2.7.

2.2.1 Preparation of liver samples

The liver samples were thawed in ice and approximately 1 g of liver was weighed and put in a glass homogenising tube. A 0.1 M K-phosphate homogenising buffer was added until 5 ml of solution was obtained. The solution was homogenised using a motorised homogeniser (Potter-Elvehjem type) with at least ten passes with a teflon pestle. The homogenate was transferred to centrifugation tubes and centrifuged at 10000 *x* g at 4ºC for 30 min. The supernatant (PMS[5] fraction) was collected with a pipette, carefully avoiding the pellet and the floating lipid layer, transferred to new centrifugation tubes and centrifuged at 48000 *x* g at 4ºC for 120 min. The supernatant (cytosol

[5] Postmitochondrial supernatant

fraction) was removed and frozen at -80ºC and not analysed in this context. The pellet (microsomal fraction) was re-suspended in 1,5 ml re-suspending buffer and homogenised using a glass-teflon homogeniser. The homogenate was transferred to Eppendorf tubes and frozen at -80ºC for analysis for microsomal protein content (2.2.2) and EROD activity (2.2.3).

2.2.2 Protein

The microsomal protein content was analysed quantitatively by a modified method of Lowry et al. (1951). The analysis is based on the two-step reaction of protein with an alkaline copper tartrate solution and Folin reagent. The blue coloured end product can be measured at 750 nm. A standard curve was made using dilutions of Bovine gamma globulin protein standard in Tris buffer (0,125 – 1,00 mg/ml).
10 µl of diluted sample (the resuspended pellet obtained by the preparation of the liver samples) or standard were added to a microtiter plate. Then 25 µl of alkaline copper tatrate solution and 200 µl of diluted Folin reagent was added and the plate was mixed by the mixing function in the plate reader. After incubation for 15 minutes the absorbance was read in the plate reader at 750 nm. The protein concentrations in the samples could then be determined by reference to the standard curve.

2.2.3 EROD analysis

The EROD analysis was performed according to Eggens & Galgani (1992), which is a modified version of the method described by Burke and Mayer (1974), using a plate-reader. The reaction solution was made by diluting a stock solution of 0,2 mM 7-ethoxyresorufin in dimethylsulfoxide (DMSO) in 0,1 M K-phosphate buffer and the absorbance at 450 nm was measured. A standard curve of 8 standard solutions (0 – 0,64 µM) was made of a 1 mM stock solution of resorufin in DMSO diluted in K-phosphate buffer. The starting concentration (highest standard concentration) of 0,64 µM was obtained by adding 50 µl of 10 µM resorufin in DMSO to 5,2 ml K-phosphate buffer and the absorbance was read at 572 nm. The other standard solutions were then made by dilution of this solution in K-phosphate buffer. A 2,4 mM NADPH stock solution was made of 20 mg NADPH in 10 ml K-phosphate buffer. As ethoxyresorufin and resorufin are sensitive to light, the EROD analysis was protected from direct light.
If necessary the samples were diluted in K-phosphate buffer. 275 µl of standard was added to 16 of the wells in a microtiter plate (duplicates of each standard). 50 µl of buffer and 50 µl of sample (6 replicates) were added to the rest of the wells, 10 µl of 0,32 µM resorufin standard was added to half of the wells containing sample (three of the sample replicates). 200 µl of 7-ethoxyresorufin solution (reaction solution) and, to initiate the reaction, 25 µl NADPH solution was added to all the wells except for those containing standards. The plate was read immediately in a plate reader at exitation wavelength of 530 nm and emission wavelength of 590 nm and the EROD values could be calculated. The calculation of EROD results was done using the formula (Nilsen et al., 1998):

$$\text{pmol resorufin/min/mg protein} = F_S/\text{min} \times R/F_R \times 1/V_S \times 1/C_S$$

F_S/min = Increase in fluorescence per minute.
R = Amount of resorufin added as internal standard (pmol).
F_R = Increase in fluorescence due to resorufin standard.
V_S = Sample volume (ml).
C_S = Protein concentration of sample (mg/ml).

EROD analysed by HPLC

The sculpin results were generally negative, which shows that the method used was not sensitive enough for the sculpin analysis. The sculpin samples were, therefore, reanalysed at the National Veterinary Institute in Norway by HPLC as described in Ruus et al., 2002, with some modifications.

The 1,5 ml incubation mixture contained 0,1 M sodium phosphate buffer (pH 7,8), 5 µl 7-ethoxyresorufin(0,5 mM dissolved in DMSO) and 10 µl NADPH (10 mM). Reactions were initiated by adding 100 µl of microsomal protein solutions (corresponding to appr. 0,4 mg protein) and stopped with 1,5 ml of ice-cold methanol. The incubation time was 20 min. The vials were centrifuged at 3100 rpm for 30 min and the supernatant was transferred to HPLC vials. Resorufin was then quantified against known standards by the use of HPLC (5 µl injections, mobile phase: 40:60 v/v acetonitrile:water, flow:1.0 ml/min). The column used was a Symmetry® C18 (3.9 x 150 mm, 5 µm; Waters, Milford, MA, USA) and resorufin was detected on a Shimadzu RF-10A XL (Shimadzu, Kyoto, Japan) fluorescence detector. Excitation was at 535 nm and fluorescence emission was measured at 585 nm.

2.2.4 CYP1A protein

CYP1A protein content was measured by NIVA using an enzyme-linked immunosorbent assay (ELISA).

100 µl of coating buffer was added to the A1-D1 wells of the 96 well microtiter plates whereas 100 µl of reference sample was added to the E12-H12 wells. To the rest of the wells 100 µl of diluted sample was added (sample 1 in A2-D2, sample 2 in E2-H2, sample 3 in A3-D3 etc.) The plates were sealed and incubated at 4ºC over the night.

Then the plates were washed three times with TTBS (Tris-buffered saline solution (TBS) with 0,05% Tween-20). A 300 µl blocking solution (TBS with 1% Bovine Serum Albumin (BSA)) was added to all wells, and the plates were incubated for 30-60 min. at room temperature. The plates were again washed three times with TTBS. Then 100 µl of primary antibody (Anti-fish (CP226)) diluted 1:1000 in TTBS with 0,1% BSA, was added to all the wells and the plates were sealed and incubated at 4ºC over the night. The plates were washed three times with TTBS and 100 µl of secondary antibody (Sigma goat anti rabbit IgG HRP conjugated (GAR-HRP)) diluted 1:3000 in TTBS with 0,1% BSA, was added to all wells. The plates were sealed and incubated at 4ºC for 6-8 hours. After washing 5 times in TTBS, 100 µl of TMB-plus solution (0,04 % O-phenylene-diamine in 150 mM phosphate, 50 mM citrate buffer, pH 5,7, with 0,012 % hydrogen peroxide) was added to all the wells for color development, and the plates were incubated in the dark for 8-12 min. before adding the stop solution (1 M H_2SO_4). The absorbance was read in a platereader at 450 nm.

2.2.5 Preparation of bile samples

The gall bladder, with contents, had been frozen in cryo tubes, and the first step was to remove the bile from the bladder. This was done with a syringe with which the content of the bladder was transferred into new Eppendorf tubes. Several of the gall bladders, however, contained insufficient amounts of bile to be analysed.

For the preparation of the bile samples to be analysed, using HPLC with fluorescence detection, 0,05 ml of deionized water, 20 µl of sample, 20 µl of β-glucuronidase/aryl sulfatase, and 10 µl internal standard (triphenylamin) was added to the sample tubes. After mixing on a whirl mixer the samples were incubated at 37ºC for one hour. Then the samples were mixed again and centrifugated

at 4000 *x* g for ten minutes. The supernatant was transferred to HPLC vials and put in a freezer (-20ºC) until analysis the next day.

The remaining bile (if any) was analysed for relative biliverdin content by measuring the absorbance at 380nm of bile diluted in ethanol, using a spectrophotometer.

2.2.6 PAH-metabolites

The HPLC detector used in analysing the prepared bile samples, was a Waters High Performance Liquid Chromatograf consisting of: Waters 600 pump controller, Waters 470 Scanning Fluorescence Detector, Waters 717 Autosampler, Waters 490 Programmable multi-wavelength detector and a Waters column oven. 25 µl of sample is injected into the column. The mobile phase consists of acetonitril (ACN, Rathburn HPLC-grade), starting with 40% ACN in H_2O changing to 100% ACN during the running of one sample. The temperature of the column is 35ºC and there is a continuing degassing with helium during the analysis. The fluorescence detector works with exitation at 282 nm and an emission at 375 nm.
A standard curve of 11 standards, containing the metabolites that were analysed: 2-OH-naphtalene, 1-OH-fenantrene, 1-OH-pyrene, 3-OH-B(a)P and the internal standard: triphenylamin, was run prior to the samples, and one of the standards was run between every ten samples. The results reflect with retention times and peak highs the presence and concentrations of the different metabolites, compared to the standard curve and the level of the internal standard (triphenylamin). To six of the samples, however, which were prepared one day later than the others, internal standard was not added, and the results were found by running the standard curve again and comparing the values of the samples with the standard curve without adjusting for the internal standard.

2.2.7 Vitellogenin in blood samples

Analysis of vitellogenin in cod

Blood samples from cod were analysed using **competitive ELISA** (enzyme-linked immunosorbent assay) as described in Scott & Hylland (2002).
The 96 well microtiter plates were coated by adding 100 µl of standard cod antigen (Vtg), in the concentration 50 ng/ml, to the wells in the microtitre plate, except for the blanks and NSB (Non-Specific Binding) wells, to which only coating buffer was added. The plates were sealed and incubated at 4ºC over night.
Then the plates were washed three times with TTBS (Tris-buffered saline solution (TBS) with 0,05% Tween-20). A 250 µl blocking solution (1% Bovine Serum Albumin (BSA) in TBS without Tween-20) was added to block any remaining protein-binding sites in the wells, and the plates were incubated for 60 min. at room temperature. The plates were again washed three times and were then ready for primary antibody incubation. The samples were diluted 1:10 and 1:100 in TBS with 0,1% BSA. 100 µl TBS was added the blanks and 50 µl of TBS to the NSB wells. To all the other wells 50 µl standard or diluted sample were added. For each sample, an aliquot of sample, diluted 1:10, was added to 4 wells and the sample diluted 1:100 was added to 4 other wells. Primary antibody 50 µl of polyclonal cod antibody (CS-1)[6] was added to each well, except for the blanks. The plates were sealed and incubated at 4ºC over night.
The plates were washed three times with TTBS. Then 100 µl of secondary antibody (goat anti rabbit/HRP conjugate) was added at a dilution 1:15000 in TBS with 0,1% BSA. Only TBS with

[6] Polyclonal (rabbit) antibody against antigen of Atlantic cod (*Gadus morhua*).

0,1% BSA was added to the blanks. The plates were sealed and incubated for 6 hours at 4ºC. After washing 5 x 1,5 min. in TTBS the plates were ready for color development. 100 µl of TMB-plus solution (0,04 % O-phenylene-diamine in 150 mM phosphate, 50 mM citrate buffer, pH 5,7, with 0,012 % hydrogen peroxide) was added to all the wells and incubated in the dark for 8-12 min. before adding the stop solution (1 M H_2SO_4). The absorbance was read in a plate-reader at 450 nm and the concentration of vitellogenin was calculated using the standard curve.

Analysis of vitellogenin in sculpin and dab

Since there was no sculpin and dab antibody serum available, sculpin and dab samples had to be tested against the available antibody serums (Arctic char, sea-bream, wolffish and cod) to see how the sculpin and dab antigens reacted against them. For this purpose a **capture ELISA** test was used (Scott & Hylland, 2002; Specker & Anderson, 1994).

Optimalization

To find the optimal concentrations of antibody, 4 microtiter plates were coated by adding 100 µl of samples diluted 1:1000 in coating buffer. The samples were taken from three sculpins and three dabs: One reproductionally mature female, one less reproductionally mature female and one male. For each plate two columns of each of the selected samples were added. The plates were covered with plate-sealer and incubated at 4ºC over the night. Then the plates were washed three times with PBS (phosphate buffered saline solution) with 0,05% Tween and blocking solution (2% Bovine Serum Albumin (BSA) in PBS) was added. After an incubation for 60 min. at room temperature and an additional washing procedure, 100 µl of the four different antibody serums diluted in 1% BSA in PBS were added to the four different plates. To each row on the plate were added different dilutions of the antibody, the dilutions doubling for each row from 1:1000 (row A) to 1:128 000 (row H). The plates were sealed and incubated at 37ºC for 2 hours and after an additional washing procedure the secondary antibody could be added. 100 µl of goat anti rabbit/HRP conjugate diluted 1:3000 in 1% BSA in PBS was added to each well and the plates were sealed and incubated at 37ºC for 1 hour. Then the plates were washed five times with PBS solution with Tween and the developing procedure was done in the same way as for the competitive ELISA except that the developing reaction lasted 14 min. for all the plates before adding the stop solution. The plates were read in the plate-reader at 450 nm and from the results the best suitable primary antibody and the best suitable dilution could be chosen.

Parallellism

The chosen antibody serums were CS-3 (wolffish) and CS-1 (cod). To test, if the reaction of sculpin and dab were parallell to the cod, one reproductionally mature female and one reproductionally immature male of the three species were chosen. Two plates were coated with samples from those individuals with two columns for each individual. The samples were diluted, the dilution doubled for each row from 1:1000 (row A) to 1:128000 (row H). A capture ELISA test was performed, as described above, using the CS-3 antibody as primary antibody for one plate at a dilution of 1:32000 and the CS-1 antibody as the primary antibody for the other plate at a 1:16000 dilution. The secondary antibody was Goat anti Rabbit/HRP conjugate diluted 1:12000 for both plates. The results were plotted to see if the shape of the curves of sculpin and dab were parallel to the cod curve. If the curves were parallel, the cod standard curve could be used as a standard to get the relative concentrations of vitellogenin in dab and sculpin.

3 Results

Table 3.1 shows the number and size of the fish caught, along with the Condition Factor Index (CFI), Liversomatic Index (LSI) and Gonadosomatic Index (GSI). Sculpins from three periods were analysed: January, March-May and July, while cod and dab were analysed from two periods: April(-May) and July and flounder was only from February. Each fish species from one location was analysed (either Kaldbak or Kirkjubø) except dab, which was analysed from two locations (Kaldbak and Kirkjubø) in July. The individual data for the fish are given in attachment 3.

The length of the sculpins varied between 16,5–32,5 cm, and the weight between 66–600 g. The cod was from 35-58 cm and weighed 212-4050 g. The length and weight of the dabs varied between 24,5-38 cm and 200-750 g, respectively and the flounders were between 29-42 cm and 250-1334 g.

Table 3.1 Mean lenght and weight of fish caught in Kaldbak and Kirkjubø from January to July 2002.

Species	Location	Date	n		Length, cm	Weight, g	CFI[a]	LSI[b]	GSI[c]
Sculpin	Kaldbak	Jan. '02	4	Female	29,5±1,9	496±43,2	1,9±0,3	4,1±1,7	12,3±7,0
			2	Male	28,5±0,7	353±29,7	1,5±0,0	1,6±0,9	1,9±0,6
		Mar.-May '02	9	Female	25,0±5,0	283±145	1,6±0,3	1,3±0,3	1,6±1,5
			3	Male	24,5±2,3	230±94,9	1,5±0,3	1,2±0,1	1,0±0,6
		July '02	8	Female	25,6±3,8	289±111	1,6±0,2	2,1±1,4	1,0±0,5
			2	Male	27,0±5,7	384±306	1,7±0,4	1,8±0,6	1,1±0,4
Cod	Kaldbak	Apr.-May '02	6	Female	44,6±9,8	965±624	1,0±0,1	2,4±2,7	0,5±0,3
			6	Male	42,0±4,8	699±206	0,9±0,1	1,3±0,8	1,5±1,8
		July '02	6	Female	37,5±9,1	577±422	0,9±0,0	1,4±0,5	0,4±0,4
			6	Male	42,8±16,7	1226±1476	1,1±0,1	1,9±0,9	0,3±0,6
Dab	Kaldbak	July '02	6	Female	30,1±3,4	311±87,8	1,1±0,2	1,5±0,5	2,5±2,6
			3	Male	28,0±0,5	226±5,3	1,0±0,0	1,7±0,4	0,4±0,3
	Kirkjubø	Apr. '02	4	Female	35,3±3,0	595±179	1,3±0,2	1,7±0,2	14,2±3,3
			4	Male	29,5±1,7	262±62,4	1,0±0,1	0,9±0,2	0,6±0,4
		July '02	11	Female	32,0±2,3	371±93,7	1,1±0,1	1,7±0,7	1,2±0,5
			2	Male	30,5±1,4	340±48,1	1,2±0,0	1,5±0,1	1,0±1,4
Flounder	Kirkjubø	Feb. '02	0	Female	-	-	-	-	-
			6	Male	32,8±4,8	530±403	1,3±0,3	2,7±1,8	10,2±9,2

[a] Condition Factor index: total weight/total length^3
[b] Liversomatic Index: (liver weight/total weight)x100
[c] Gonadosomatic Index: (gonad weight/total weight)x100

As can be seen on the standard deviations there is great variation in the weights of the different groups, and for the cods also in the length. This is due to some large individuals which skew the sample pool. For the sculpins the variation in the weight can be due to the very variable stomach content which in some specimens could be a significant part of the weight. The length is therefore a better variable to look at when comparing the size of the specimens. Grafs of lengths versus weights for the different species can be seen in attachment 4.

The Gonadosomatic Index (GSI) is the weight of the gonads divided by the whole weight of the fish multiplied by 100, and the seasonal course of the GSI can be used as a measure of the reproductional cycle of the fish (Sabarowski et al., 1997).

Figure 3.1 and Table 3.1 shows the GSI of the fish sampled and the periods with increased gonad development can be distinguished. The sculpins from January, the flounders from February and the dabs from April had increased gonadal development, whereas in cod, only males from April-May had a slightly increased gonadal development. The GSI was significantly higher in females than in males dab from April-May (ANOVA P=0,0002), but in sculpins from January the difference between males and females is not significant.

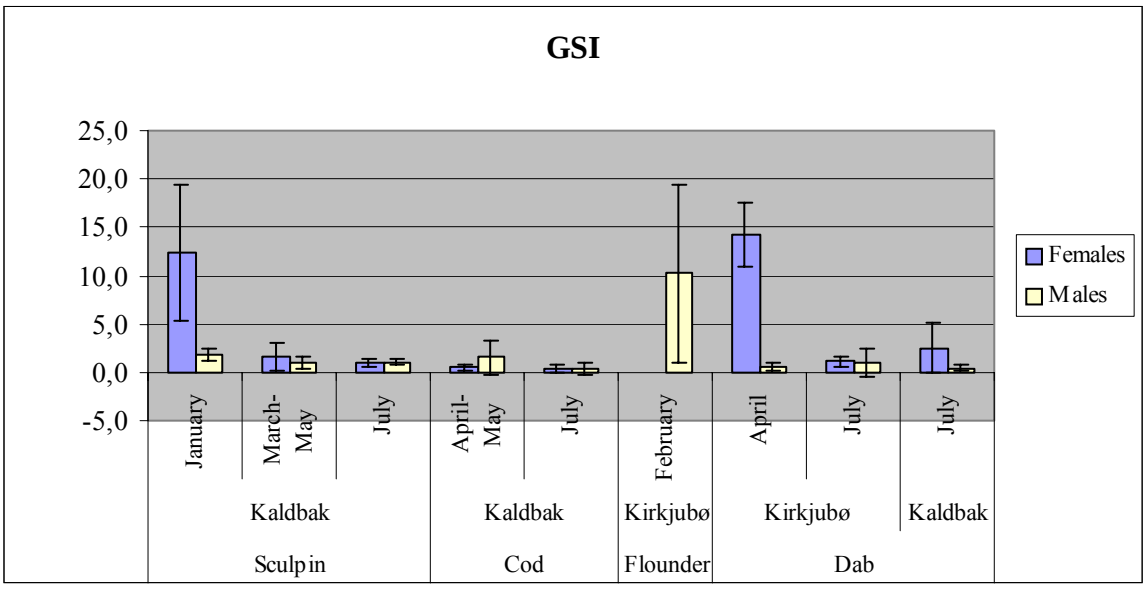

Figure 3.1 GSI in fish species from Kaldbak and Kirkjubø

3.1 P4501A induction

Induction of the P4501A (CYP1A) system was measured as catalytic activity by the 7-ethoxyresorufin-*O*-deethylase (EROD) assay, and in sculpin and cod also as CYP1A protein content.

3.1.1 CYP1A activity

Table 3.2 and Figure 3.2 show the mean values of EROD activity in the fish samples. As seen on the standard deviations there are very large variations in the results.

EROD activity was analysed in sculpin from Kaldbak in January, March-May and July. Of the fish species analysed sculpin showed the lowest EROD activities. The single values ranged from 0,0 to 52,2 pmol/min/mg protein in females and from 0,1 to 14,3 pmol/min/mg protein in males. The females had lowest mean activity in January and highest in July. Males had highest activity in January, the values being at the female July level, while male March-May and July levels were at the female January level. The number of samples is however low, and the variability high, and the difference in EROD activity between females and males is not statistically significant in any of the seasons (P>0,05).

Table 3.2 EROD activity in fish from Kaldbak and Kirkjubø

Species	Location	Sampling period	Gender	n	EROD (pmol/min/mg protein)	
					mean±std.dev	range*
Sculpin	Kaldbak	January	Female	4	0,4±0,2	(0,2-0,7)
			Male	2	7,1	(0,1-14,1)
		March-May	Female	9	2,1±4,6	(0,0-14,3)
			Male	3	0,2±0,1	(0,1-0,2)
		July	Female	8	7,2±18,2	(0,1-52,2)
			Male	2	0,3	(0,3-0,4)
Cod	Kaldbak	April-May	Female	6	65,2±110,0	(1,3-286,8)
			Male	6	64,3±78,2	(0,3-218,5)
		July	Female	6	42,0±54,9	(2,8-151,9)
			Male	6	36,5±28,9	(2,0-83,6)
Dab	Kaldbak	July	Female	6	139,2±159,8	(3,8-420,8)
			Male	3	27,5±12,2	(19,7-41,5)
	Kirkjubø	April	Female	4	3,2±1,5	(1,5-5,1)
			Male	4	83,0±27,7	(43,1-106,0)
		July	Female	11	199,4±263,5	(30,6-868,3)
			Male	2	94,9	(67,2-122,7)
Flounder	Kirkjubø	February	Male	6	23,8±15,2	(1,2-37,3)

*min-max values

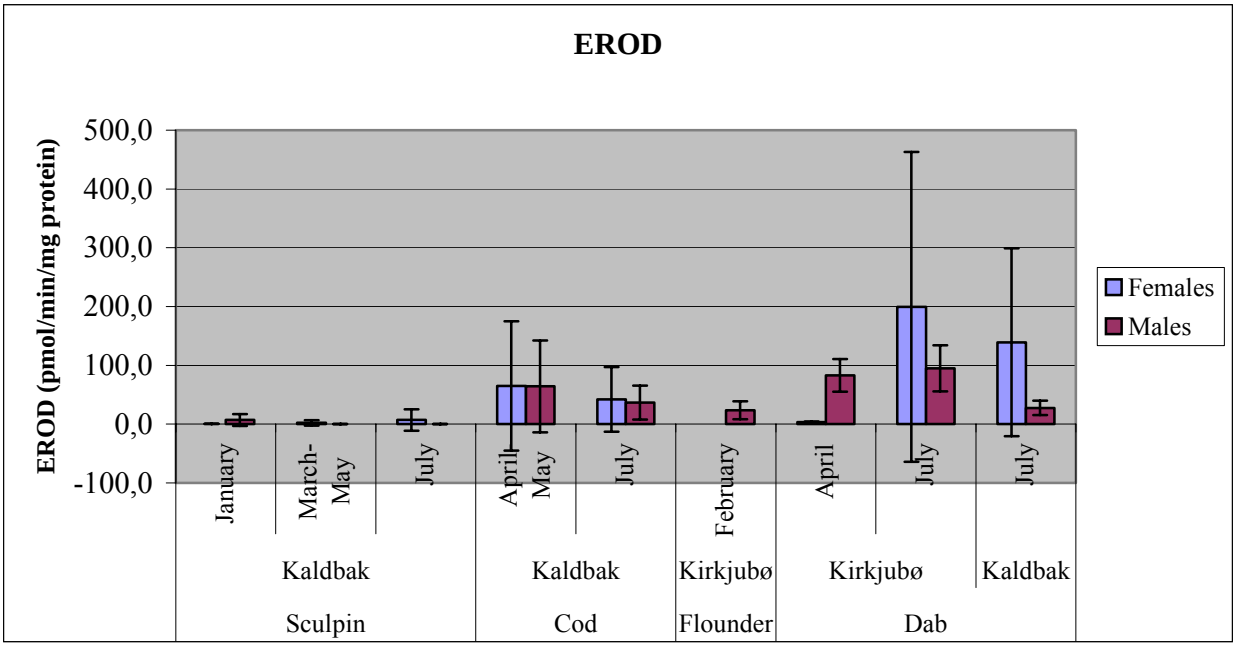

Figure 3.2 Mean EROD activity in fish from Kaldbak and Kirkjubø

EROD activity was analysed in cod from Kaldbak in April-May and July. In cod there appeared to be no difference in EROD activity between females and males. The levels seemed to be higher in April-May than in July, but not significantly higher. The levels were higher than in sculpins, ranging from 0,3-218,5 pmol/min/mg protein in males and 1,3-286,8 pmol/min/mg protein in females.

Generally, the highest EROD activities were found in dab, with the highest mean activity in females from Kirkjubø in July. The highest single value of 868,3 pmol/min/mg protein was found in a female dab caught in Kirkjubø in July. Hwever, the values show large variation and the lowest values for dabs in this period on this location was 30,59 pmol/min/mg protein, in a female. The female dabs seemed to have lower activity in April than in July, (although not significantly lower) the activity in males was at the same level both periods. The EROD activity in dab ranged from 1,5-868,3 pmol/min/mg protein in females and 19,7-122,7 pmol/min/mg protein in males.

In July dabs were analysed from two stations: Kaldbak and Kirkjubøur, and in both females and males the mean activities were higher in Kirkjubø than in Kaldbak. The differences between locations were not significant in either of the sex groups, but in males the difference was close to significant (P=0,06).

Flounder was only analysed from Kirkjubø in February and all specimens were males. The EROD activity ranged from 1,2-37,3 pmol/min/mg protein with a mean activity of 23,8 pmol/min/mg protein.

3.1.2 CYP1A protein

CYP1A protein was analysed in sculpin and cod. The results are shown in Table 3.3 and the number of individuals analysed (n) is in some of the groups lower than the number sampled, because of lack of liver tissue.

Table 3.3 CYP1A protein content in sculpin and cod from Kaldbak

Species	**Location**	**Sampling period**	**Gender**	**n**	**CYP1A (abs/mg protein)**	
					mean±std.dev	range
Sculpin	Kaldbak	January	Female	4	0,084±0,04	0,045-0,146
			Male	2	0,162±0,03	0,141-0,183
		March-May	Female	9	0,159±0,03	0,132-0,212
			Male	3	0,182±0,04	0,148-0,226
		July	Female	3	0,128±0,02	0,109-0,152
			Male	2	0,150±0,01	0,145-0,154
Cod	Kaldbak	April-May	Female	6	0,058±0,01	0,036-0,073
			Male	5	0,068±0,06	0,054-0,068
		July	Female	5	0,067±0,01	0,058-0,081
			Male	6	0,077±0,02	0,053-0,098

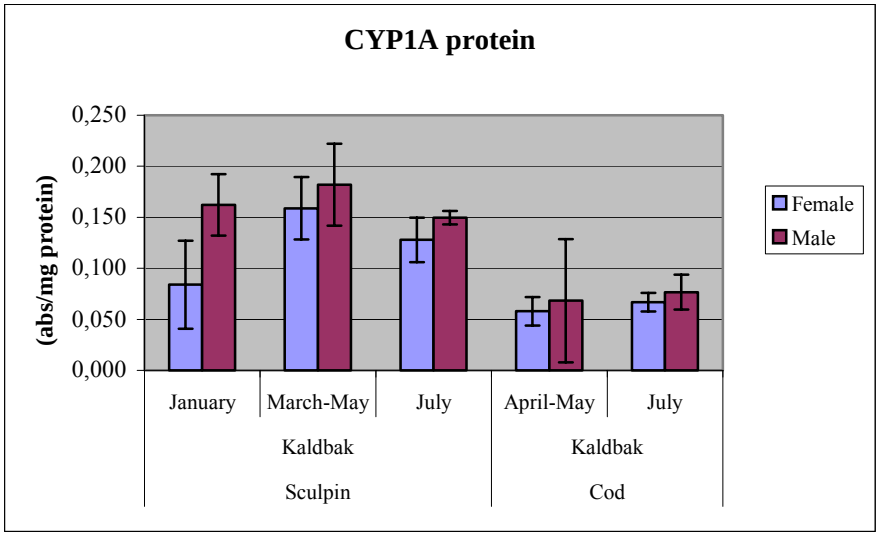

Figure 3.3 Content of CYP1A protein in sculpin and cod.

As shown above the mean values of CYP1A protein are generally higher in sculpins than in cod. For both cod and sculpins the mean values were lower in females than in males, but the difference between sexes was not significant (P>0,05).

3.1.3 EROD vs. CYP1A protein

Since the EROD and CYP1A protein analyses are measurements of the catalytic activity and concentration of the same proteins the two parameters would be expected to be correlated, if the catalytic activity has not been destroyed or suppressed. Figure 3.4 and Figure 3.5 show EROD activity versus CYP1A protein activity in sculpins and cod respectively.

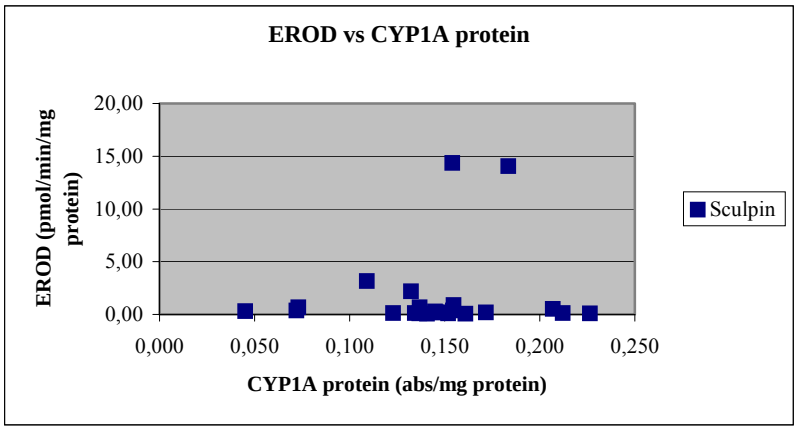

Figure 3.4 The correlation between EROD activity and CYP1A protein in sculpin.

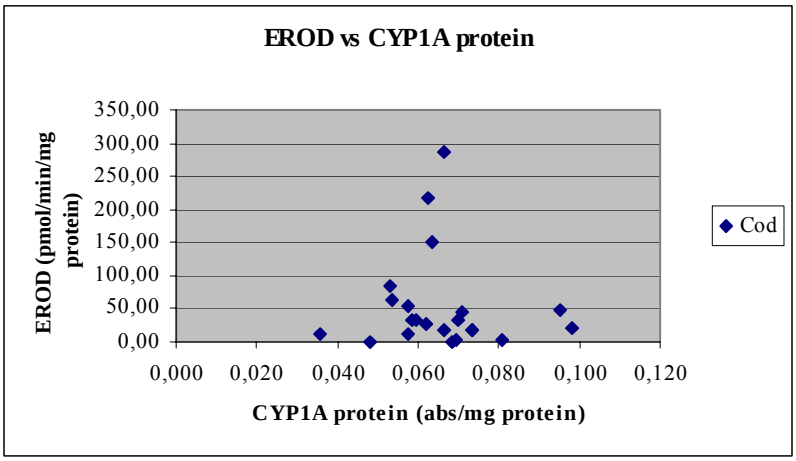

Figure 3.5 The correlation between EROD activity and CYP1A protein in cod.

As seen in the figures the two parameters do not seem to be correlated in either of the species (r^2=0,003 in cod and 0,018 in sculpin).The low EROD results in sculpin compared to the CYP1A protein results indicate that the catalytic activity has been destroyed in the sculpin samples and hence that the analysis of CYP1A protein is a better indicator of CYP1A induction in these samples than EROD.

3.1.4 CYP1A induction versus Gonadosomatic Index

The CYP1A induction can be affected by the gonadal maturation (Krüner & Westernhagen, 1999; Lange et al., 1998; 1999; Edwards et al, 1988; Lindström-Seppä & Stegeman, 1995). This is most likely due to the presence of sex steroids, which have been found to suppress the MFO expression (Förlin et al.,1984; Stegeman & Woodin, 1984). Hence the MFO activity will be low in pre-spawning and spawning females, when the GSI is high.

To investigate the correlation between these parameters, the EROD activity has been plotted versus the GSI for the different species in their spawning seasons. Figure 3.6 shows the EROD activity versus GSI in flounder from February.

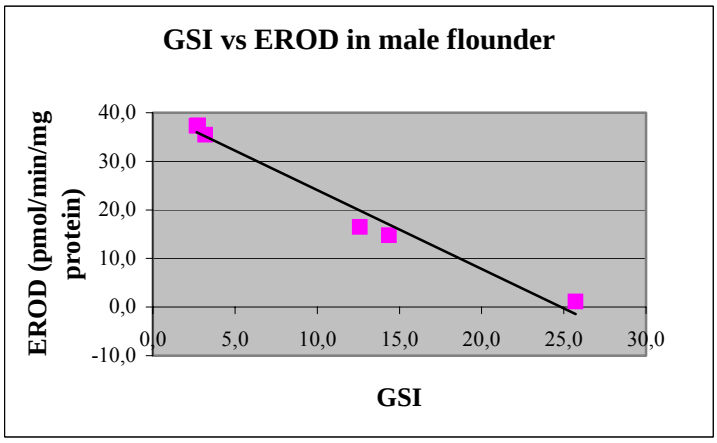

Figure 3.6 EROD versus GSI in male flounder in February 2002

As the figure shows there is a significant negative correlation between EROD and GSI in flounder (P=0,0002; r^2=0,98) (Figure 3.6).

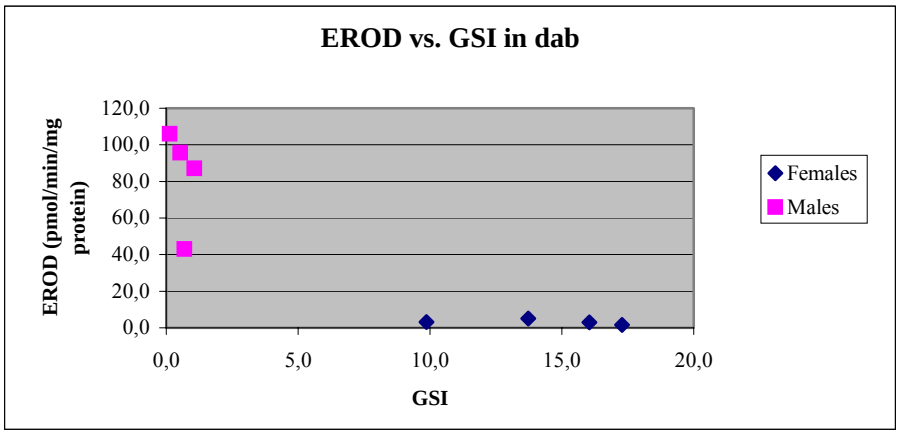

Figure 3.7 EROD versus GSI in dab from April 2002

In dab from April the results of EROD activity versus GSI are grouped into males and females, where the females have high GSI and low EROD activity and males have low GSI and high EROD activity (Figure 3.7). The values of EROD and GSI in female dab are not significantly correlated, although the EROD activity is low when the GSI is high and vice versa. The difference in EROD activity in female dab in April and July is not significant (P>0,05), while the GSI is significantly higher in April than in July (P=0,0000).

In sculpins the CYP1A protein content has been plotted against GSI instead of EROD, as these results seem to be more reliable than the EROD results as a measure of the MFO activity in sculpins. The CYP1A protein content and GSI are significantly correlated when lumping females and males (r^2=0,93 and P=0,002) and for females only (r^2=0,96 P=0,02).

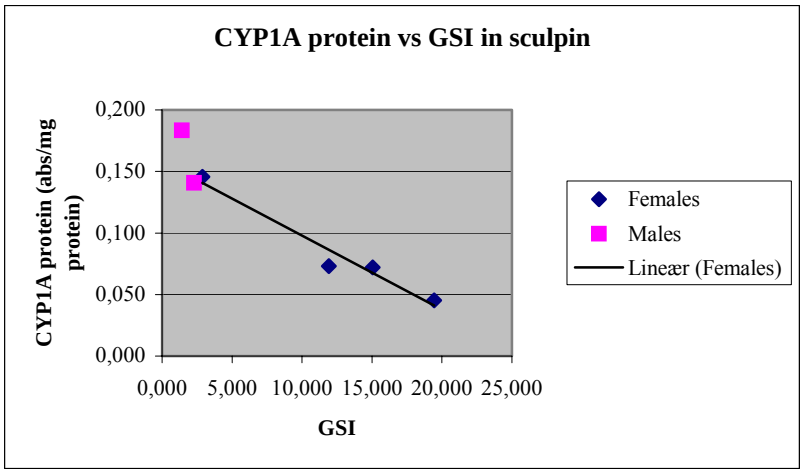

Figure 3.8 CYP1A content versus GSI in sculpins from January 2002

3.2 PAH metabolites

Of the PAH metabolites analysed only 1-OH-pyrene was found in non-negligible amounts. Table 3.4 and Figure 3.9 show the content of 1-OH-pyrene found in the fish.

Table 3.4 1-OH-pyrene in fish from Kaldbak and Kirkjubø

Species	**Location**	**Sampling period**	**Gender**	**n**	**1-OH-pyrene** (µg/kg)			**n**	**1-OH-pyrene** norm. (µg/kg/abs 380nm)
					mean±std.dev	range			mean±std.dev
Sculpin	Kaldbak	January	Female	4	43,7±24,1	(15,2-72,8)		4	2,81±0,7
			Male	1	18,3	-		1	1,9
		March-May	Female	4	18,7±12,2	(4,5-33,7)		3	0,9±0,6
			Male	2	5,3±0,8	(4,7-5,9)		1	0,5
		July	Female	7	21,4±29,3	(1,8-81,0)		4	0,3±0,4
			Male	1	23,0	-		1	1,5
Cod	Kaldbak	April-May	Female	6	14,0±12,7	(4,6-39,2)		6	0,7±0,4
			Male	6	10,5±3,6	(5,0-15,0)		4	0,6±0,4
		July	Female	3	10,4±10,8	(4,0-22,8)		3	0,6±0,5
			Male	5	11,1±12,6	(3,5-33,4)		4	0,9±1,1
Dab	Kaldbak	July	Female	6	14,1±17,9	(3,8-50,1)		5	0,2±0,2
			Male	3	27,8±24,3	(13,2-55,8)		3	0,4±0,2
	Kirkjubø	April	Female	2	2,4±0,3	(2,2-2,6)		1	0,7
			Male	3	8,3±9,2	(2,9-18,9)		2	3,7±5,0
		July	Female	7	4,8±4,3	(0,9-11,2)		5	0,5±0,6
			Male	2	2,0±1,1	(1,2-2,7)		1	0,4
Flounder	Kirkjubø	February	Male	1	322,0	-		1	6,1

Several of the gall bladders contained too small amounts of bile to be analysed, and, therefore, the numbers of individuals analysed (n) is somewhat lower in most of the groups than the number sampled.

As the concentration of metabolites in bile can be influenced by differences in bile density, the results were normalized by dividing the results by the absorbance at 380nm. The normalized results are shown in Table 3.4 (grey shaded), but as this resulted in even less number of samples (n) because of small amounts of bile fluid, those results are not treated further.

Only one of the flounders had enough bile to be analysed and the 1-OH-pyrene concentration was much higher in this individual than other groups. The concentration in flounder was 322,0 µg/kg which is nearly 3,5 times higher than the second highest single value of 72,8 µg/kg found in a female sculpin in January. However when looking at the normalized data, the difference between the level in flounder and in sculpins in January is not that big, indicating that the bile fluid in this particular flounder is highly concentrated.

Disregarding the flounder, the highest values of 1-OH-pyrene were found in sculpins, ranging from 4,5-72,8 µg/kg. In male groups the numbers analysed were only 1 or 2 and although the mean values seem to be higher than in the female groups the male values were within the range of the females.

The range of 1-OH-pyrene in cod was between 3,5-39,2 µg/kg, and there seems not to be any difference between males and females or season.

In dab the values of 1-OH-pyrene ranged between 1,2-18,9 in Kirkjubø and between 3,8-55,8 in Kaldbak. There seemed not to be any difference between the values in males and females in any of the groups.

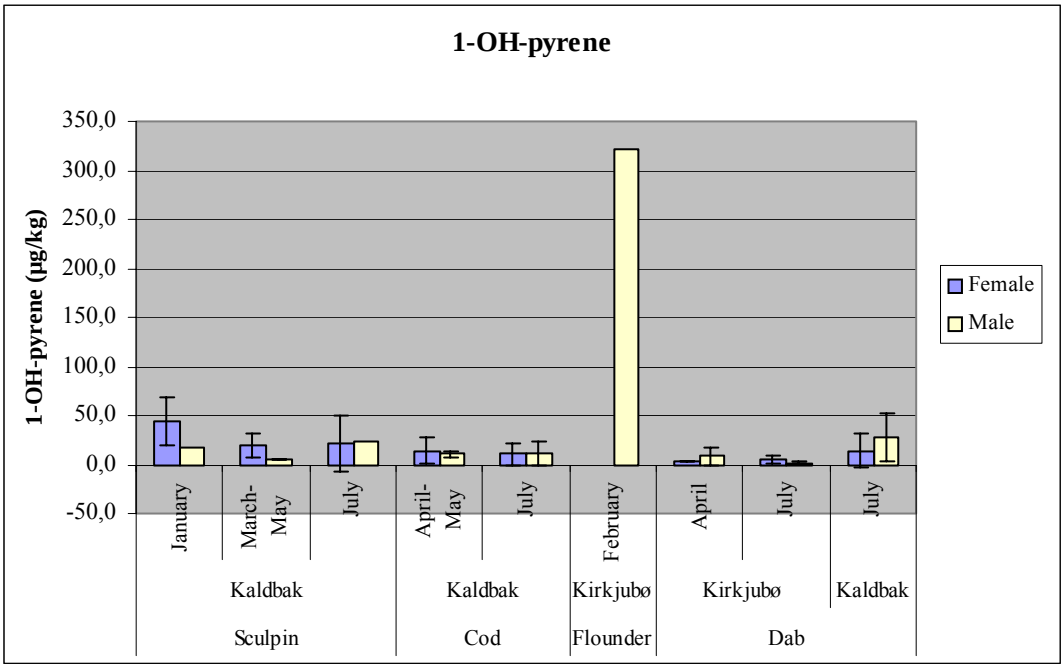

Figure 3.9 Mean concentrations and standard deviations of 1-OH-pyrene in the fish samples

In July dab from both Kaldbak and Kirkjubø was analysed and the 1-OH-pyrene content was significantly higher in dabs from Kaldbak than in dab from Kirkjubø ($P<0,05$).

3.2.1 CYP1A induction versus pyrene

PAH compounds metabolised by the cytochrome P450 enzyme system may be excreted by the organism via the bile. Hence, if the PAH exposure has occurred recently a correlation between CYP1A induction and concentration of PAH metabolites in bile should be expected.

The correlation between EROD or CYP1A protein and 1-OH-pyrene in the fish groups, not affected by spawning, are shown in Table 3.5 and Table 3.6.

Table 3.5 Correlation between EROD and 1-OH-pyrene for different fish groups

EROD vs pyrene			f(x)	r^2	P(2 tail)
Cod	Kaldbak	April-May	-0,0108x + 12,994	0,01	0,74
		July	-0,104x + 16,294	0,18	0,29
Dab	Kaldbak	July	-0,0515x + 23,91	0,13	0,34
	Kirkjubø	July	0,0164x + 1,9834	0,41	0,06

Table 3.6 Correlation between CYP1A protein and 1-OH-pyrene for different fish groups

CYP1A protein vs pyrene			f(x)	r^2	P(2 tail)
Sculpin	Kaldbak	March-May	0,0015x + 0,1455	0,56	0,09
		July	0,0003x + 0,1198	0,23	0,52
Cod	Kaldbak	April-May	-0,0006x + 0,0671	0,34	0,07
		July	0,0007x + 0,0654	0,23	0,27

The PAH metabolite content in the bile and the CYP1A induction in the liver does not seem to be correlated for the species caught in either of the locations. Only the correlation between EROD and OH-pyrene in dab from Kirkjubø in July was nearly significant (r^2=0,41 P=0,06) (Figure 3.10), as well as the correlation between CYP1A protein and OH-pyrene in sculpin from Kaldbak in March-May (r^2=0,56 P=0,09) (Figure 3.11).

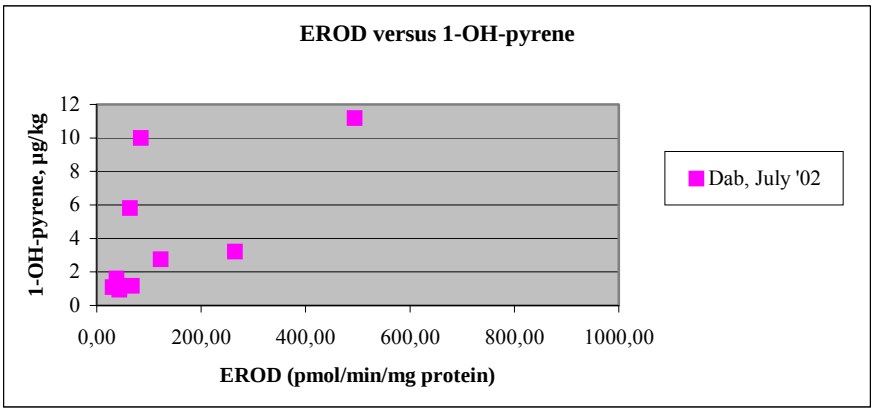

Figure 3.10 The correlation between 1-OH-pyrene and EROD activity in dab from Kirkjubø

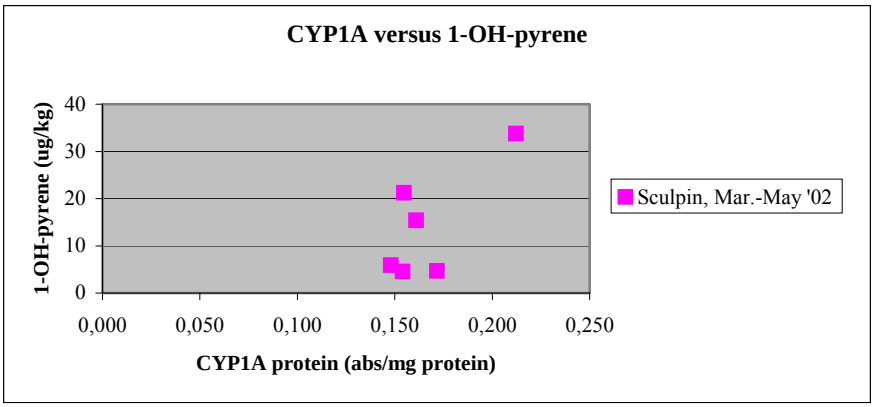

Figure 3.11 The correlation between 1-OH-pyrene and CYP1A protein content in sculpin from Kaldbak in March-May.

3.3 Vitellogenin

The results from the vitellogenin analyses in cod are given in Table 3.7 and Figure 3.12. Two samples from the group of females in April-May were discarded because their optical density values were below the standard curve, both when diluted 100x and 10x. These should have been reanalysed undiluted, but this was not done.

The levels seem to be higher in July than in April-May and the variations seem to be larger in July. There was no significant difference between females and males.

Table 3.7 Vitellogenin in cod from Kaldbak

Sampling period	**Gender**	**n**	**Vtg** (ng/ml)	
			mean±std.dev.	(range)
April-May	Female	4	1978±347	1563-2394
	Male	6	1758,3±452	1163-2445
July	Female	6	4183±4001	784-8790
	Male	6	2878±2117	798-6070

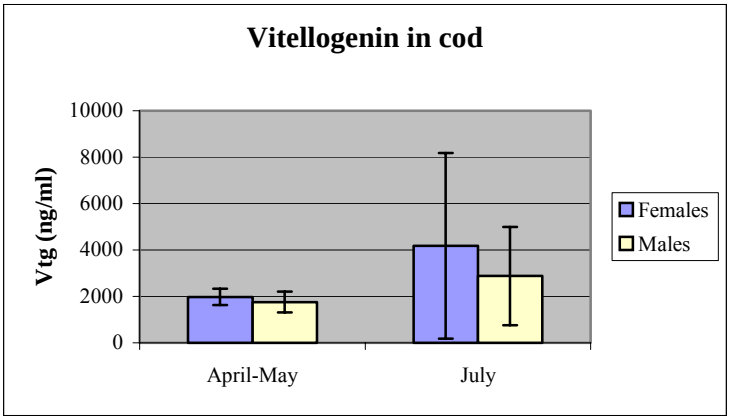

Figure 3.12 Vitellogenin in cod from Kaldbak

Since there was no sculpin and dab antibody serum available, only cod were analysed for vitellogenin. Sculpin and dab antigens were however tested against the available antiserums to see which of them gave the best reaction. Against the two antigens which gave the best response (cod and wolffish) the sculpin and dab antigens were tested along with cod antigens to see if the response matched the cod response and a relative value could be measured. The response in dab did match the cod response, whereas the sculpin response did not. The results are given in attachment 5. Relative vitellogenin, values therefore, can be measured in dab using cod antibody, but this was not done because of lack of time and resources.

4 Discussion

The analyses of biomarkers show the level of contamination in fish to PAHs in the coastal zone of the Faroe Islands.

Sampling
Initially the plan was to sample sculpin and dab in Kaldbak and Kirkjubø respectively, over three seasons in one year. This to be able to determine seasonal differences. Unfortunately, we were only able to get few samples in the first sampling period and we only caught flounder. After changing sampling methods in the second sampling period we were able to catch dab with a by-catch of other species (flounder and cod). Therefore, only sculpins are sampled in all three seasons, whereas dab and cod are only sampled in spring and summer. To reduce the number of samples the only flounders analysed were those from Kirkjubø in late winter (February). This means that data on seasonal variation are limited for cod and dab. Dab was analysed from both locations in July making it possible to compare the two locations.

Variation
The results generally show large variations within the groups. Especially the EROD results vary within the groups, but also the data on concentration of pyrene-metabolites and the vitellogenin from July. The large variations are probably, in part, due to the small sample sizes. The samples size are between 6 and 12 individuals in the groups for each species, at each location, each season. These have furthermore been divided into females and males. Larger sample sizes would facilitate statistical analyses. Vandermeulen & Mossman (1995) also found large variation in the groups when analysing for MFO activities in winter flounder (Pleuronectes americanus) although they had 30 individuals (males and females) in their groups. They explained the variation as individual physiological differences or differences in inductive response.

Variability in metabolite content in bile can be due to differences in the feeding status of the fish (Beyer, 1996) resulting in differences in bile density. Normalising for bile density reduced variations for some groups, but in other groups the variations became larger. However, many of the fish did not have a sufficient amount of bile to allow the measurement of the absorbance at 380nm. The sample numbers for normalized data became very small, making it difficult to compare them to the un-normalized data. It has been shown that errors are introduced by normalizing the data (Aas et al., 2000b). The coefficient of variation (Std.dev./mean) increases by normalisation with respect to biliverdin concentrations, and this is more pronounced for groups with low PAH exposure.

GSI
As the sampling was performed only 1-3 times a year for each species it is difficult to distinguish between different reproductive periods. According to Sabarowski et al. (1997) the reproductive cycle in dab can be classified into four periods: Pre-spawning, spawning, post-spawning and resting. The pre-spawning period is characterised by an increase in ovary weight (and thereby in GSI), whereas the spawning period is characteized by a decrease in GSI due to the release of eggs. The beginning of the spawning period is defined as the maximum GSI of females. In the post-spawning period the gonads re-develope an remain in a state of resting until the pre-spawning period (Sabarowski et al., 1997).

When we see an increase in GSI for one species, this could reflect both the pre-spawning and spawning period since we do not have monthly samplings and are not able to determine the maximum level of GSI. According to Joensen & Tåning (1969) the spawning period in flounder and sculpin is in from winter to early spring, while spawning in dab starts in April (peaks in May). This means that sculpin from January and flounder from February should be spawning and dab from April should be at the beginning of the spawning period. However, monthly measurements of GSI in dab from the same area as in this study have been done by Dam (2000) in 1996-97. This study showed that the spawning season seemed to be in March (or February) in 1996 and even earlier in 1997 (January - February). Hence the time of spawning can vary between years. Our results however show a GSI of approximately 14 in female dab in April. This is at the same level as the peak in GSI shown by Sabarowski et al. (1997) indicating that the dabs from April in our study were at or near at their peak in GSI.

Cod did not show elevated GSI in females in any of the two sampling periods. This is to be expected, because the cod in the coastal stage is believed to be immature. The GSI of males in spring (April-May) is however slightly elevated indicating that some of the individuals have reached maturity. The red variety of cod (reyðfiskur) used in this study normally lives near the coast until it reaches 1-1½ years of age, when it migrates to spawn and live among the cod on the continental shelf. The size of the fish is around 15-38 cm when it is between 1 and 2 years old (Steingrund, pers. comm.). The age of the fish has not been determined in this study, but the cod used was 35-72 cm indicating that some of them are older than 1-2 years and may have reached maturity.

CYP1A induction

The EROD activity was generally very low in sculpins. When using the fluorescence plate-reader method, most of the results were inconclusive. This was also observed by Stephensen et al. (2000), who analysed EROD activity in sculpins on Iceland.Others have found elevated levels of EROD in sculpin (e.g Ruus et al., 2002). The lack of correlation between EROD activity and CYP1A protein in sculpins indicates that the catalytic activity has been destroyed or suppressed. The loss of catalytic activity could be due to non-optimal freezing, transport and storage conditions (Stephensen et al., 2000) since EROD activity has been shown to be sensitive to such conditions (Förlin & Andersson, 1985) or due to other contaminants such as heavy metals (Goksøyr et al., 1989; Beyer et al., 1997; Sandvik et al., 1997). These can have effects on the heme synthesis resulting in distorted forms of cytochromes P450 with no enzymatic activity. The distorted forms of cytochrome P450 will however still be detected by immunochemical analysis of CYP1A protein (Goksøyr et al., 1989). Also some organochlorines can inhibit the catalytic activity of induced P-450 (Gooch et al., 1989). If the lack of catalytic activity is due to the freezing, transport and storage conditions, the sculpins seem to be more sensitive than the other species analysed in this project, since all the fish were treated the same way and the other species showed higher EROD activity.

The EROD activity in flounder at reference stations have been reported to be 91$\pm$41 pmol/min/mg protein (Addison & Edwards, 1988) and 39$\pm$19 pmol/min/mg protein (Stegeman et al., 1988) in Langesundfjorden and 4$\pm$1 pmol/min/mg protein (Beyer et al., 1996) in Sørfjorden in Norway. Our results in flounder are comparable to Stegeman et al. (1988) although one should bear in mind that our results are influenced by spawning.

The EROD results in female dab from July, both from Kaldbak and Kirkjubø, exceed these levels. In the other groups the results seem to be comparable. However EROD has been found to be 3-4

times higher in dab than in flounder when sampled under same conditions (Krüner & Westernhagen, 1999). Taken this into consideration the dab results seem to be comparable to the results of Stegeman et al. (1988). Ruus et al. (2003) reported EROD in dab levels ranging from 123-529 pmol/min/mg protein. Our levels are much lower. However, our results have large variations, and some of the individual results are in the high end of or above this range. The higher EROD activity in male than in female dab earlier reported (Krüner & Westernhagen, 1999; Lange et al., 1999; Goksøyr et al., 1992) was not found in this study.

Ruus et al. (2003) reported EROD concentrations in cod from reference stations in the range 9-95 pmol/min/mg protein. Our results in cod are at the same level or lower, except for three individual values, which are above that range. A study of the pollution status of the harbour in Tórshavn[7] included analyses of EROD activity in cod (the coastal stage "reyðfiskur") in June, and found a mean concentration of 68 pmol/min/mg protein (Dam & Danielsen, 2002). Comparing this to our results from July shows, as would be expected, that the EROD activity in cod from Kaldbak is lower, and the cod here is less exposed to EROD inducers than cod from the harbour of Tórshavn.

No correlation was found between the EROD activity and CYP1A protein content in cod and sculpin. As mentioned above, this indicates that the low EROD activities in sculpin are due to catalytically inactive enzymes, which also explains the lack of correlation between the EROD measurements and the CYP1A protein. It is more puzzling that there was no correlation between these parameters in cod, as several individuals had high EROD activities but relative low CYP1A protein content. Goksøyr et al. (1989) found good correlation between EROD activity and ELISA CYP1A protein in dab, but not in flounder and plaice (Pleuronectes platessa).

The EROD activity was generally higher in females than in males in the species analysed, except in the spawning season, during which the males had higher activity than the females. This is not in accordance with the general findings that males have higher EROD activity than females. For the CYP1A protein contents, on the other hand, the mean values were higher in males than in females in all the groups analysed.

CYP1A induction versus GSI

The CYP1A induction was influenced by female gonadal development in flounder, dab and sculpin. An inverse relationship has been shown between GSI and EROD in the periods of developing ovaries in flounder (Khan & Payne, 2002) and in dab (Lange et al., 1998) and this was also found in this study for dab and flounder. In April the EROD of female dab was only 3,8% of that in males, and 1,6% of the activity in females in July. In male dab the April activity was 87% of that in July. The GSI in males was significantly lower than in females in April. This is however normal as shown by Saborowski at al. (1997) where maximum GSI in male dab did not exeed 3,0 at any of the monthly sampling dates during the reproduction cycle. Lindström-Seppä & Stegeman (1995) found that GSI in pre-spawning flounder was three times higher in females than in males and EROD activity in females was only 3,5% of that in males. This is similar to our results and can be explained by the fact that hormonal factors such as estradiol are supressing CYP1A induction in female liver (Lindström-Seppä & Stegeman, 1995).

Cod did not show elevated GSI in females in either of the two sampling periods, which is expected as the cod living near the shores is believed to be immature. The GSI of males in spring (April-

[7] The capital of the Faroe Islands

May) is however slightly elevated indicating that some of the individuals have reached maturity. The red variety of cod (reyðfiskur) used in this study normally lives near the coast until it reaches 1-1½ years of age, and the size is around 15-38 cm (Steingrund, 2002). The age of the fish was not determined in this study, but the cod used was 35-72 cm indicating that some of them are older than 1-2 years and may have reached maturity.

PAH metabolites

1-OH-pyrene was the only metabolite detected in bile of the fish analysed. OH-pyrene is the major biotransformation product of pyrene in teleosts (Beyer, 1996). Only one of the flounders had sufficient amount of bile to be analysed and the concentration of OH-pyrene found was markedly higher than in the other species. Since we only have one measurement it is impossible to say if the OH-pyrene content is higher in flounders in general or only in this individual. The EROD activity was low in this individual, and combined with the findings of a high density of biliverdin in the bile sample, this indicates that the pyrene metabolites do not stem from a recent exposure but are rather a result of concentration of the bile fluid and of PAH metabolites in it.

The pyrene metabolite concentration in the sculpin, cod and dab were in the lower end compared to data from Norway in 1997-2001 (Ruus et al., 2003) (normalized data). In the study of the pollution status of the harbour of Tórshavn PAH metabolites were also analysed in cod. The results showed a mean concentration of1-OH- pyrene of 66 µg/kg (Dam & Danielsen, 2002) and are, as would be expected, at a much higher level than our results, which had a mean around 11µg/kg in July.

EROD versus pyrene

No correlation between EROD activity and OH-pyrene content in bile was found in any of the species analysed, but in Kirkjubø there was a some correlation in July, although this was not significant. Correlation between the two parameters would indicate recent contamination of PAH (Ruus et al., 2003). Aas et al. (2000a) found significant correlation between EROD activity and PAH metabolites in bile in cod exposed to crude oil. The lack of correlation, found in our study, could be due to other inducing agents, as EROD activity, in addition to PAH, can also be induced by other planar molecules such as dioxins and planar PCBs.

Vitellogenin

Vitellogenin (Vtg) levels were only measured in cod. This was the only species analysed where antibodies were available. The vitellogenin concentrations did not differ significantly between sexes or seasons. This is what would be expected as the fish are believed to be immature and no gonadal maturation should be going on in the females. The vitellogenin levels are however very high. Hylland and Haux (1997) analysed vitellogenin in cod exposed to sewage effluents and seawater. The vitellogenin in their unexposed control group did not exceed 100ng/ml whereas our mean results are from around 2000-4000 ng/ml, with individual concentrations ranging from 800 ng/ml to almost 9000 ng/ml. Mean levels of vitellogenin in cod from the harbour of Tórshavn was 4948 ng/ml (Dam & Danielsen, 2002) which is only slightly higher than the results from July and covered a similar range of vtg-concentrations as in the present study.

Locations

Comparison of the results from the two locations show that the OH-pyrene concentrations in dab from July were higher in Kaldbak than in Kirkjubø. For EROD activity the mean values were higher in Kirkjubø than in Kaldbak. This is in accordance with the observed lack of correlation between EROD activity and level of OH-pyrene, a finding that indicates that the PAH contamination that created the pyrene metabolites is not due to recent exposure, and that the EROD activity may have been induced by other contaminants, for instance PCBs.

5 Conclusion

The present investigation shows the level of several biomarkers in different fish species. Conclusions on the suitability of the different species as test organisms for the different biomarker analyses can also be drawn from the results. A larger number of individual analyses than in this study, are, however, required in order to determine the baseline levels of the biomarkers, as the results showed large variation.

The levels of CYP1A induction and the content of 1-OH-pyrene in bile, seem to be at levels comparable to or lower than the levels reported from clean reference sites in Norway, although there is large variation in the results with some high individual levels.

It is therefore suggested that one of the species analysed in the present study is subjected to further investigations, where a larger number of individuals are analysed and where effort is focused on minimizing the influence of parameters that affect the variability. To minimize the effects from influencing parameters the sampling should be performed once a year in a season not influenced by gonadal development.

The species best suited for further analyses is dab (*Limandalimanda*) or cod (*Gadus morhua*). Dab seems to be the most sensitive of the species analysed. The MFO measurements in dab are however markedly influenced by the spawning season and show different levels for males and females. Therefore, if dab is to be used, the sampling should be performed in the non-spawning (resting) period (July or Aug.-Sept.).

Cod are immature when living near the coast and not influenced by seasonal differences in the reproduction cycle or difference between the sexes. In favour of cod is also that in this study all the biomarker analyses have been performed on cod, because no vitellogenin and CYP1A protein data are available for dab near the Faroese coast at present.

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Attachments