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# Biomarkers in monitoring

## A review

Photo: Thomas Lang, BFA-Fi, Cuxhaven, DE

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# REPORT

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Abstract

Biological effects are increasingly used to monitor impacts of contaminants in marine ecosystems. Biomarkers have been defined as "biochemical, cellular, physiological or behavioural variations in the tissue or body fluids or at the level of whole organism that provide evidence of exposure to chemical pollutants, and may also indicate a toxic effect". The biomarkers reviewed here were PAH bile metabolites, cytochrome P4501A, glutathione *S*-transferase, markers for DNA damage (adducts, alkaline unwinding, Comet assay), micronucleus formation, peroxisomal proliferation, acetyl cholinesterase inhibition, metallothionein, vitellogenin and delta-aminolevulinic acid dehydratase. Focus for the review was dose-response relationships, confounding factors, links to population effects, baseline values, assessment criteria and quality assurance for the relevant biomarker. Whereas correlative links to population-relevant effects have been found for some biomarkers, e.g. biomarkers for DNA damage, most biomarkers are generally more useful as markers for possible impacts elsewhere in ecosystems.

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| 1. biomarker | 1. biomarker |
| 2. overvåking | 2. monitoring |
| 3. offshore | 3. offshore |
| 4. økologiske effekter | 4. ecological impact |



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# Biomarkers in monitoring

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# Preface

The project was initiated by OLF (Unn Orstein) to produce a review of methods relevant to water column monitoring with a focus on interpretation and ecological links. Laurence Pinturier, Total, has been OLF's contact with the project group. The project has been shared between NIVA and IRIS with Ketil Hylland, NIVA, as lead author.

Rogaland Research were responsible for the review of PAH metabolites (Lars-Petter Myhre with inputs from Ketil Hylland) the Comet assay (Renée Bechmann) and micronucleus analyses (Jan Fredrik Børseth and Janina Barsiene). NIVA was responsible for editing, DNA adducts, vitellogenin, metallothionein and ALA-D (Ketil Hylland), cytochrome P4501A activity (Anders Ruus and Ketil Hylland), acetyl cholinesterase inhibition (Anders Ruus and Merete Grung), MXR and peroxisomal proliferation (Merete Grung) and glutathione *S*-transferase (Knut-Erik Tollefsen).

Oslo, 9.3 2006

*Ketil Hylland*

# Contents

# Summary

Biological effects are increasingly used to monitor the influence of contaminants in marine ecosystems. Biological effects may conveniently be divided into community or population responses, bioassays and biomarkers. While community responses are widely used to address impacts on benthic communities, biomarkers have been used to investigate effects in fish or mussels. This review concerns biomarkers with a focus on methods that have been used in monitoring effluents from offshore activities. The methods included were PAH bile metabolite concentration, cytochrome P4501A activity, glutathione *S*-transferase activity, two methods to address DNA damage (DNA adducts and the Comet method), micronucleus formation, multixenobiotic response, acetyl cholinesterase inhibition, vitellogenin concentration, metallothionein concentration and delta-aminolevulinic acid dehydratase inhibition. Histological assays, including lysosomal stability, were not included in this review.

Biomarkers may be viewed as "biological responses that can be related to an exposure to, or toxic effect of, an environmental chemical or chemicals". This is a very wide definition, since "biological responses" can refer to responses at any hierarchical level, from molecular processes to the ecosystem. This project used the following definition: "biochemical, cellular, physiological or behavioural variations in the tissue or body fluids or at the level of whole organism that provide evidence of exposure to chemical pollutants, and may also indicate a toxic effect". In addition, methods were selected for their previous use in monitoring effluents from offshore activities or for their potential in this context.

For each method, the review focused on dose-response relationships, confounding factors, links to effects at population level, baseline levels, assessment criteria and quality assurance.

From previous experience and existing data, all the reviewed biomarkers could conceivably be relevant for use in monitoring offshore effluent environmental impacts. Although it is not possible to establish direct links between responses in any of the biomarkers and population effects, there is support through correlation for a predictive ability for some biomarkers in that regard, e.g. DNA adducts and the Comet assay. Although presumably not critical in adult fish, vitellogenin induction will indicate levels of estrogens that may have population knock-on effects through changes in sex ratio. Similarly, micronucleus formation in mussels indicates the presence of substances that may cause chromosomal damage to other marine organisms and conceivably affect their offspring. Responses in other biomarkers included herein, i.e. cytochrome P4501A activity, metallothionein, AChE and ALA-D, will in most cases indicate contaminant-induced changes, but there are no clear links to population effects. Two of the biomarkers reviewed, peroxisomal proliferation and multixenobiotic resistance, have not been used to a large extent in monitoring and should be further reviewed when more data are available.

Baseline values and assessment criteria could be established for some species for limited range of the methods, i.e. PAH bile metabolites, cytochrome P4501A activity (tentative), vitellogenin, metallothionein and ALA-D.

Quality assurance programmes have until now included PAH bile metabolites, cytochrome P4501A activity, vitellogenin, metallothionein and ALA-D. There have also been some international intercalibration activities for AChE.

# 1. Introduction

## 1.1 Background – biomarkers

There is a need to identify whether anthropogenic activities affect natural ecosystems, both because humans have a moral responsibility not to damage the environment and because environmental deterioration may be in conflict with other human uses of the ecosystem. For management purposes there is a need both to be able to quantify the distribution, bioaccumulation and biomagnification of contaminants as well as their effects on marine organisms.

Methods to identify biological effects can be conveniently grouped as shown in Table 1. In addition to the methods shown, biological components are also included in sensors (biosensors) or sensors may be used to monitor biological processes in real-time.

Table 1. A grouping system for biological effects methods.

| designation | examples | positive properties | negative properties |
|---|---|---|---|
| ecosystem | none | no generally accepted methods | |
| population/community<br><br>(large samples of organisms from defined areas) | benthic community<br>rocky shore community | ecologically relevant | strongly affected by non-contaminant parameters<br>difficult to standardise<br>large natural variability |
| bioassays<br><br>(sample or extract from the field is tested in biological system in lab) | Acartia 96-hr test<br>Corophium sediment test<br>Skeletonema 96-hr test<br>fish hepatocyte cytoxicity test | easy to standardise<br>ecologically relevant endpoints | robust species (not ecologically relevant)<br>not contaminant-specific |
| biomarkers<br><br>(health assessment of individual organisms) | EROD<br>DNA adducts<br>metallothionein<br>AChE<br>ALA-D<br>Comet assay | easy to standardise<br>related to individual health<br>contaminant-related<br>species similarities (some methods) | limited ecological relevance<br>species differences (some methods) |

There are several definitions for “biomarkers”. According to Peakall (1994) biomarkers are “biological responses that can be related to an exposure to, or toxic effect of, an environmental chemical or chemicals”. This is a very wide definition, since “biological responses” can refer to responses at any hierarchical level, from molecular processes to the ecosystem. Other definitions are more specific, such as that of Mayer et al. (1992) which states that biomarkers are “quantifiable biochemical, physiological, or histological measures that relate in a dose- or time-dependent manner to the degree of dysfunction that contaminants have produced”. The non-governmental organisation English Nature has defined biomarkers as:

“**biochemical, cellular, physiological or behavioural variations in the tissue or body fluids or at the level of whole organism that provide evidence of exposure to chemical pollutants, and may also indicate a toxic effect**”.

This definition complies well with the selection of methods for this review. In addition, methods were selected for their previous use in monitoring effluents from offshore activities or for their potential in this context.

Any biological effect method should ideally have a number of characteristics, outlined in e.g. Stegeman *et al.* (1992):

1 The assay to quantify the biomarker should be sensitive, reliable, and relatively simple;
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 adaptation; and finally,
6 changed levels of the biomarker should be correlated with the "health" or "fitness" of the organism.

Very few of the methods used actually comply with all those requirements, but it can be useful to keep such an "ideal" in mind while reviewing different techniques.

## 1.2 The need for baseline data and assessment criteria

Although it is generally agreed that biological effects results should not be used or assessed singly there are currently no generally agreed frameworks for such assessments. Current activities within OSPAR (WKIMON) may generate a framework; the results from that activity are due in 2006. Until such a framework is available, biomarker methods have to be assessed singly. This means that in any given study, there should be criteria agreed on *a priori* as to whether the result is "an effect" or not. In the scientific literature, and in biomonitoring, this has generally been achieved through hypothesis testing – asking the question "is group B different from the reference or control group"? There could however be biological reasons to state that only biomarker responses at least 5 or 10 times those measured in a reference group would be indicative of an impact.

Depending on the method, there are two strategies for the assessment of single biomarker responses:

- observed biomarker responses can be compared against a global criterion or
- observed biomarker responses must always be compared to responses in a reference group sampled at the same time.

An assessment is obviously simpler if the former strategy is possible; this will however only apply to methods and species for which there is extensive knowledge of all factors that may affect the response (in addition to contaminants). In most cases this will also be methods for which there are limited species differences.

The latter strategy must be chosen for methods for which there is not sufficient information about confounding factors, for which it is not possible to correct for such factors or for which exogenous factors may affect the response in non-predictable ways (but similarly at exposed and reference locations).

## 1.3 Links between biomarkers and ecologically relevant endpoints

As indicated above there is not a direct link between most biomarker responses and population effects, although it is generally thought that biomarker responses will be predictive of such impacts in one way or another. It is this elusive link that ecotoxicologists have been searching for during the past two decades.

There are however good reasons why this link is so elusive; marine organisms that may be weakened for some reason or other (e.g. contaminants) will not be as apparent as e.g. a weakened fish eagle or polar bear. It will simply disappear, eaten because avoidance responses were too slow or starved to death and then eaten. The implications are that we are always sampling the healthiest part of any population in field campaigns.

It is by definition not possible to measure early and late effects in the same individuals and ecologically relevant parameters (increased mortality, reduced reproductive output) can not be determined under field conditions for most species. One possible way to establish links is through multi-year, -method and –species research studies, but it will still only be causality within a population. There is really only one such situation in the world, the work by NOAA in Puget Sound, recently reviewed by Myers et al. (2003). In their work they have been able to show causality between (petrogenic) PAHs in sediment, PAH-metabolites in bile, cytochrome P4501A induction, DNA adduct formation and development of liver cancer (neoplasms) in flatfish. Since they have been able to include many species they have also been able to see that cancer develops in some species, but not in others. A second possible approach is to perform long-term mesocosm experiments under semi-natural conditions. Such studies will obviously be open to the criticism that fish (or other organisms) were unable to migrate as they normally would. Furthermore, due to space limitations it is never possible to have an appropriate replication in such systems. The most well-known such study was done by RIKZ in the Netherlands. They exposed flounder and plaice to contaminated and reference sediments for two years (Vethaak et al., 1996). From this extensive mesocosm experiment they were able to support a suggested link from PAH in sediment to liver cancer development (Eggens et al., 1996). Some of the steps in between did not fit the model, possibly due to adaptation.

A follow-up issue is the question of whether it is really necessary to find direct links between a response in a fish species and the population of that very same fish, as suggested under item (6) above (the ability to relate a response to health or fitness of the organism under study). A response in the fish indicates that contaminants are present in the ecosystem at sufficient levels to modulate normal metabolism in this species. Other species or life-stages in the ecosystem may be more strongly affected and the biomarker response is simply a marker for this risk. That said, there is obviously a need to qualify such an assessment in terms of which other species could be at risk.

This discussion will be continued for each of the biomarkers in the following chapters.

## 1.4 Quality assurance

If biomarker results are to be comparable from year to year and between laboratories it is imperative that both internal and external quality assurance procedures are in place. International organisations, e.g. OSPAR, AMAP, ICES, MEDPOL, have focussed on this issue over the past decade.

Over the past 10 years there has been considerable international activity to harmonise protocols and intercalibrate biomarker methods, primarily in the framework of the BEQUALM, first an EU-funded project and now a self-funding venture (http://www.bequalm.org).

# 2. PAH metabolites in bile

## 2.1 Introduction

Analyses of PAH metabolites in fish bile have been used as a biomarker of exposure to PAH contamination since the early 1980s. The presence of metabolites in bile (and in urine) is the final stage of the biotransformation process whereby lipophilic compounds are transformed to a more soluble form and then passed from the organism in bile or urine. This review will focus on metabolites in bile since this is the method that has been most widely used and is most relevant for offshore monitoring.

As a biomarker of exposure, measuring PAH metabolites in bile has many advantages over other techniques that require sophisticated tissue preparation protocols. The pretreatment of bile samples requires relatively simple dilution steps prior to analysis by direct fluorescence measurement. The bile is diluted in methanol : distilled water (1:1) and fluorescence is measured with a fluorometer. Fixed wavelength fluorescence is a suitable screening method for samples while HPLC/F or GC-MS SIM is utilized for qualitative and quantitative measures.

Bile is generally stored in the gall bladder prior to episodic release into the esophagus where bile salts have a function to perform as part of the digestive process. This period of storage permits a degree of accumulation of metabolites and hence an increase in their concentration. The periodic release of bile does however introduce a variable into the technique, which must be accounted for. The feeding status of fish has been shown to influence both the volume and the density of the bile (Collier and Varanasi, 1991).

The ability of fish to biotransform PAHs into less lipophilic derivatives means that reliance on the detection of parent PAHs alone may lead to an underestimation of the *in vivo* exposure level of PAH in the fish. PAH metabolite detection, on the other hand, represents a quantification of the flux of PAHs streaming through the fish's body. From a toxicological point of view, flux information is more relevant for estimating the actual biotic stress due to PAH exposure, than the body burden data of the unmetabolised parent PAH compounds in tissues (most often liver). Despite this, body burden measurements are still more commonly used within monitoring studies than metabolite determination.

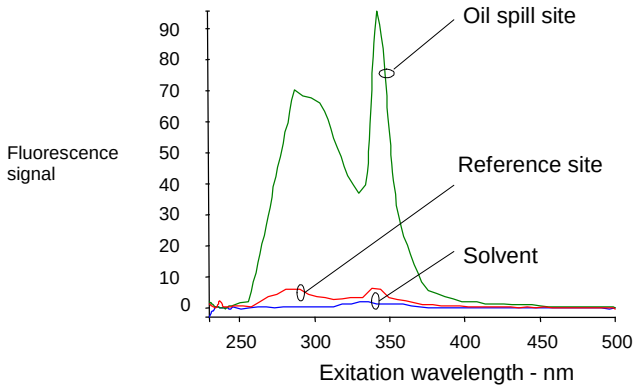


Figure 1. Synchronous fluorescence screening of bile samples from fish at a reference site compared with fish taken at a site polluted with an oil slick. Data courtesy of Endre Aas, IRIS-Akvamiljø.

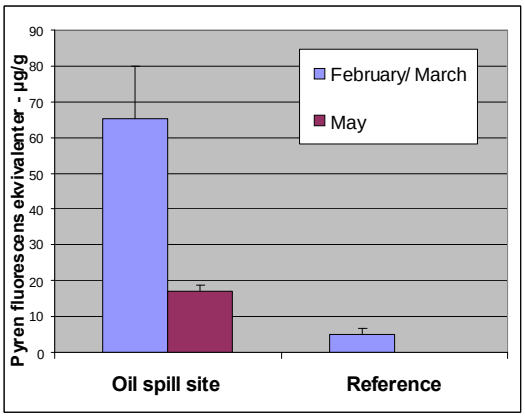


Figure 2. Fixed fluorescence detection of bile samples from fish at a reference site versus bile from fish at a field site polluted with an oil slick. Data courtesy of Endre Aas, IRIS-Akvamiljø.

## 2.2 Dose-response (species specific)

The PAH compounds are metabolised rapidly in the organisms and it is the endpoint of this metabolisms that is measured in the bile. The compounds are measured using chemical analysis. A consistent dose-response relationship has been demonstrated in laboratory studies between PAH exposure and the subsequent presence of metabolites in bile (Beyer et al.,1997; Sandvik et al., 1997; Aas et al., 2000). To establish a good dose-response relationship in field studies it is necessary to focus on aspects that influence the excretion of bile.

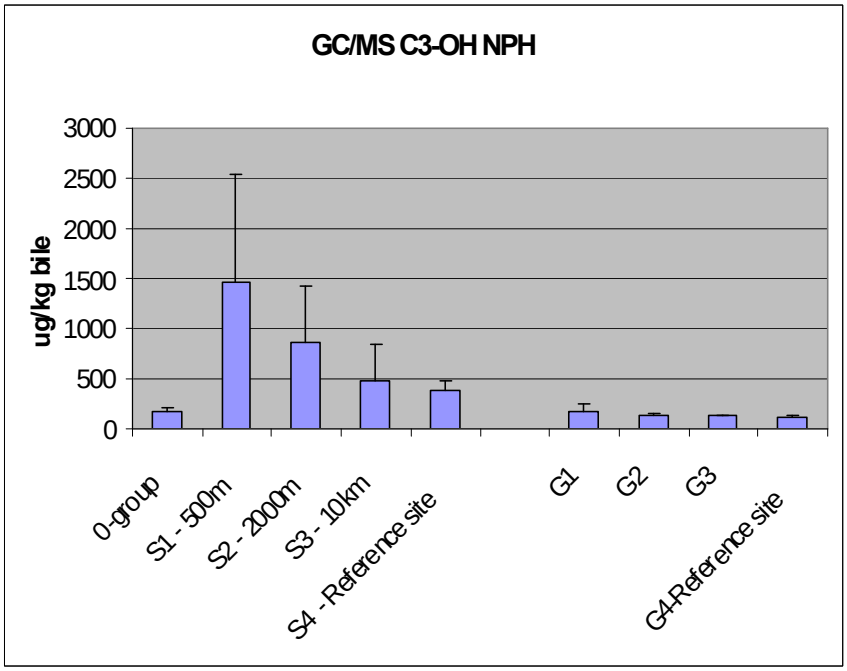


Figure 3. GC/MS quantification of metabolites of alkylated naphthalene in bile from fish caged downstream from the discharge of produced water at the Tampen field (BECPELAG field study in 2001). Stations G1-G4 are situated in the southern North Sea (Aas et al., in press).

The method requires that bile is available in the gall bladder. Since the fish renew bile as part of normal metabolism and excrete it during digestion, it is important to know about the dietary status of the organism to establish a dose- response relationship. If the fish feed just before sampling, the gall bladder may become more or less empty. After the gall bladder has been emptied it will fill up and metabolites will be concentrated up to a plateau level corresponding to the exposure regime. Consequently the time since last digestion is important for the dose-response relationship. Fish generally have a very efficient metabolic excretion of most PAHs and it has been shown that most of the PAH will excreted after 2 – 8 days following exposure. This means that the PAH metabolites determined in bile will represent exposures on the scale of days and, at most, two weeks.

It has been shown in several field and laboratory studies that there is a good correlation between PAH exposure and bile metabolites (see Table 3). Because of the rapid metabolism and the correlation between bile content and digestive status it is difficult to make a dose-response relationship that can be used to quantify the exposure. Work has been done to try to correlate bile metabolite concentration to digestive status, by correlating it to the amount of protein or biliverdin in the bile. Absorbance at 380 nm is also used (similar to biliverdin) (Hylland, unpublished). This normalisation is not standardised because it has been shown to only explain parts of the variability, but it is recommended to be part of the explaining factors in the interpretation of results. In laboratory studies it is normal to stop the feeding some days before sampling to ensure the bile quality. In field sampling this can be taken into account by letting the fish go some days in tanks before sampling, but this has some logistical challenges.

## 2.3 Species sensitivity

The responsiveness of bile metabolites towards pollution is shown to be good in several species. In this overview we have focused on cod, haddock, polar cod and sheepshead minnows (the latter is a model species from the American continent). The background level differs from species to species so it is important to establish good baseline before using new species. It may be expected that species with fatty livers, i.e. most gadiids, may metabolise PAHs more slowly as more will partition into fat, but this has not been documented experimentally.

## 2.4 Relevance of other factors

As mentioned above, food availability will affect the concentration of PAH metabolites in bile. In an assessment of data for more than 500 individual cod sampled through five years of national monitoring, variables such as size/age and sex explained some variability in multiple regression models (Ruus et al., 2003). This could be due to different feeding preferences, but also endogenous processes. In addition, the fat-content of the liver (measured as liver-somatic index, LSI) came out as significant, presumably because fat decreases the availability of PAH to the cellular compartments of liver cells.

## 2.5 Links

PAH metabolites are biomarkers of exposure. Consequently it is only possible to link the PAH metabolites in bile indirectly to effects by correlate it to other expected effects that will appear with exposure to PAHs. But it is important to bear in mind that the occurrence of PAH metabolites in bile indicate a biotransformation process of the corresponding PAHs in the organism. Thus any implications for fitness related to induction of enzymes, such as EROD, are equally appropriate if the final products of this process can be measured in the bile. Establishing links between bile metabolites and fitness parameters will require a similar approach to that taken for the previous biomarkers, chronic, low dose exposures in the laboratory and the examination of endpoints related to growth and reproduction in association with regular analysis of bile profiles. Manipulation of feeding regimes as part of this process may throw additional light on the relationship.

## 2.6 Background responses

Baseline levels of PAH metabolites have been established for many of the species relevant for monitoring in Norwegian coastal and offshore waters. From Ruus et al. (2003) values for the relevant species are: (all values standardised to absorbance at 380 nm) Atlantic cod: 0.6-4 µg/kg bile, flounder 27-89 µg/kg bile, dab 3.1-34 µg/kg bile, plaice 0.4-3 µg/kg bile (all quantified using HPLC separation and fluorescence detection and quantification). Standardisation at 380 nm is used to remove variability due to bile salts.

## 2.7 Assessment criteria

It is possible to establish global criteria for individual PAH metabolites. Baseline data for individual species may be used to test against to determine whether fish have been exposed to PAHs. As mentioned above, some variation in PAH metabolites in bile appear to be related to

sex and size/age (Ruus et al., 2003), knowledge of which should be included in the sampling design.

## 2.8 Quality assurance

A general protocol outlining analytical strategies and their strengths as well as weaknesses has recently become available (Ariese et al., 2005). There have been international intercalibration exercises for the determination of PAH-metabolites in fish bile, arranged in collaboration between an EU-project and QUASIMEME[1]. Reference bile samples were generated as part of the aforementioned EU project and are now available at IRMM, JRC, Geel, Belgium (http://www.irmm.jrc.be/html/homepage.html).

[1] QUASIMEME – organisation that offers quality assurance for chemical endpoints; http://www.quasimeme.org

Table 2. Overview of field and laboratory studies – PAH metabolites measured by fixed fluorescence.

| Species | Substance (lab/field) | Test concentrations/area | Exposure time | Metabolite | Baseline | control or reference | exposed /control | reference/comments |
|---|---|---|---|---|---|---|---|---|
| Cod (Gadus morhua) | Feral fish | Barents Sea | Baseline | | | | | Aas et al., 2003 |
| Cod (Gadus morhua) | Feral fish | Egersund | Baseline non polluted area | Naph type | 5.3 ug/ml | | | Klungsøyr et al. 2003 |
| | | | | Pyren type | 0.8 ug/ml | | | |
| | | | | BaP type | 0.4 ug/ml | | | |
| Cod (Gadus morhua) | Feral fish | Sleipner | Baseline polluted area? | Naph type | 6.1 ug/ml | | | Klungsøyr et al. 2003 |
| | | | | Pyren type | 1.0 ug/ml | | | |
| | | | | BaP type | 0.5 ug/ml | | | |
| Cod (Gadus morhua) | Feral fish | Statfjord | Baseline polluted area? | Naph type | 5.9 ug/ml | | | Klungsøyr et al. 2003 |
| | | | | Pyren type | 0.9 ug/ml | | | |
| | | | | BaP type | 0.3 ug/ml | | | |
| Cod (Gadus morhua) | Feral fish | Frøy, ceased installation 10 000 m (ref) 2000 m - 200 m | Baseline polluted area? | Naph type | | 3.9 ug/ml | 1.1 - 1.1 | Beyer et al. 2003 |
| | | | | Pyren type | | 0.6 ug/ml | 1.1 - 0.9 | |
| | | | | BaP type | | 0.3 ug/ml | 0.9 - 0.9 | |
| Cod (Gadus morhua) | Feral fish | Barents sea | Baseline | Naph type | 2,15 ug/g | | | Sundt, 2002 |
| | | | | Pyren type | 1,63 ug/g | | | |
| | | | | BaP type | 0,69 ug/g | | | |
| Cod (Gadus morhua) | Feral fish | Barents sea | Baseline | Naph type | 5,8 ug/g | | | Aas & Børseth, 2002 |
| | | | | Pyren type | 1,7 ug/g | | | |
| | | | | BaP type | 0,8 ug/g | | | |
| Cod (Gadus morhua) | Laboratory | 1 ppm crude oil Statfjord B | 14 days | | | | | Aas et al., 2002 |
| Cod (Gadus morhua) | Laboratory | 0.06 - 0.25 - 1 ppm Oil | average 3, 7, 14, 24 days | Naph type | | 3,9 ug/g | 7,5 - 23,7 - 31,4 | Skadsheim et al., 2004 |
| | | | | Pyren type | | 2,6 ug/g | 3,6 - 10,6 - 13 | |
| | | | | BaP type | | 1,0 ug/g | 1,7 - 2,4 - 2,2 | |
| Cod (Gadus morhua) | Laboratory | 0.06 - 0.25 - 1 ppm Oil | average 3, 17, 31 day | Naph type | | *53.1 ug/g* | 0.7 - 2.3 - 2.9 | Skadsheim et al., 2004 |
| | | | | Pyren type | | 7.0 ug/g | 1 - 2.9 - 3.3 | |
| | | | | BaP type | | 1.0 ug/g | 1.1 - 1.5 - 1.5 | |
| Cod (Gadus morhua) | Laboratory | Oil 0.06 - 0.25 - 1 ppm | 30 days | Naph type | | 7.1 fi | 5.1 - 9.5 - 227.5 | Aas et al. 2000 |
| | | | | Pyren type | | 2 fi | 6.4 - 12.7 - 43.3 | |
| | | | | BaP type | | 0.8 fi | 2.3 - 3.6 - 9.6 | |

| Species | Substance (lab/field) | Test concentrations/area | Exposure time | Metabolite | Baseline | control or reference | exposed /control | reference/comments |
|---|---|---|---|---|---|---|---|---|
| Cod (Gadus morhua) | Laboratory | PW Oseberg, 1:1000 - 1:200 - 0.2 ppm oil - 0.2 ppm oil + PAHmix | 15 days | Naph type | | 12.6 ug/ml | 1.3 - 2.5 - 3.6 - 5.4 | Sundt, 2004 |
| | | | | Pyren type, | | 4 ug/ml | 1.7 - 3.7 - 4.1 - 17.8 | |
| | | | | BaP type, | | 1.8 ug/ml | 1.3 - 1.8 - 1.5 - 2.4 | |
| Cod (Gadus morhua) | Field, Caged | North Sea - Statfjord, 10000 m - 2000m - 500 m German bight G | 5.5 weeks | Naph type | 7.5 ug/ml | 0,7 | 1.7 - 1.9 - 2.1 | Aas et al., in press |
| | | | | Pyren type | 3.1 ug/ml | 0,7 | 1.2 - 1.5 - 1.6 | |
| | | | | BaP type | 1.2 ug/ml | 0,8 | 1.2 - 1.1 - 1.2 | |
| Cod (Gadus morhua) | Field, Caged | German bight G4 (Ref) G1 - G2 - G3 | 5.5 weeks | Naph type | 7.5 ug/ml | 0,4 | 0.9 - 0.9 - 1.6 | Aas et al., in press |
| | | | | Pyren type | 3.1 ug/ml | 0,5 | 0.8 - 0.9 - 1.7 | |
| | | | | BaP type | 1.2 ug/ml | 0,7 | 0.8 - 1 - 1.3 | |
| Cod (Gadus morhua) | Field, Caged | North Sea - Troll, 1000 m - 500m | 6 weeks | Naph type | 4.6 ug/ml | 1,4 | 1.7 - 2.5 | Børseth et al., 2004 |
| | | | | Pyren type | 2.4 ug/ml | 0,9 | 1.1 - 1.3 | |
| | | | | BaP type | 0.9 ug/ml | 1,1 | 1.1 - 1.3 | |
| Cod (Gadus morhua) | Field, Caged | North Sea - Tampen, 10000 - 2500 - 1000 – 500 | 6 weeks | Naph type | | 8.8 ug/ml | 1.0 - 1.5 - 1.2 - 1.2 | Hylland et al., 2005 |
| | | | | Pyren type | | | | |
| | | | | BaP type | | 1.4 ug/ml | 0.9 - 0.7 - 0.8 - 0.9 | |
| Haddock (Melanogrammus aeglefinus) | Feral fish | Egersund | Baseline non polluted area | Naph type | 5.1 ug/ml | | | Klungsøyr et al. 2003 |
| | | | | Pyren type | 1.4 ug/ml | | | |
| | | | | BaP type | 0.7 ug/ml | | | |
| Haddock (Melanogrammus aeglefinus) | Feral fish | Sleipner | Baseline polluted area? | Naph type | 6.8 ug/ml | | | Klungsøyr et al. 2003 |
| | | | | Pyren type | 1.9 ug/ml | | | |
| | | | | BaP type | 0.8 ug/ml | | | |
| Haddock (Melanogrammus aeglefinus) | Feral fish | Statfjord | Baseline polluted area? | Naph type | 11.2 ug/ml | | | Klungsøyr et al. 2003 |
| | | | | Pyren type | 2.5 ug/ml | | | |
| | | | | BaP type | 0.7 ug/ml | | | |
| Haddock (Melanogrammus aeglefinus) | Feral fish | Barents sea | | Naph type | 2.52 ug/g | | | Sundt, 2004 |
| | | | | Pyren type | 1.69 ug/g | | | |
| | | | | BaP type | 0.77 ug/g | | | |
| Haddock (Melanogrammus aeglefinus) | Feral fish | Barents sea | | Naph type | 2.0 ug/g | | | Aas & Børseth, 2004 |
| | | | | Pyren type | 1.3 ug/g | | | |
| | | | | BaP type | 0.6 ug/g | | | |
| Haddock | Feral fish | Frøy, ceased installation | Baseline polluted | Naph type | | 5.6 ug/ml | 1.3 - 2.2 | Beyer et al., 2003 |

| Species | Substance (lab/field) | Test concentrations/area | Exposure time | Metabolite | Baseline | control or reference | exposed /control | reference/comments |
|---|---|---|---|---|---|---|---|---|
| (Melanogrammus aeglefinus) | | 10 000 m (ref) 2000 m - 200 m | area? | Pyren type | | 1.4 ug/ml | 1.4 - 0.7 | |
| | | | | BaP type | | 0.75 ug/ml | 1.8 - 0.6 | |
| | | | | | | | | |
| Sheepshead minnow | Laboratory | North sea oil A 0.1 - 0.4 - 0.7 ppm | 5 weeks | Naph type | | 6916 | 2.3 - 6.2 - 9.3 | Bechmann et al. 2004 |
| | | | | Pyren type | | 569 | 2.5 - 5 - 6.3 | |
| | | | | BaP type | | 107 | 4 - 13.1 - 19.2 | |
| Sheepshead minnow | Laboratory | North sea oil B 0.1 - 0.9 - 5.6 ppm | 6 weeks | Naph type | | 18164 | 1.8 - 4.3 - 12.5 | Bechmann et al. 2004 |
| | | | | Pyren type | | 438 | 5.6 - 12.6 - 30.8 | |
| | | | | BaP type | | 110 | 12.6 - 42.7 - 123.9 | |
| Sheepshead minnow | Laboratory | 2 - 14 - 214 ppb | 5 weeks | Naph type | | 267280 | 0.9 - 2.2 - 18.6 | Bechmann et al. 2004 |
| | | | | Pyren type | | 9926 | 0.9 - 1.5 - 9.6 | |
| | | | | BaP type | | 5152.7 | 3 - 17.4 - 207 | |
| Polar cod (Boreogadus saida) | Laboratory, feral fish 2001, 2002 | 1.5 ppm StatfjA oil , baseline, control | 14 days | Naph type | 16.0 ug/g | 2 | 16,9 | Sundt & Bechmann, 2004 |
| | | | | Pyren type | 0.9 ug/g | 5,5 | 74,4 | |
| | | | | BaP type | 0 ug/g | 0 | 1,8 | |

Table 3. PAH-metabolites in marine fish – measured by GC-MS.

| Species | Substance (lab/field) | Test concentrations | Exposure time | Metabolite | Baseline | control or reference | exposed/control | reference |
|---|---|---|---|---|---|---|---|---|
| Cod (Gadus morhua) | Feral fish | Barents sea | baseline | Naph sum | 150,6 ng/g | | | Aas & Børseth, 2002 |
| | | | | Phen sum | 61,2 ng/g | | | |
| | | | | Pyren | 4,6 ng/g | | | |
| Cod (Gadus morhua) | Feral fish | Barents sea | baseline | Naph sum | 1285 ng/g | | | Sundt, 2004 |
| | | | | Phen sum | 220 ng/g | | | |
| | | | | Pyren | 3.5 ng/g | | | |
| Cod (Gadus morhua) | Feral fish | Egersund | Baseline non polluted area | Naph sum | 2005.1 ng/g | | | Klungsøyr et al. 2003 |
| | | | | Phen sum | 230.2 ng/g | | | |
| | | | | Pyren | 3.9 ng/g | | | |
| Cod (Gadus morhua) | Feral fish | Sleipner | Baseline polluted area? | Naph sum | 1296.1 ng/g | | | Klungsøyr et al. 2003 |
| | | | | Phen sum | 197.8 ng/g | | | |
| | | | | Pyren | 0 ng/g | | | |
| Cod (Gadus morhua) | Feral fish | Statfjord | Baseline polluted area? | Naph sum | 1361.7 ng/g | | | Klungsøyr et al. 2003 |
| | | | | Phen sum | 351.1 ng/g | | | |
| | | | | Pyren | 4.0 ng/g | | | |
| Cod (Gadus morhua) | Laboratory | 0.06 - 0.25 - 1 ppm Oil | average 3, 7, 14, 24 days | Naph sum | | 2549 ng/g | 4.6 - 13.4 - 23.6 | Skadsheim et al., 2004 |
| | | | | Phen sum | | 691 ng/g | 7.7 - 22.9 - 34.9 | |
| | | | | Pyren | | 27 ng/g | 7.3 - 16.2 - 25.1 | |
| Cod (Gadus morhua) | Laboratory | 0.06 - 0.25 - 1 ppm Oil | average 3, 17, 31 day | Naph sum | | 5702 ng/g | 4 - 13.3 - 12,7 | Skadsheim et al., 2004 |
| | | | | Phen sum | | 377 ng/g | 10,5 - 40,3 - 48,7 | |
| | | | | Pyren | | 5 ng/g | 8,6 - 63 - 88,4 | |
| Cod (Gadus morhua) | Field, Caged | North Sea - Statfjord, 500 - 2000 - 10000 m | | Naph sum | | 1150 ng/g | 3.0 - 2.0 - 1.3 | Aas et al., in press |
| | | | | Phen sum | | 340 ng/g | 3.5 - 2.7 - 2.5 | |
| | | | | Pyren | | - | - | |
| Cod (Gadus morhua) | Field, Caged | North Sea - Troll, 1000 m - 500m | 6 weeks | Naph sum | 1515.1 ng/g | 1,1 | 1.1 - 1.2 | Børseth et al., 2004 |
| | | | | Phen sum | 327.2 ng/g | 1,6 | 2.1 - 2.0 | |
| | | | | Pyren | 173.2 ng/g | 1,2 | 0.9 - 1.2 | |
| Cod (Gadus morhua) | Field, Caged | North Sea - Tampen, 10000 - 2500 - 1000 - 500 | 6 weeks | Naph sum | | 965.3 ng/g | 0.9 - 1.7 - 0.9 - 1 | Hylland et al., 2005 |
| | | | | Phen sum | | 934.5 ng/g | 1.4 - 3 - 1.8 - 1.5 | |
| | | | | Pyren | | 3.7 ng/g | 0 - 0 - 0.5 - 0.0 | |
| Cod (Gadus morhua) | Field, Caged | North Sea - | 5.5 weeks | Naph sum | 228 ng/g | 0,2 | 0.9 - 1.1 - 0.9 | Aas et al., in press |

| Species | Substance (lab/field) | Test concentrations | Exposure time | Metabolite | Baseline | control or reference | exposed/control | reference |
|---|---|---|---|---|---|---|---|---|
| | | Statfjord, 10000 m – 2000m - 500 m | | Phen sum | 482 ng/g | 2,0 | 3 - 4.5 - 6.7 | |
| | | | | Pyren | 28 ng/g | 10,2 | 29.5 - 31.1 - 41.5 | |
| Cod (Gadus morhua) | Field, Caged | German bight G4 (Ref) G1 - G2 - G3 | 5.5 weeks | Naph sum | 228 ng/g | 0,8 | 1 - 1 - 1.9 | Aas et al., in press |
| | | | | Phen sum | 482 ng/g | 1,0 | 0.7 - 0.8 - 0.8 | |
| | | | | Pyren | 28 ng/g | 0,0 | 0 - 0 - 0 | |
| Haddock (Melanogrammus aeglefinus) | Feral fish | Egersund | Baseline non polluted area | Naph sum | 1346.9 ng/g | | | Klungsøyr et al. 2003 |
| | | | | Phen sum | 526.8 ng/g | | | |
| | | | | Pyren | 5.7 ng/g | | | |
| Haddock (Melanogrammus aeglefinus) | Feral fish | Sleipner | Baseline polluted area? | Naph sum | 1111.5 ng/g | | | Klungsøyr et al. 2003 |
| | | | | Phen sum | 331.5 ng/g | | | |
| | | | | Pyren | 10.4 ng/g | | | |
| Haddock (Melanogrammus aeglefinus) | Feral fish | Statfjord | Baseline polluted area? | Naph sum | 1279.7 ng/g | | | Klungsøyr et al. 2003 |
| | | | | Phen sum | 331.9 ng/g | | | |
| | | | | Pyren | 3.1 ng/g | | | |
| Haddock (Melanogrammus aeglefinus) | Feral fish | Barents sea | | Naph sum | 1474 ng/g | | | Sundt, 2004 |
| | | | | Phen sum | 165 ng/g | | | |
| | | | | Pyren | 0 | | | |
| Polar cod (Boreogadus saida) | Laboratory, feral fish 2001, 2002 | 1.5 ppm StatfjA oil , baseline, control | 14 days | Naph sum | 1330 ng/g | 1,3 | 114 | Sundt & Bechmann, 2004 |
| | | | | Phen sum | 538 ng/g | 0,9 | 90 | |
| | | | | Pyren | 52 ng/g | 14,6 | 60 | |

# 3. Cytochrome P4501A activity (EROD)

## 3.1 Introduction

EROD (7-Ethoxyresorufin-*O*-deethylase) is a specific cytochrome P450 reaction where ethoxyresorufin is the substrate (Burke and Mayer 1974). Cytochrome P450 1A catalyse the deethylation of 7-ethoxyresorufin to resorufin. CYP activity in microsome fractions can be quantified from the amount of resorufin produced.

The cytochrome P450 system is a large superfamily of enzymes with several hundred forms comprising more than 250 different families, further divided into subfamilies. The CYP system is a highly diversified set of proteins and is found in bacteria, plants, lower eukaryotes and in animals. Members of the P450 subfamily CYP1A are particularly important in the metabolism of many pollutants. In the case of planar molecules, such as polycyclic aromatic hydrocarbons (PAHs) isoenzymes of CYP1A are responsible for the insertion of oxygen into the molecule, which is the first oxidative step in the biotransformation process (termed 'phase I'; Williams, 1974). The introduced oxygen creates a functional group for attachment of larger polar molecules of endogenous origin in a 'phase II' reaction (which includes e.g. glutathione *S*-transferases).

In addition to being substrates for biotransformation, planar compounds, such as PAHs, can also interact with cytochrome P450 1A as inducers, by binding to the cytosolic Ah (aryl hydrocarbon)-receptor. EROD is a tool used to quantify this induction. The induction of cytochrome P450 enzymes in fish liver was first suggested as an indicator of environmental contamination in the 1970s by Payne (1976). It has later gained widespread use, and the results of the most relevant studies for this review will be addressed in the following.

## 3.2 Dose-response

Whyte *et al.* (2000) rank chemicals according to the level of EROD activity they induce in treated or exposed fish when compared with untreated or control fish. Contaminants that induce EROD less than 10-fold above control levels are considered "weak" inducers, 10- to 100-fold are "moderate" inducers, and chemicals that elicit > 100-fold induction are considered "strong" inducers. Dioxins, planar PCBs and PAHs (benzo[a]pyrene) are categorised as "strong" inducers.

Over 25 studies have observed induction of hepatic EROD by benzo[a]pyrene in 15 species of fish (Whyte *et al.* 2000). However, in the present overview, results for Gadoid species are focussed on, due to their relevance in offshore monitoring studies in the Norwegian sector.

Table 4. Dose-response, background response and sensitivity in gadoid fish.

| species | substance(s) | lowest-highest concs | exposure time | baseline/control (level/activity) | induction (fold) | reference |
|---|---|---|---|---|---|---|
| Experimental studies | | | | | | |
| Polar cod Boreogadus saida<br>juvenile | Crude oil (Oseberg C) | ~200 mg/kg (i.p. inj.) | 10 and 21 d post inj. | ~30 pmol/min/mg | ~8 and ~2.5 (245 and 80 pmol/min/mg) | (George et al. 1995) |
| Polar cod Boreogadus saida<br>male | Crude oil (Oseberg C) | ~200 mg/kg (oral) | 21 d post exposure | 28 pmol/min/mg ± 6 (n=12) | ~5 (132 ± 14 pmol/min/mg) | (George et al. 1995) |
| Polar cod Boreogadus saida<br>female | Crude oil (Oseberg C) | ~200 mg/kg (oral) | 21 d post exposure | 8 pmol/min/mg ± 2 (n=14) | ~5 (42 ± 6 pmol/min/mg) | (George et al. 1995) |
| Polar cod Boreogadus saida<br>juvenile | β-naphthoflavone | 50 mg/kg (i.p. inj.) | 21 d post inj. | ~30 pmol/min/mg | ~12.5 (380 pmol/min/mg ) | (George et al. 1995) |
| Cod, Gadus morhua<br>juvenile | 2,3,7,8-TCDD | 0.008 mg/kg oral dose twice, d 0 and d 4 | 9 and 17 d post exposure | 55.4 (d 9) and 91.4 (d 17) pmol/min/mg | ~4 and ~3 (230 and 277 pmol/min/mg) | (Hektoen et al. 1994) |
| Cod, Gadus morhua<br>juvenile | PCB-105 | 10 mg/kg oral dose twice, d 0 and d 4 | measure at d 9 and d 17 | 55.4 (d 9) and 91.4 (d 17) pmol/min/mg | 1.5 and 1.2 | (Bernhoft et al. 1994) |
| Cod, Gadus morhua<br>juvenile | β-naphthoflavone | 100 mg/kg (i.p. inj. at d 0 and d 4) | measure at d 7 | 84 pmol/min/mg ± 8 (n=5) | ~13 (1074 ± 340 pmol/min/mg) | (Goksoyr et al. 1987) |
| Cod, Gadus morhua | β-naphthoflavone | 100 mg/kg (2 i.p. inj.) | measure 3-4 d after last injection | 40 pmol/min/mg | ~72 (2870 pmol/min/mg) | (Goksoyr et al. 1991) |
| Cod, Gadus morhua<br>juvenile | Crude oil (North Sea) | 0.06 – 1 ppm | 30 days | ~2 pmol/min/mg | ~ 2- 5.5 (~ 4 – 11 pmol/min/mg) | (Aas et al. 2000) |
| | | | | | | |
| Field studies | | | | | | |
| Rockling, Ciliata mustella | Crude oil (Gullfaks; M.V. Braer spill, Shetland) | 85000 tons spill<br>129 ± 38 ng /g dry wt. of PAHs (selected 2- and 3-ring) detected in muscle. | 3 months after spill | ~160 pmol/min/mg ± 50 | ~9 (1480 pmol/min/mg) | (George et al. 1995) |
| Roundnose grenadier, Coryphaenoides rupestris | i.a. PAHs and PCBs | | | 260 ± 20 (Male)<br>~170 (Female)<br>pmol/min/mg | ~2 (530 ± 70 (male) and ~350 (female) pmol/min/mg) | (Lindesjoo et al. 1996) |
| Burbot, Lota lota | Bleached-kraft mill | | | 20 pmol/min/mg | 1.7, not sign. | (Kloeppersams and |

| species | substance(s) | lowest-highest concs | exposure time | baseline/control (level/activity) | induction (fold) | reference |
|---|---|---|---|---|---|---|
| (Freshwater) | effluents (i.a. dioxins) | | | ± 10 | (34 ± 23 pmol/min/mg) | Benton 1994) |
| Hake, Urophycis spp. | Pollution (PAH) from oil platforms (Gulf of Mexico) <100m from platforms | | | 10.9 ± 6.4 and 11.7 ± 10.5 pmol/min/mg (>3000 m from platforms) | <1 (10.6 ± 3.8 and 10.5 ± 7.1 pmol/min/mg) | (McDonald et al. 1996) |

Table 4 continued.

| ***species*** | ***substance(s)*** | ***exposure time*** | ***baseline/control (level/activity)*** | ***induction (fold)*** | ***reference*** |
|---|---|---|---|---|---|
| **Caging studies** | | | | | |
| Cod, *Gadus morhua* **juvenile** | i.a. PAH and PCB from industry (smelters) | 4 weeks | ~55 pmol/min/mg (farthest from source) | ~3 (closest to source) | (Goksoyr *et al.* 1994) |
| Cod, *Gadus morhua* **juvenile** | i.a. PAH and PCB from industry (smelters) | 3 months | 16 ± 3 pmol/min/mg (farthest from source) | ~2 (closest to source) | (Beyer *et al.* 1996) |

## 3.3 Blue mussel

Cytochrome P450-related activity is less well described in mussels or other invertebrates compared to fish. The assay most commonly used is benzo[a]pyrene hydroxylase (BaPH), for which there is some experience from both research and monitoring (e.g. Michel et al., 2001).

## 3.4 Species sensitivity

As will be apparent from the previous section, PAH-related induction of metabolising enzymes (e.g. cytochrome P4501A) is expressed to a much larger extent in fish compared to most invertebrates. It is not entirely clear whether this is because of the assay (e.g. substrate) or whether it is the biochemistry of the organisms. High bioaccumulation of PAHs in, for example, mussels compared to fish support the latter view.

## 3.5 Relevance of other factors

Several factors have been shown to affect hepatic EROD, both endogenous and exogenous. The most important endogenous factors for most fish species are developmental stage (juvenile-mature), gender, reproductive status and age, all of which can be controlled through sampling design. In addition, environmental temperature has been shown to affect EROD (Sleiderink et al., 1995; Lange et al, 1999). Seasonal cycles in EROD induction have been observed for e.g. rainbow trout (Förlin & Haux 1990), flounder (Nissen-Lie 1997; Hylland et al., 1998), salmon (Larsen et al., 1992), most likely due to both to changes in water temperature and reproductive cycles (which it is not really possible to separate in the field).

Several species have baseline EROD activities within the same order of magnitude among different studies/measurements and also show greater than 10-fold EROD induction after contaminant exposure (Whyte *et al.* 2000). These are, however, mostly freshwater species. Developmental stage of the fish is very important. The main age-related factors are time of exposure/accumulation, food selection and reproductive stage.

The mechanism for CYP1A suppression in spawning females is related to 17β-estradiol ($E_2$) (or xenoestrogen) levels. The hormone controls the induction of vitellogenin (VTG; egg yolk protein) production during gonadal recrudescence. Some of the inter-gender differences during spawning can be attributed to increased levels of CYP isoenzymes in males rather than suppression of levels in females.

Dietary factors can be important for the induction of CYP1A. Firstly, of course, AhR ligands can be presented to the organism through the food. Secondly, proper nutrition is a prerequisite for enzyme systems to function properly. Hylland et al. (1995) reported an elimination of EROD response (i.e. to control levels) in BaP-treated flounder deprived of food for one month.

## 3.6 Links

Cytochrome P4501A (CYP1A) activity is one of the parameters which have been included in a causality model of how PAHs in sediments may cause effects on fish that lead to the development of liver cancer (neoplasia) (Myers et al., 2003). There is therefore evidence for a correlation between CYP1A activity, DNA adduct formation and development of neoplasia. Changes in EROD may also affect normal steroid metabolism, but evidence for such effects is tenous.

## 3.7 Background responses

Baseline levels of EROD in four marine species have been estimated from results derived from the Norwegian monitoring programme (Ruus et al., 2003). The baseline value for Atlantic cod has been set to 9-95 pmol/min/mg protein including fish from the Norwegian west coast and if only fish from the Barents sea had been included, the values would have been 9-25 pmol/min/mg protein. For flounder, baseline values are in the range 10-43 pmol/min/mg protein, for dab 123-529 pmo/min/mg protein and for plaice 33-146 pmol/min/mg protein. The fish were sampled from reference locations (i.e. no known local sources of contamination) in the autumn, the data includes males and females and the water temperature at the sampling locations was 9-11°C.

## 3.8 Assessment criteria

As many factors are known to influence EROD and it is not possible to correct for all in the design, it is advisable always to include an appropriate reference group in studies that include EROD as an endpoint. Experience suggests that an EROD value in most marine species above twice the upper limit of baseline values indicate an ecosystem influenced by planar organic contaminants.

## 3.9 Quality assurance

Cytochrome P4501A is possibly the most widely used biomarker. There have been two international intercalibrations for the method, both within BEQUALM. The intercalibrations have pinpointed variability relating to most steps in the analytical process, excepting possibly the enzyme kinetic analysis itself. It is imperative that laboratories have internal quality assurance procedures, e.g. use internal references samples with all batches of analyses.

# 4. Glutathione *S*-transferase activity

## 4.1 Introduction

Glutathione *S*-transferase enzymes (GSTs) are a group of cytosolic enzymes that are involved in phase-II biotransformation of a large number of pollutants (Lindstrom-Seppä et al., 1986). These enzymes catalyse the conjugation of glutathione (GSH) to lipophilic compounds with electrophilic centres in order to neutralise and excrete chemicals capable of causing toxic effects. To date, several groups of enzymes (isoenzymes) have been reported and number of isoenzymes and their characteristics vary between different animals and tissues (Fitzpatrick and Sheehan, 1993; Gallagher *et al.*, 1996; Martinez-Lara *et al.*, 1997; Angelucci *et al.*, 2000; Novoa-Valinas *et al.*, 2002). Glutathione *S*-transferase is one the most efficient phase-II biotransformation pathways for potentially toxic chemicals in vertebrates and invertebrates and has been characterized in various species including polychaetes, clams, gastropods, mussels, crustaceans and fish (see Table 5 for details).

Many of the GST isoenzymes can be induced by pollutants and increased hepatic GST activity has been reported in several studies after exposure to chemicals (Perez-Lopez *et al.*, 2002). The induction of GST has thus been suggested to be adopted for use as a biomarker for exposure to chemicals such as such as PAHs, PCBs and Dioxins (Van der Oost *et al.*, 2003).

Total GST level is commonly measured by means of a catalytic assay involving the conversion of the substrate 1-chloro-2,4-dinitrobenzene (CDNB). The activity of individual isoenzymes, which is expressed as specific catalytic activity, may in a similar fashion be measured with the use of isoenzyme specific substrates. Although GST activity has been documented in various tissues, determination of GST is predominantly performed on tissues that display high biotransformation activity. This applies in particular to use of liver tissues in vertebrates and the hepatopancreas/digestive gland in invertebrates, although gill GST is of importance as the first line defence towards waterborne pollutants. Measurement of GST is most commonly performed on the cytosolic fraction (CF) obtained after high speed centrifugation.

## 4.2 Dose-response

Dose-response relationships are commonly obtained for the induction of total GST activity following exposure to phase-II substrates under controlled laboratory conditions (experimental studies), sampling of wild fish (field studies) and caging of fish (caging deployment studies). Although many pollutants are documented to induce GST activity, proper dose-response relationships are difficult to derive from the reported data due to lack of measured concentrations. In general, GST is only weakly inducible with an induction factor of up three times in laboratory studies and up to five times in field studies. GST activity is inhibited compared to control groups in several studies with pure chemicals and mixtures as well as under field experiments. Table 5 shows the dose-response relationships for selected fish and invertebrates after exposure to pure chemicals and environmental mixtures. This biomarker has been measured in both fish and invertebrates. It may be both up- and down regulated as a result of contaminant exposure.

Table 5. Dose-response, background response and induction of GST in fish.

| species | Tissue | substance(s) Field/caging locality | concentration | exposure time (days) | *baseline/control* (nmol/min/mg protein) | induction (%) | reference |
|---|---|---|---|---|---|---|---|
| **Experimental studies** | | | | | | | |
| | Liver CF | Phenanthrene | 0.5, 1, 2 uM (nominal) | 14, 28 | 650±70 (14d) 640±30 (28d) | 0 (14d, L-CF) 0 (28d, L-CF) | (Jee and Kang, 2005) |
| Olive Flounder, *Paralichthys olivaceus* | Gill CF | Phenanthrene | 0.5, 1, 2 uM (nominal) | 14, 28 | 290±20 (14d) 360±50 (24d) | +300 (14d) +258 (28d) | (Jee and Kang, 2005) |
| | Kidney CF | Phenanthrene | 0.5, 1, 2 uM (nominal) | 14, 28 | 230±20 (14d) 250±30 (28 d) | 0 (14d) 0 (28d) | (Jee and Kang, 2005) |
| Atlantic cod *Gadus morhua* | Liver S9 | 4-t-butylphenol 4-n-pentylphenol 4-n-hexylphenol 4-n-heptylphenol | 0,02-80 μg/kg (nominal, oral) | 28 | 952 ± 153 | -20% (only lowest concentration) | (Hasselberg *et al*., 2004) |
| Pacific herring, *Clupea pallasi* | Liver CF | WSF crude oil | ∑PAH 9.7-99.3μg/L. (measured, unstable) | 2.4, 4 | 101.1± 8.5 (2.4d) 103.8±3.6 (4d) | +69 (2.4d) 0 (4d) | (Kennedy and Farrell, in press) |
| Rainbow trout Onchorynchus mykiss | Liver CF | Nonylphenol | 66 and 220, μg/l (nominal) | 7,14,21 | 68.8 ± 9.7 (7d) 83.7 ± 11.5 (14d) | +200 (7d, 220 μg/l) +127 (7d, 66 μg/l) -24 (21d, 66μg/l) | (Uguz *et al*., 2003) |
| Rainbow trout *Onchorynchus mykiss* | Liver CF | BNF CB-77 CB-126 (i.p. injection) | 50mg/kg (BNF) 0.1, 1, 5 mg/kg (CB-77, CB-126) (nominal) | 6 | 310±96 | -18 (BNF) +75 (CB-77, 0.1) +55 (CB-77, 1) +23 (CB-77, 5) +47 (CB-126, 5) +74 (CB-126, 1) +23 (CB-126, 5) | (Huuskonen *et al*., 1996) |
| Rainbow trout *Onchorynchus mykiss* | Liver CF | Arochlor-1254 (i.p. injection) | 5 mg/kg (nominal) | 63 | 493±147 | -23 (BNF) +29 (CB-77, 0.1) +8 (CB-77, 1) -25 (CB-77, 5) -31 (CB-126, 5) -18 (CB-126, 1) +2 (CB-126, 5) | (Huuskonen *et al*., 1996) |
| **Field studies** | | | | | | | |
| Flounder *Platichthys flesus* | Liver S9 | Southern Baltic Sea | n.a. | Feral | 86.6±39.8 | +48% | (Napierska and Podolska, 2005) |
| | | | | | | | |
| **Cage deployment studies** | | | | | | | |
| Atlantic cod *Gadus morhua* | Liver CF | Troll Oil field, North Sea | n.a. | 42 | ~750 | Approx. +13% (1000 m station) | (Børseth and Tollefsen, 2004) |

| species | Tissue | substance(s) Field/caging locality | concentration | exposure time (days) | *baseline/control (*nmol/min/mg protein*)* | induction (%) | reference |
|---|---|---|---|---|---|---|---|
| Atlantic cod *Gadus morhua* | Liver CF | Statfjord B, North Sea | n.a. | 42 | ~950 | Approx. -29% (males, 1000 m station) | (Hylland *et al.*, 2005) |

n.a. – not analysed, i.p.-intraperitoneal, WSF - water soluble fraction of crude oil, CF – cytosolic fraction, S9 – S9 fraction obtained after high speed centrifugation.

Table 6. Dose-response, background response and induction of GST in invertebrate species.

| species | Tissue | substance(s) Field/caging locality | concentration | exposure time (days) | *baseline/control (*nmol/min/mg protein*)* | induction (%) | reference |
|---|---|---|---|---|---|---|---|
| **Experimental studies** | | | | | | | |
| Mediterranean mussels *Mytilus galloprovincialis* | Gill S9 | BaP (feed) | 50 mg B[a]P:kg d.w. (theoretical) | 28 | ~170 | Approx. -9 | (Akcha *et al.*, 2000) |
| *Lymnaea palustris* | Whole Body CF | Atrazine | 5, 25, 125 µg/L (nominal) | 21 | ~1040-1125 | -16,4 (5 µg/l)<br>-14,9 (25 µg/l)<br>-14,3 (125 µg/l) | (Baturo and Lagadic, 1996) |
| Shore crab *Carcinus maenas* | Hepatopancreas S9 | Cypermethrin | 50, 200, 500ng/l (nominal)<br>Variable measured levels | 7 | ~38 | +46 (50 ng/l)<br>+27 (200 ng/l)<br>+53 (500 ng/l) | (Gowland *et al.*, 2002) |
| **Field studies** | | | | | | | |
| *Nereis diversicolor* | Digestive Gland S9 | Cádiz bay (Spain) | n.a. | Feral | 25.4±3.6 | +200 | (Perez *et al.*, 2004) |
| *Scrobicularia plana* | Digestive Gland S9 | Cádiz bay (Spain) | n.a. | Feral | 392±69 | 0 | (Perez *et al.*, 2004) |
| **Cage deployment studies** | | | | | | | |
| Blue mussels *Mytilus edulis* | Hepatopancreas S9 | Loch Leven (Scotland) | Mussel concentration ∑PAH 15-927 ng/g w.w. | Variable (study design) | 10.2 | +456 | (Gowland *et al.*, 2002) |
| Green lipped mussels *Perna viridis* | Digestive Gland S9 | Various harbours, (China) | Mussel concentration 0-300 ng PAH/g d.w. 0-90 ngBaP/g d.w. | 30 | 61-95 | Approx. +40 | (Cheung *et al.*, 2001) |
| | Gill S9 | Various harbours, (China) | Mussel concentration 0-300 ng PAH/g d.w. 0-90 ngBaP/g d.w. | 30 | 90-120 | Approx. +45 | (Cheung *et al.*, 2001) |

n.a. – not analysed, i.p.-intraperitoneal, WSF - water soluble fraction of crude oil, CF – cytosolic fraction, S9 – Sp fraction obtained after high speed centrifugation, BNF – beta-naphtoflavone; CB - chlorobifenyl.

## 4.3 Species sensitivity

No studies are available that address sensitivity of GST to exposure to pollutants directly, although it seems that GST are induced to a higher degree in invertebrates than in vertebrates. This difference in sensitivity may be caused by the lower constitutive levels of GST in invertebrates compared to vertebrates. Up to 8 times differences in basal levels of GST has been documented in fish species (Förlin *et al.*, 1995), thus suggesting that interspecies differences in GST induction sensitivity may also be relevant for fish. Laboratory studies and field studies with English sole (*Parophrys vetulus*) and starry flounder (*Platichthys stellatus*) confirm that susceptibility towards contaminant-associated hepatic lesions is partly related to differences in constitutive levels of GST in these fish (Collier et al., 1992).

## 4.4 Relevance of other factors

GST activity is constitutively expressed and basic activity may be affected by various factors related to the physiological state of the organisms such as food availability, diet gender and maturation status. Several reports document that GST activity may be affected by co-exposure to other pollutants, algal toxins, and but also to infections by pathogen organisms such as *Listeria monocytogene.* All these factors may potentially influence the ability to determine effects in the field either as a result of inhibition of GST, leading to false negatives, or to induction of GST, leading to false positives. Table 7 depicts factors that are known to influence GST activity in fish and vertebrates.

Table 7. Relevance of other factors.

| Species | Tissue | Factor | Response | Comment | Reference |
|---|---|---|---|---|---|
| Rainbow trout *Onchorynchus mykiss* | Liver CF | Food deprivation | Decreased GST activity | Reduced to 50% control activity | (Blom *et al.*, 2000) |
| Various fish species | Liver CF | Diet composition | No change | GST activity is not affected by diet composition. | (DeKoven *et al.*, 1992; Guderley *et al.*, 2003; Morrow *et al.*, 2004) |
| Blue mussels, *Mytilus edulis* | Digestive Gland & Gill S9 | Seasonal variation | Variable response | ~200% difference between high and low activity throughout year | (Manduzio *et al.*, 2004) |
| Sockeye salmon (*Oncorhynchus nerka*) | Liver S9 | Maturation status | Decrease | GST decrease at spawning | (Kennish *et al.*, 1992) |
| Flounder *Platichthys flesus* Bullrout *Myoxocephalus scorpius* | Liver S9 | Gender | GST levels dependent on gender | Males display higher GST activity than females | (Ruus *et al.*, 2002; Napierska and Podolska, 2005) |
| Arctic Charr, *Salvelinus alpinus.* | Liver CF | Chemical co-exposure | Inhibition of GST | TBT inhibited GST activity 37-60 % | (Padros *et al.*, 2003) |
| Mediterranean mussels *Mytilus galloprovincialis*. | Gill CF | Chemical co-exposure | Inhibition of GST | Retene inhibited GST activity | (Gravato *et al.*, 2004) |
| Atlantic salmon, *Salmo salar* | Liver CF | Exposure to toxic algae | Induction of GST | Paralytic shellfish poisoning toxins induce GST (~250%) | (Gubbins *et al.*, 2000) |
| Carp *Cyprinus carpio* | Liver Kidney Gill CF | Bacterial infections | Inhibition of GST | Listeria monocytogenes infection reduce GST activity | (Chambras *et al.*, 1999; Dautremepuits *et al.*, 2002) |

## 4.5 Links

Since GST activity is a biomarker for exposure rather than effect, direct linkage between alteration in GST levels and effects on health, population parameters and community composition are limited. Alterations in GST activity may affect the ability to detoxify potentially toxic chemicals and consequently affect these parameters indirectly. Several studies support this assumption:

Loss or inhibition of hepatic GST activity may increase susceptibility to liver neoplasms and to DNA damage by environmental or endogenous chemicals that are normally detoxified by GST (Stalker *et al.*, 1991; Kirby *et al.*, 1995; Gravato and Santos, 2003). Laboratory and field studies by Collier et al. (1992) suggest that the prevalence in contaminant-associated liver lesions is related to a combination of induction of Phase-I biotransformation pathways causing bioactivation of certain PAHs and lower constitutive levels of detoxification activity by GST. In European flounder (*Platichthys flesus L.*) that has developed liver carcinomas, expression of multi-xenobiotic resistance (MXR), phase-I and Phase-II biotransformation activities (including GST) were induced as clonal adaptation to pollution exposure (Köhler *et al.*, 1998; Koehler *et al.*, 2004).

GST levels were found to increase in parallel with DNA adducts in livers of gold-lined sea bream (*Rhabdosargus sarba*) after intraperitoneal exposure to benzo[*a*]pyrene (Xu *et al.*, 2001). In vitro studies with hepatocytes from rainbow trout show that GST activity is unregulated and DNA integrity, DNA damage and prevalence of apoptotic cells were increased after exposure to wastewater (Faverney *et al.*, 2001; Risso-de Faverney *et al.*, 2001). GST induction was found to increase in parallel with lipid peroxidation and DNA damage in the digestive glands of the mangrove mussel (*Mytella guyanensis*) after exposure to polluted waters (Torres *et al.*, 2002). In other studies, GST levels were found to be correlated to exposure to PAHs such as benzo[*a*]pyrene, but DNA adducts were not (Akcha *et al.*, 2000; Bocquene *et al.*, 2004).

## 4.6 Background responses

Background responses vary within animal groups and individual species. Table 8 indicates ranges and average/median levels of GST activity for selected vertebrates and invertebrates.

## 4.7 Assessment criteria

There is insufficient information about factors that affect GST to develop assessment criteria at this time.

## 4.8 Quality assurance

The analysis of GST activity using CDNB is straightforward and most laboratories use the procedures outlined by Habig et al. (1974). Laboratories that analyse for the protein must establish in-house quality assurance procedures.

Table 8. Background levels of GST in different tissues of vertebrates and invertebrates.

| Animal group | Species | Tissue | Background response (nmol/min/mg protein) |
|---|---|---|---|
| Vertebrates | Olive Flounder, *Paralichthys olivaceus* | Liver CF | 640-650 (average 645) |
| | | Gill CF | 290-360 (average 325) |
| | | Kidney CF | 230-250 (average 240) |
| | Rainbow trout, *Onchorynchus mykiss* | Liver CF | 68.8-493 (median 310) |
| | Atlantic cod *Gadus morhua* | Liver CF | 750-952 (median 950) |
| | Flounder *Platichthys flesus* | Liver S9 | 86.6 |
| | Pacific herring, *Clupea pallasi* | Liver CF | 101.1-103.8 (average 102) |
| Invertebrates | Mediterranean mussels *Mytilus galloprovincialis* | Gill S9 | 170 |
| | Blue mussels *Mytilus edulis* | Hepatopancreas S9 | 10.2 |
| | Green lipped mussels *Perna viridis* | Digestive Gland S9 | 61-95 (average 78) |
| | | Gill S9 | 90-120 (average 105) |
| | Shore crab *Carcinus maenas* | Hepatopancreas S9 | ~38 |
| | *Nereis diversicolor* | Digestive Gland S9 | 25.4 |
| | *Scrobicularia plana* | Digestive Gland S9 | 392 |
| | *Lymnaea palustris* | Whole body CF | 1040-1125 (average 1083) |

# 5. DNA damage

## 5.1 Introduction

It is of vital importance to the survival of the cell and of the organism that DNA is kept intact and unchanged. All cells therefore have an extensive system of quality control and repair involved in the replication and transcription of DNA. Since normal cellular processes will generate damage to DNA continuously, any impairment of repair systems will obviously also potentially result in DNA damage. In addition to intracellular processes, there are immunological mechanisms (in vertebrates) that will identify and kill cells with DNA modifications that may be deleterious to the organism (i.e. cancer). DNA damage may simply cause cell death, but may also lead to carcinogenic changes in the cell, structural damage to chromosomes and mutagenic effects.

There are three main direct or indirect mechanisms that may lead DNA damage – breaks, adducts and impaired repair. There are a range of methods in ecotoxicology that address breaks and adduct formation, but few have been established to assess repair mechanisms. A distinction is commonly made between methods that identify chromosomal breaks and those that are used to quantify all breaks in DNA. It is also possible to distinguish between one- and two-strand breaks in the latter category. Micronucleus formation (see section 7) is the most widely used method to address chromosomal breaks that result in the formation of satellite DNA. The two most widely used assays for breaks are alkaline unwinding and the Comet assay. The former will be discussed briefly in this section, the Comet assay will be treated in more detail in section 6.

## 5.2 DNA repair

Although presumably critical in the maintenance of DNA integrity, DNA repair has not been extensively studied in fish. The results found by Willett et al. (2001) may indicate part of the problem – they found no detectable nucleotide excision repair (NER) in hepatocytes from two catfish species following fairly harsh UV-treatment. There are however other studies in which such activity has been detected, e.g. Walter et al. (2001), David et al. (2004). This is obviously a field which needs further research.

## 5.3 Alkaline unwinding

As mentioned above, different methods are being used to address DNA damage in aquatic organisms. Different methods may yield results which differ by many orders of magnitude (Collins 2005) and it is obviously critical that comprehensive documentation is available for each study. Alkaline unwinding assays can be set up to distinguish between single and double breaks and have been used in fish (Shugart 1988, Everaarts 1995, Liepelt et al. 1995, Feng et al. 2003) and in mussels (Accomando et al. 1991, Nacci et al. 1992, Ching et al. 2001). The principle of the technique is the same as that used in the Comet assay, but the latter has the advantage of measuring effects on single cells. There is not really sufficient information to review alkaline unwinding in terms of sensitivity or dose-dependency for species relevant to North Sea monitoring. Results from alkaline unwinding have failed to relate to contaminant exposure in e.g. mussels (Siu et al. 2003).

## 5.4 DNA adducts

There is a considerable number of ecotoxicological studies that have used DNA adducts as a measure of genotoxic effects. There is also a range of different methods and units to present results, despite the existence of an internationally acknowledged manual (Reichert et al., 1999). In the tables below, only studies that use the Nuclease P1 enrichment technique have been considered.

### 5.4.1 Dose-response

There is a limited number of toxicokinetic studies with DNA adducts or alkaline unwinding as endpoints. It is also difficult to use results from studies earlier than the mid-1990s due to obvious developments in both experimental and analytical protocols. The majority of studies use fish or mussels sampled in the field. In general there is only limited knowledge of exposure history in field studies.

There is not always a straightforward dose-response relationship. In some studies, e.g. Ericson et al. (1999), decreased concentrations of DNA adducts were found in intestine at dietary exposures higher than 150 µmol PAH-mixture/kg, presumably due to cytotoxic effects. Adduct concentrations in the liver of that study remained high even at higher levels (but did not increase further). An overview of field and laboratory studies with fish can be found in Table 9 and Table 10, studies with mussels can be found in Table 11 and Table 12.

### 5.4.2 Species baseline and sensitivity

Species differences in DNA adduct concentrations may derive from different capacities of phase-1 or phase-2 metabolism, as suggested for two flatfish species by Anulacion et al. (1998) or by differences in the rate of which adducts are cleared from tissues, as suggested for two species of catfish by Ploch et al. (1998).

### 5.4.3 Time-course of adduct development

The time-course of adduct development has been studied both following dietary exposure and exposure through water.

In a study by Ericson et al. (1999), DNA adducts were found to be significantly elevated in PAH-fed pike (*Esox lucius*) after 3 weeks (the first sampling point) and then increased beyond the last feeding (day 50) until day 83 for adducts in intestine, liver and gills. There were still significantly (> 100-fold) increased concentrations of DNA adducts in the three tissues after 128 days (2.5 months after last exposure). A second study with pike explored shorter time scales and dose-dependency. Following a single i.p. injection of a PAH-mixture, adducts increased in liver until day 9, but then dropped to day 12. In the same experiment, adducts increased in intestine as well and levelled off on day 9. In an accompanying feeding experiment, a single bolus with a PAH-mixture resulted in maximally increased adduct levels in intestine already after three days, decreasing on day 12. The largest mean increase of hepatic adducts was seen on day 6.

In a study reported by Aas et al. (2000), DNA adducts in the liver of Atlantic cod were apparent after only 3 days at the highest exposure level, dosed through water (0.94 mg/L dispersed oil; 0.6 ppb PAH). The adduct levels increased until 30 days, at the time of which there was also a significant increase in adducts in a second group exposed to 0.04 mg/L dispersed oil.

In the American freshwater species mummichog (*Fundulus heteroclitus*), DNA adducts increased in all tissues (liver, anterior kidney and spleen) until 32 days after a single injection of benzo[*a*]pyrene (12 mg/kg) (Rose et al., 2001). Levels of adducts decreased significantly by day 96 (Rose et al., 2001).

### 5.4.4 Relevance of other factors

**Fish**

Carpenter et al. (1995) found weak temperature effects *in vitro* (higher adduct levels at higher temperatures), but generally similar adduct formation when assays were run at acclimation temperature.

The results found by Akcha et al. (2003) show that there may indeed be detectable levels of DNA adducts in all dab populations in an area, but also indicated that there could possibly be a sex difference in the sensitivity to adduct formation.

External agents other than PAHs may also cause adduct formation. Armstrong et al. (2002) found that UV-radiation would cause DNA damage (although not measured as adducts) in medaka (*Oryzias latipes*). Other contaminants, e.g. CBs, may also cause adducts as shown by e.g. Dubois et al. (1995).

Some studies have also shown that DNA adducts will increase at different rates in different tissues (e.g. Ericson et al., 1999). The liver is the main tissue used in most cases and there is not sufficient data to suggest that other tissues be used at the moment. A study is ongoing to investigate this possibility with Atlantic cod (Balk, personal communication).

There is no clear evidence that spawning affect DNA adducts in other tissues or for seasonality in the formation or concentration of DNA adducts.

**Mussels**

Not surprisingly, DNA adducts in blue mussels have been shown to have a seasonal variability (Skarphedinsdottir et al. 2005), presumably due to spawning activity and variations in food availability (i.e. fat content) through the year. In the same study evidence was presented that intertidal mussels have higher concentrations of DNA adducts than subtidal mussels, not correlated to carcinogenic PAHs in the tissues, but to a larger extent to total PAH (Skarphedinsdottir et al. 2005).

There may also be differences between organs in the response to contaminants, as found by Pisoni et al. (2004), in which there was good agreement between results for gills and hepatopancreas as concerned the reference location, but results for contaminated locations varied between sampling campaigns.

### 5.4.5 Links

DNA adducts have been used as an intermediate between chemical or biochemical endpoints, i.e. PAH-metabolites and cytochrome P4501A activity, and histological changes in tissues, i.e. neoplasia or cancers (Myers et al., 2003). There is thus evidence that increased DNA adducts are linked to cancer in fish, but still unclear whether this will actually decrease growth or reproductive output. Recent data has indicated that adduct formation is found at concentrations similar to those that will cause reproductive effects in marine organisms (Bechmann, Sanni & Smit, personal communication).

### 5.4.6 Background responses

Aas et al. (2003) investigated background levels in a range of marine species from the Norwegian Sea, i.e. polar cod (*Boreogadus saida* ), daubed shanny (*Leptoclinus maculatus*), sea tadpole (*Careproctus reinhardti*), Atlantic spiny lumpsucker (*Eumicrotremus spinosus*), black seasnail (*Paraliparis bathybius*), Arctic rockling (*Gaidropsarus argentatus*), doubleline eelpout (*Lycodes eudipleurostictus*), polar sculpin (*Cottunculus microps*), Greenland halibut (*Reinhardtius hippoglossoides*), Atlantic cod (*Gadus morhua*) and capelin (*Mallotus villosus*). Levels were below detection limit (from 0.4 to 1.3 nmol adducts/mol DNA) for most of individuals and species. Polar sculpin was the only species for which all sampled individuals had detectable adduct levels (mean+/- SD: 1.74 +/- 1.04 nmol adducts/mol DNA). Balk (unpublished) has indicated that background level of DNA adducts in the liver of haddock (*Melanogrammus aeglefinus*) is in a range similar to that found for other fish species, but with a distinct zone on the sheet found in all individuals. This zone is then subtracted for all to quantify contaminant-related adducts (Balk, unpublished).

In blue mussels the baseline concentration of adducts appear to be below detection limit for the normally used $^{32}$P post labelling method (Skarphedinsdottir et al., 2005). This means that any adducts present were found at concentrations so low as to be inseparable from the analytical control.

### 5.4.7 Assessment of DNA adduct results

DNA adducts is one of the methods for which it is possible to set global reference values (using baseline values). Data as those generated by Aas et al. (2003) could be used to establish such limits, which lie in the range 0.5-1.5 nmol adduct/mol DNA for most, but not all, species. Although DNA adducts is the one biomarker methods closest to manifest disease (liver cancer) it is still not feasible to set absolute levels since other factors also affect the levels of liver cancers in fish (if that would be an acceptable “ecologically relevant” endpoint). Presumably the most useful approach is to indicate to which extent DNA adduct values in a given study exceed baseline values. A lower trigger level would be twice the baseline value.

### 5.4.8 Quality assurance

The measurement of DNA adducts through $^{32}$P-postlabelling is a complex analysis with many steps, many of which are crucial to the final result (Dolcetti et al., 2002). As the results above indicate, results from different laboratories are not necessarily directly comparable, most commonly because they use different protocols or because they express results differently.

DNA adducts was included as one component in the EU-funded BEQUALM project and an intercalibration between different laboratories was performed by ITM, Stockholm University (led by Lennart Balk). There are plans to include DNA adducts in the ongoing BEQUALM activity, led by CEFAS (UK; project office) and NIVA (responsible for biomarkers).

Table 9. Overview of field studies in which DNA adducts were analysed using nuclease P1 enrichment (fish).

| *species* | *ecosystem* | *tissue* | *substance(s)* | *lowest-highest concs* | *baseline/control (level/activity)* | *induction (fold)* | *reference* |
|---|---|---|---|---|---|---|---|
| brown bullhead (Ameirus nebulosus) | freshwater | liver | field | | 42 nmol/mol | 25-30 | (Pinkney et al. 2004) |
| mummichog (Fundulus heteroclitus) | freshwater | liver | field (creosote) | | <2 nmol/mol DNA | >100 | (Rose et al. 2000) |
| mummichog (Fundulus heteroclitus) | freshwater | anterior kidney | field (creosote) | | <2 nmol/mol DNA | >100 | (Rose et al. 2000) |
| mummichog (Fundulus heteroclitus) | freshwater | spleen | field (creosote) | | <2 nmol/mol DNA | >100 | (Rose et al. 2000) |
| mummichog (Fundulus heteroclitus) | freshwater | pooled blood | field (creosote) | | <2 nmol/mol DNA | >50 (only one sample) | (Rose et al. 2000) |
| perch (Perca fluviatilis) | freshwater | liver | field (pulp mill) | 2 km and 8 km from source | <0.5 nmol/mol DNA | >30 | (Ericson & Larsson 2000) |
| perch (Perca fluviatilis) | freshwater | intestine | field (pulp mill) | 2 km and 8 km from source | <0.5 nmol/mol DNA | >50 | (Ericson & Larsson 2000) |
| perch (Perca fluviatilis) | freshwater | liver | field (creosote) | various distances from source | 0.2 nmol/mol DNA | >100 | (Ericson et al. 1999) |
| perch (Perca fluviatilis) | freshwater | liver | field (smelter) | various distances from source | 5 nmol/mol DNA | 3 | (Ericson et al. 1998) |
| perch (Perca fluviatilis) | freshwater | gill | field (smelter) | various distances from source | 0.3 nmol/mol DNA | 5 | (Ericson et al. 1998) |
| perch (Perca fluviatilis) | freshwater | head kidney | field (smelter) | various distances from source | 1 nmol/mol DNA | 5 | (Ericson et al. 1998) |
| perch (Perca fluviatilis) | freshwater | trunk kidney | field (smelter) | various distances from source | 0.8 nmol/mol DNA | 10 | (Ericson et al. 1998) |
| Atlantic cod (Gadus morhua) | marine | liver | field (smelter) | various distances from source | <0.5 nmol/mol DNA | none | (Næs et al. 1999) |
| Atlantic cod (Gadus morhua) | marine | liver | field (smelter) | various distances from source | <0.5 nmol/mol DNA | >100 | (Aas et al. 2003) |
| Atlantic cod (Gadus morhua) | marine | liver | caging-offshore, 5 wk | gradient from Statfjord | 0.3 nmol/mol DNA | 1.5-2¤ | Balk et al., in press |
| corkwing wrasse (Symphodus melops) | marine | liver | field (smelter) | various distances from source | 27 nmol/mol DNA | >100 | (Aas et al. 2003) |
| dab (Limanda limanda) | marine | liver | field | no assoc sed pah | 0.1 adducts/10-8 nucleotides¤ | >10 | (Akcha et al. 2003) |

| *species* | *ecosystem* | *tissue* | *substance(s)* | *lowest-highest concs* | *baseline/control (level/activity)* | *induction (fold)* | *reference* |
|---|---|---|---|---|---|---|---|
| dab (Limanda limanda) | marine | liver | field | no assoc sed pah | 4 adducts/10-8 nucleotides | 6 | (Lyons et al. 2000) |
| dab (Limanda limanda) | marine | liver | field | no difference between sites | 0.2-0.4 adducts/10-8 DNA nucleotides | none | (Akcha et al. 2004) |
| flounder (Platichthys flesus) | marine | liver | field | | 4 adducts/10-8 nucleotides | 20 | (Lyons et al. 2004) |
| flounder (Platichthys flesus) | marine | liver | field | | 10 adducts/10-8 nucleotides | 3 | (Lyons et al. 1999) |
| grass goby (Zosterisessor ophiocephalus) | marine | liver | field | field | 0.055 adducts/10-8 DNA nucleotides | 2* | (Venier & Zampieron 2005) |
| grass goby (Zosterisessor ophiocephalus) | marine | intestine | field | field | 0.371 adducts/10-8 DNA nucleotides | 7-8* | (Venier & Zampieron 2005) |
| grass goby (Zosterisessor ophiocephalus) | marine | gill | field | field | 0.068 adducts/10-8 DNA nucleotides | approx 30 | (Venier & Zampieron 2005) |
| haddock (Melanogrammus aeglefinus) | marine | liver | field-offshore production | three areas | 4 nmol/mol DNA (Egersund bank) | 4.5 | Hylland et al., 2006 |
| Lipophrys pholis | marine | ovaries/ testes | field-oil spill | | n/a | 3 | (Lyons et al. 1999) |
| Lipophrys pholis | marine | liver | field-oil spill | | n/a | 1,2 | (Lyons et al. 1997) |
| Lipophrys pholis | marine | gill | field-oil spill | | n/a | 2* | (Lyons et al. 1997) |
| sculpin (Myoxocephalus scorpius) | marine | liver | field-harbours | field | 1 nmol/mol DNA | 15-30 | Stephensen et al., 2000 |

Table 10. Overview of laboratory studies in which DNA adducts were analysed using nuclease P1 enrichment (fish).

| *species* | *ecosystem* | *tissue* | *substance(s)* | *lowest-highest concs* | *exposure time* | *baseline/control (level/activity)* | *induction (fold)* | *reference* |
|---|---|---|---|---|---|---|---|---|
| Atlantic cod (Gadus morhua) | marine | liver | dispersed oil | 1 mg/L dispersed oil | 2 weeks | 1.6 nmol/mol DNA | 10 | (Aas et al. 2003) |
| Atlantic cod (Gadus morhua) | marine | liver | dispersed oil | 0.94 mg/L dispersed oil | 3, 16, 30 | not provided | 110 nmol/mol¤ | (Aas et al. 2000) |
| Atlantic cod (Gadus morhua) | marine | liver | dispersed oil | 0.04 mg/L dispersed oil | day 3, 16, 30 | not provided | 10 nmol/mol¤ | (Aas et al. 2000) |
| brown bullhead (Ameiurus nebulosus) | freshwater | liver | injection | 20 mg BaP/kg | 1, 3, 7, 14, 45 d | 0.6 fmol adduct/mg DNA | 6¤ | (Ploch et al. 1998) |
| channel catfish (Ictalurus nebulosus) | freshwater | liver | injection | 20 mg BaP/kg | 1, 3, 7, 14, 45 d | 0.5 fmol adduct/mg DNA | 1.8¤ | (Ploch et al. 1998) |
| English sole (Pleuronectes vetulus) | marine | liver | manipulated sediments | PAH gradient in sediments | 2 wk, 5 wk | 5 nmol/mol DNA | >100 (5wk), >30 (2 wk) | (French et al. 1996) |
| flounder (Platichthys flesus) | brackish water | leukocytes | injection | 10, 50 mg BaP/kg | 10 d | n/a | >80 (pooled) | Malmström et al., 2000 |
| flounder (Platichthys flesus) | brackish water | liver | injection | 10, 50 mg BaP/kg | 10 d | n/a | >40 | Malmström et al., 2000 |
| flounder (Platichthys flesus) | marine | liver | PAH-mix/diet | 1, 5, 50 mg/kg | 1 mo, 6 mo | n/a | 2 add/10-8 (5 mg/kg), 6 add/10-8 (50 mg/kg) | Reynolds et al., 2003 |
| killifish (Fundulus grandis) | freshwater | liver | injection | 5 mg BaP/kg | 2, 4, 8, 14 d | <0.1 adducts/10-8 nucleotides | >15 (all time-points) | (Willett et al. 1995) |
| killifish (Fundulus grandis) | freshwater | liver | injection | 1, 5, 15, 50 mg BaP/kg | 4 d | <0.1 adducts/10-8 nucleotides | >50 (15, 15 mg/kg) | (Willett et al. 1995) |
| mummichog (Fundulus heteroclitus) | freshwater | pooled blood | BaP/injection | 15 mg/kg BaP | 8, 16, 32. 96 d | <2 nmol/mol DNA | >20 (32 d) | (Rose et al. 2001) |
| mummichog (Fundulus heteroclitus) | freshwater | anterior kidney | BaP/injection | 14 mg/kg BaP | 8, 16, 32. 96 d | 6 nmol/mol DNA | >10 (32 d) | (Rose et al. 2001) |
| mummichog (Fundulus heteroclitus) | freshwater | spleen | BaP/injection | 13 mg/kg BaP | 8, 16, 32. 96 d | 10 nmol/mol DNA | 4 (32 d) | (Rose et al. 2001) |
| mummichog (Fundulus heteroclitus) | freshwater | liver | BaP/injection | 12 mg/kg BaP | 8, 16, 32. 96 d | 11 nmol/mol DNA | 10 (32 d) | (Rose et al. 2001) |
| pike (Esox lucius) | freshwater | liver | BaP,BkF,DMC/diet | 0.180 mmol/kg PAH-mix, single | 1, 3, 6, 9 d | <0.5-2.8 nmol/mol DNA | >150 (6 d) | (Ericson & Balk 2000) |

| *species* | *ecosystem* | *tissue* | *substance(s)* | *lowest-highest concs* | *exposure time* | *baseline/control (level/activity)* | *induction (fold)* | *reference* |
|---|---|---|---|---|---|---|---|---|
| | | | | feed | | | | |
| pike (Esox lucius) | freshwater | intestine | BaP,BkF,DMC/diet | 0.1 mmol/kg PAH-mix x 5 | 21, 59, 66, 83, 128 d | <0.5 nmol/mol DNA | >100 after 59 d | (Ericson & Balk 2000) |
| pike (Esox lucius) | freshwater | intestine | BaP,BkF,DMC/diet | 0.180 mmol/kg PAH-mix, single feed | 1, 3, 6, 9 d | <0.5-1.5 nmol/mol DNA | >150 (6 d) | (Ericson & Balk 2000) |
| pike (Esox lucius) | freshwater | liver | BaP,BkF,DMC, injection | 0.120 mmol/kg PAH-mix, single injection | 1, 3, 6, 9, 12 d | <0.5-2.8 nmol/mol DNA | >15 (9 d) | (Ericson & Balk 2000) |
| pike (Esox lucius) | freshwater | liver | BaP,BkF,DMC/diet | 37.5, 150, 300, 600 µmol/kg PAH-mix | 9 d | <0.5-2.8 nmol/mol DNA | >100 (100, 200 µmol/kg) | (Ericson & Balk 2000) |
| pike (Esox lucius) | freshwater | intestine | BaP,BkF,DMC/diet | 37.5, 150, 300, 600 µmol/kg PAH-mix | 9 d | <0.5-1.5 nmol/mol DNA | >100 (100 µmol/kg) | (Ericson & Balk 2000) |
| pike (Esox lucius) | freshwater | brain | BaP,BkF,DMC/diet | 0.1 mmol/kg PAH-mix x 5 | 21, 59, 66, 83, 128 d | <0.5 nmol/mol DNA | >100 after 59 d | (Ericson & Balk 2000) |
| pike (Esox lucius) | freshwater | liver | BaP,BkF,DMC/diet | 0.1 mmol/kg PAH-mix x 5 | 21, 59, 66, 83, 128 d | <0.5 nmol/mol DNA | >100 after 59 d | (Ericson & Balk 2000) |
| pike (Esox lucius) | freshwater | intestine | BaP,BkF,DMC, injection | 0.120 mmol/kg PAH-mix, single injection | 1, 3, 6, 9, 12 d | <0.5-1.5 nmol/mol DNA | >5 (9, 12 d) | (Ericson & Balk 2000) |
| pike (Esox lucius) | freshwater | gill | BaP,BkF,DMC/diet | 0.1 mmol/kg PAH-mix x 5 | 21, 59, 66, 83, 128 d | <0.5 nmol/mol DNA | >100 after 59 d | (Ericson & Balk 2000) |
| polar cod (Boreogadus saida ) | marine | liver | dispersed oil | 1 mg/L dispersed oil | 2 weeks | 1.2 nmol/mol DNA | 10 | (Aas et al. 2003) |
| rainbow trout (Oncorhynchus mykiss) | freshwater | liver | effluent pulp mill | 2% dilution | 50 d | <0.5 nmol/mol DNA | >10 | (Lindesjoo et al. 2002) |
| zebrafish (Danio rerio) | freshwater | liver | BaP/water | 0.2 mg/kg - 3 d + 1 mg/kg - 4 d | 7 d | n/a | 4 adducts/10-8 nucleotides | (Hsu & Deng 1996) |
| zebrafish (Danio rerio) | freshwater | testis | BaP/water | 0.2 mg/kg - 3 d + 1 mg/kg - 4 d | 7 d | n/a | 0.2 adducts/10-8 nucleotides | (Hsu & Deng 1996) |
| zebrafish (Danio rerio) | freshwater | brain | BaP/water | 0.2 mg/kg - 3 d + 1 mg/kg - 4 d | 7 d | n/a | 4 adducts/10-8 nucleotides | (Hsu & Deng 1996) |

| *species* | *ecosystem* | *tissue* | *substance(s)* | *lowest-highest concs* | *exposure time* | *baseline/control (level/activity)* | *induction (fold)* | *reference* |
|---|---|---|---|---|---|---|---|---|
| zebrafish (Danio rerio) | freshwater | intestine | BaP/water | 0.2 mg/kg - 3 d + 1 mg/kg - 4 d | 7 d | n/a | 13 adducts/10-8 nucleotides | (Hsu & Deng 1996) |

Table 11. Overview of field studies in which DNA adducts were analysed using nuclease P1 enrichment (mussels).

| *species* | *tissue* | *substance(s)* | *lowest-highest concs* | *exposure time* | *baseline/control (level/activity)* | *induction (fold)* | *reference* |
|---|---|---|---|---|---|---|---|
| *Mytilus galloprovincialis* | gill | field | Venice | field | detection limit | (0.400 adducts/10-8 nucleotides) | (Venier & Zampieron 2005) |
| Mytilus galloprovincialis | gill | field | harbour | field | 0.5-1 | 5.0-10 | (Pisoni et al. 2004) |
| Mytilus galloprovincialis | hepatopancreas | field | harbour | field | <0.1 | >50 | (Pisoni et al. 2004) |
| Mytilus edulis | gill | field | harbours | field | 0.1-0.5 nmol/mol DNA | 2-30 (depending on season) | (Skarphedinsdottir et al. 2005) |
| Mytilus edulis | gill | field | harbours | field | | >20 (seasonally higher) | (Ericson et al. 2002) |
| Mytilus edulis | hepatopancreas | field | harbours | field | | 2-3 (less in August) | (Ericson et al. 2002) |
| Mytilus edulis | gill | caged | harbours | 6 wk | | 2 (March, but not August) | (Ericson et al. 2002) |
| Mytilus edulis | hepatopancreas | caged | harbours | 6 wk | | 2 (March, but not August) | (Ericson et al. 2002) |

Table 12. Overview of laboratory studies in which DNA adducts were analysed using nuclease P1 enrichment (mussels).

| *species* | *tissue* | *substance(s)* | *lowest-highest concs* | *exposure time* | *baseline/control (level/activity)* | *induction (fold)* | *LOEC* | *reference* |
|---|---|---|---|---|---|---|---|---|
| Mytilus edulis | gill | BaP/diet | 50, 100 mg/kg DW | 3, 10, 21 + 10 d depuration | n/a | >30 (both concentrations; 21 d) | | (Akcha et al. 2000) |
| Mytilus edulis | gill | BaP/water | 5, 50, 100 µg/L | 4 d | 1.6 nmol/mol DNA | approx 3 | 5 µg/L | (Skarphedinsdottir et al. 2003) |
| Mytilus edulis | hepatopancreas | BaP/water | 5, 50, 100 µg/L | 4 d | 1.4 nmol/mol DNA | none | none | (Skarphedinsdottir et al. 2003) |
| Mytilus edulis | gill | BaP/water | 17 µg/L | 6 d + 28 d depuration | 0.5 nmol/mol DNA | approx 7 (6 d), none (34 d) | | (Skarphedinsdottir et al. 2003) |
| Perna viridis | hepatopancreas | BaP/water | 0.3, 3, 30 µg/L | 1, 3, 6, 12, 18, 24 d | n/a | approx 6, 5, 2 (30, 3, 0.3 µg/L) 3 d, none (18, 24 d) | | (Ching et al. 2001) |
| Mytilus galloprovincialis | gill | BaP/water | 0.5-1000 µg/L | 2, 3 d | 0.07-0.12 adducts/10-8 nucleotides | approx 5 (2, 3 d) | 50 µg/L (2 d), 5 µg/L (3 d) | (Canova et al. 1998) |
| Mytilus galloprovincialis | hepatopancreas | BaP/water | 0.5-1000 µg/L | 2, 3 d | 0.05-0.06 adducts/10-8 nucleotides | approx 5 (2 d), approx 10 (3 d) | 50 µg/L (2 d), 5 µg/L (3 d) | (Canova et al. 1998) |

# 6. The Comet assay

## 6.1 Introduction

The alkaline single cell gel electrophoresis or the Comet assay is a technique for measuring DNA strand breaks and thereby DNA damage. The assay involves detection, under alkaline conditions, of cell DNA fragments which, on electrophoresis, migrate from the nuclear core, resulting in a "Comet with tail" formation Singh *et al.* (1988). The Comet assay was developed by Singh *et al.* for use on human cells but has later been applied on many species of fish and aquatic invertebrates. In contrast to other similar alkaline treatment assays the Comet assay incorporates the microscopic examination of damage to individual cell nuclei. The Comet assay works because DNA strand breaks create fragments or supercoiled DNA-loops that, when embedded in an agarose gel, migrate in an electric field. The strand breaks damage the higher order, tightly-packed structure of DNA, which also allows migration outside the region of the cell nucleus. Alkaline treatment facilitates the unwinding and denaturation of the DNA molecules, allowing for the sensitive detection of single-strand damage. In human DNA, it has been estimated that the sensitivity of the Comet to detect strand breaks is in the area of (one break in $10^{10}$ daltons). The Comet assay principles are illustrated in Figure 4.

The Comet assay is a sensitive, rapid and cost-efficient technique for the detection of DNA strand breaks, which is ideally suited as a non-specific biomarker of genotoxicity in fish and other aquatic species (Mitchelmore & Chipman, 1998). The Comet assay offers advantage over cytogenetic assays since there is no requirement for the detailed knowledge of the karyotype, thus enabling it to be easily transferred between species. The Comet assay also has the added advantage of being applicable to non-dividing cells (Dixon & Wilson, 2000). The results of Lee *et al.* (2004) indicate that there is an association between reproduction abnormalities and increased DNA strand breaks as a result of grass shrimp exposure to estuarine sediments receiving highway runoff (PAH). In our experiments we have observed that at the level where significant increase in the level of strand breaks was detected in adult mussels, reduced fitness was observed in the larvae (Baussant, 2004).

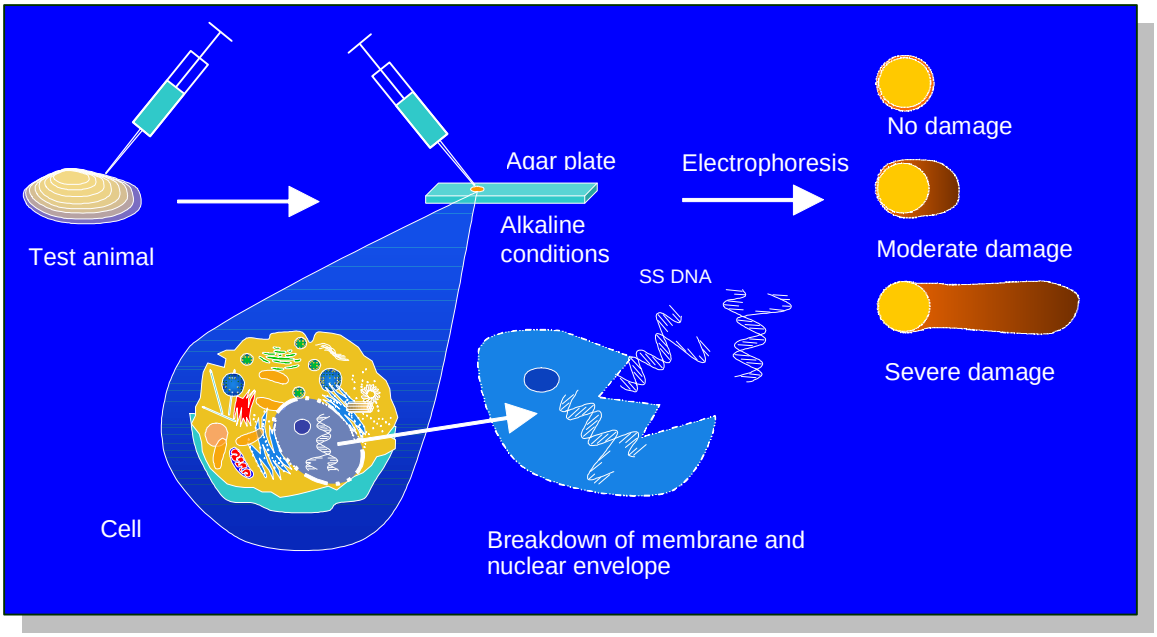


Figure 4. The principles of the Comet assay as a biomarker tool. Comets with no damage, moderate damage and severe damage are illustrated.

The level of DNA strand breaks is usually presented as the percentage of DNA migrated out of the nucleus and into the Comet tail region (% DNA in tail), but tail moments and tail lengths are also used. Figure 5 shows an example of results from thanalysis of Comet assay in haemocytes of mussels exposed to styrene (Mamaca *et al.,* 2005). The results show that significant differences between control and exposed animals can be detected both at the individual and the cell level. Only 5% of cells from control mussels had more than 12% DNA in the Comet tail, compared to 50% of the cells from styrene exposed mussels.

When mussels are exposed to low concentrations of oil and other compounds there is more overlap in response between control and exposed animals. Generally the level of DNA strand breaks in exposed cells is less than twice as high as in the corresponding control (Table 14 and Table 15). For details on methods see Aas *et al.* (2002), Taban *et al.* (2004), and Bechmann (2004) and Baussant (2004).

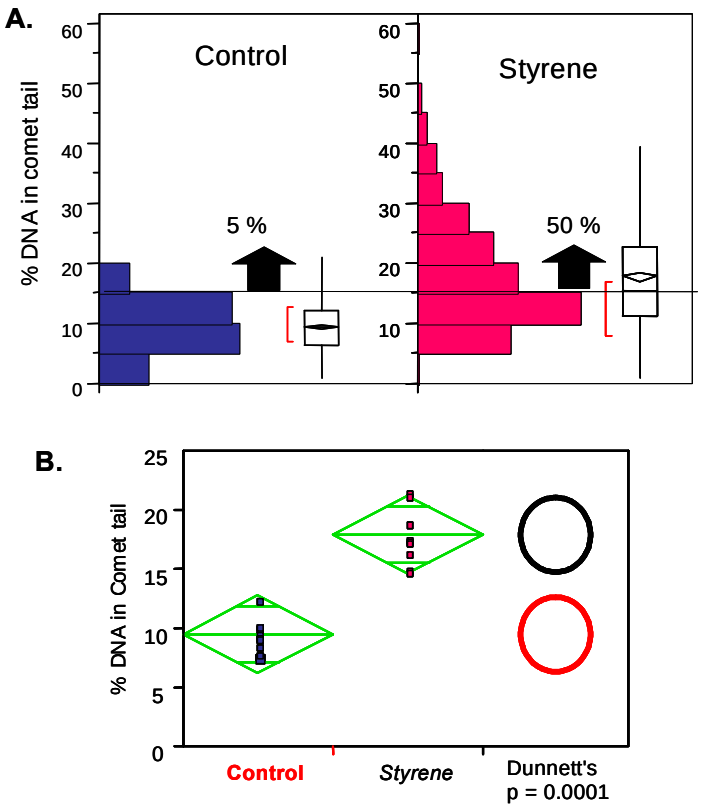


Figure 5. Example of results from the Comet assay analysis. Mussels (*Mytilus edulis*) exposed to styrene (data from Mamaca *et al.*, 2005). **A:** Frequency distribution of % DNA in the Comet tail of cells from control mussels and mussels exposed to styrene (9 mussels x 50 cells from each treatment). Cells from all the replicate mussels from each treatment are combined. **B:** Comparison of mean % DNA in the Comet tail of cells from each of the 9 mussels analysed from each treatment.

Table 13 gives an overview of studies where the Comet assay has been used for biomonitoring in the field. Mussels and different fish species have been used, and in several studies significant differences between contaminated and clean sites were detected. It seems like the Comet assay works best if caged animals are used, or if the objective is to study the effects of a spill. In some cases it was not possible to detect any increase in the level of DNA strand breaks in animals living in an environment chronically exposed to contaminants. Since laboratory results also indicate that long time exposure may reduce the response detected in the Comet assay, caging may be the best choice for monitoring off shore discharges from the oil industry (and for mussels that is also the only possibility).

Table 13. Overview of results from biomonitoring studies using the Comet on different species of aquatic invertebrates and fish collected in the field. In the "result" column symbols are used to indicate whether differences in DNA damage between contaminated and clean sites were observed: + : Significant differences in DNA damage between clean and contaminated sites, ÷ : No difference.

| Species | Type of contamination | Result | Reference |
|---|---|---|---|
| Mussel (*Mytilus edulis)* | Genotoxic waste water | ÷ | Rank (1999) |
| Mussel (*Mytilus edulis)* | PAH polluted site (Loch Leven, Scotland) | ÷ | Large *et al.* (2002) |
| Mussel (*Mytilus edulis)* | Urban /industrial contaminated site (PCB, PAH) | Caged mussels: + Indigenous mussels : ÷ | Shaw *et al.* (2002) |
| Mussel (*Mytilus edulis)* | Naval Station San Diego, PAH, Caging experiment | + | Steinert *et al.* (1998b) |
| Mussel (*Mytilus edulis)* | Caging experiment San Diego Bay, PAH | + (recovery after 24 h) | Steinert *et al.* (1998a) |
| Mussel *(Mytilus edulis)* | Reykjavik harbour PAH | + | Halldorsson *et al.* (2004) |
| *Mytilus sp.* | The French coast | ÷ | Akcha *et al.* (2004) |
| Zebra mussel (*Dreissena polymorpha*) | Caging experiment Municipal wastewater | + | Pavlica *et al.* (2001) |
| *Mytilus galloprovincialis* | Highly eutrophicated coastal lagoon | + | (Frenzilli *et al*., 2001) |
| The intertidal butterfish (*Pholis gunnellus)* | Industrial contamination | ÷ | Bombail *et al*. (2001) |
| Chub *(Leuciscus cephalus)* | River Rhone | + | Devaux et al. (1998) |
| Bullheads *(Ameiurus nebulosus)* Carp *(Cyprinus carpio)* | PAH, PCB | + | Pandrangi *et al.* (1995) |
| Smallmouth bass *(Micropterus dolomieu)* | Androscoggin River | + | (Chamberland *et al*., 2002) |
| Chub *(Leuciscus cephalus)* | Moselle River (France) | ÷ | (Flammarion *et al*., 2002) |
| California halibut *(Paralichthys californicus)* | Southern California bays and harbours | + | Brown *et al.* (2004) |
| Sacramento sucker *(Catostomus occidentalis)* | Agricultural runoff Field-caging experiments + lab | + | Whitehead *et al.* (2004) |
| Eelpout *(Zoarces viviparus)* | River Gota estuary Bunker oil spill | + | Frenzilli *et al.* (2004) |

A large number of laboratory *in vitro* and *in vivo* studies have been done using different types of cells from fish and invertebrates. There are, however, few studies that are directly relevant for the oil industry. The two most relevant references are Frenzilli *et al.* (2004) and (Hamoutene *et al.,* 2002). A recent study by Frenzilli *et al.* (2004) showed that the Comet assay can be used to study effects of oil spills on fish. Following a bunker oil (10-100 tonnes) spill Frenzilli *et al.* (2004) analysed the level of DNA strand breaks in nucleated erythrocytes of the eelpout *(Zoarces viviparus)* from the Gothenburg harbour area and a clean reference site. The results showed a high level of damaged DNA, paralleled by a peak in bile PAH metabolites, in fish from the most impacted site, 3 weeks after the oil spill. A significant recovery was observed in specimens from the spill site, 5 months later, but not in fish caught in the middle part of Gothenburg harbour, which is chronically subjected to harbour related pollution Frenzilli *et al.* (2004).

Hamoutene *et al.* exposed mussels *(Mytilus edulis)* and clams *(Mya arenaria)* to dispersions and water soluble fractions of an Arabian crude oil. Exposure to hydrocarbon fractions had no significant impact on clams. However, an increase in DNA damage was observed at $p < 0.1$ with digestive gland cells of mussels exposed to

aqueous fractions of a light crude oil (but no effect on haemocytes) (Hamoutene *et al.,* 2002).

## 6.2 The Comet assay with invertebrates

The Comet assay has proved to be a sensitive biomarker for detecting DNA strand breaks in bivalves and sea urchins exposed to oil in the laboratory. Increased levels of DNA strand breaks have been observed with increasing exposure concentration of oil (3 – 1000 µg/L oil). The significant results obtained with mussels, sea urchins and Iceland scallops exposed to relatively low concentrations of oil in the lab indicate that the comet assay may be used in biomonitoring of produced water discharges and oil spills.

The mean level of DNA strand breaks in haemocytes of control mussels and coelomocytes of control sea urchins (measured as percent DNA in comet tail) has been compared to the level of DNA strand breaks in mussels and sea urchins exposed to oil (Figure 6). All groups of oil exposed mussels and sea urchins have been grouped together to get an indication of whether comet assay has potential to be used for monitoring produced water discharges/oil spills in the field. The tested oil concentrations were in the range 3 – 1000 µg/L oil (see table 2 and 3 for details). There was a significantly higher level of DNA strand breaks in haemocytes of oil exposed mussels (14.3, n = 8) and coelomocytes of oil exposed sea urchins (16.8, n = 10) than in control cells from both species (mussels; 8.7, n = 17, sea urchins; 11.3, n = 11) (Figure 6).

It would be considerably more practical to be able to analyse frozen samples if the Comet assay should be used in off shore monitoring surveys. Scott Steinert (CSC Biomarker Laboratory, San Diego, USA) and Farida Akcha (IFREMER, France) have procedures for freezing cells in a way that apparently does not affect the results from the Comet assay (*personal communication*). This procedure needs to be established and evaluated in oil exposure experiments to determine whether freezing will reduce the sensitivity of the Comet assay in such exposures.

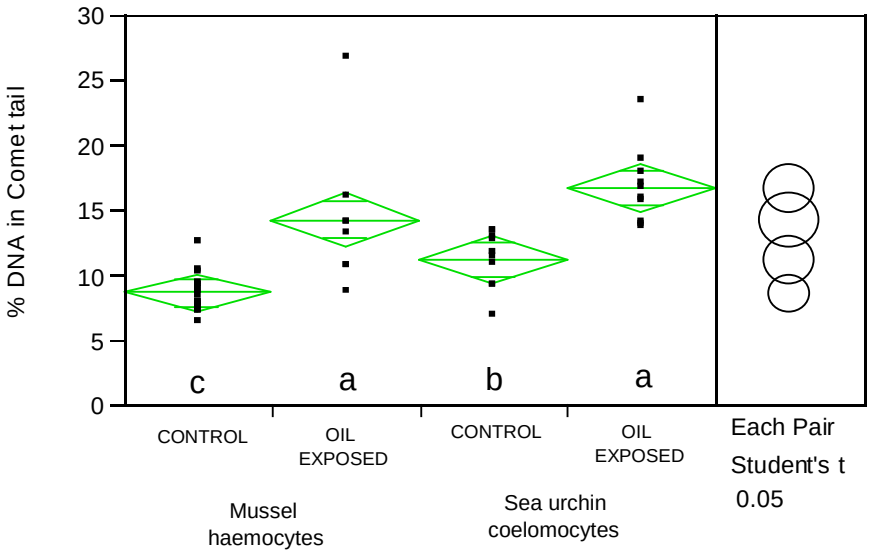


Figure 6. Laboratory controls compared to all responses in all groups of oil exposed mussels – oil concentrations in the range 3 – 1000 µg/L, including recovery after high exposure – see table x). Field values are not included. The level of DNA strand breaks in control and oil exposed animals was compared with each pair Student t-test. Groups with different letters are significantly different ($p<0.05$).

## 6.3 The Comet assay with fish red blood cells

The study by Frenzilli *et al.* (2004) showed that the Comet assay can be used to study effects of oil spills on fish. The Comet assay has been used on many species of fish, but we have not been able to find any papers where comet assay has been used on blood cells from cod. Our experience from doing comet assay on blood cells from cod is that the controls are more variable than blood cells from corkwing wrasse and invertebrates (bivalves and sea urchins). There was, however, significantly increased level of DNA strand breaks in blood cells from cod exposed to 1000 µg/l oil (Taban et al. 2000). The procedure for doing comet assay on blood cells from cod needs further optimization. However, since two other genotoxicity tests (DNA adducts and micronuclei assay) are able to detect DNA damage in oil exposed cod, it is possible that these tests are better biomarkers for biomonitoring of cod than comet assay. It may be more important to invest further effort in establishing the procedure for testing frozen cells from invertebrates using the comet assay. For the invertebrates comet assay seem to be a sensitive tool to detect exposure to genotoxic compounds also in the field.

## 6.4 Confounding factors

Akcha *et al.* (2004b) have shown that biotic (age, sex) and abiotic (sampling site and period) factors affect the extent of DNA damage measured by the Comet assay in dab *(Limanda limanda)* collected in the field. These results should also be taken into consideration when using the Comet assay on invertebrates.

After long exposures the responses measured by the Comet assay may be reduced. The results from Shaw *et al.* (2002) and Large *et al*. (2002) (and our results; Baussant, 2004) indicate that mussels may be able to adapt to the exposure. Possibly due to adaptation/induced repair mechanisms. Hence if no response is detected in wild organisms this does not necessarily mean that they have not been exposed.

## 6.5 Links

The Comet assay indicates damage to DNA. There are some data that indicate effects on reproduction at similar concentrations as those that cause increased DNA fragmentation as measured using the Comet assay (Bechmann & Sanni, unpublished), but no direct evidence as yet.

## 6.6 Background responses

The mean level of DNA strand breaks in cells from control animals has been compared (Figure 7). The mean control level of DNA strand breaks (measured as percent DNA in Comet tail) for mussel haemocytes was 8.7 (n = 17), for scallop haemocytes 10.5 (n = 5), for sea urchin coelomocytes 11.3 (n = 11) and for mussel sperm 13.8 (n = 4). The results show that the variability in the control level of DNA strand breaks is low. To detect the small changes between control and exposed animals at low exposure concentrations, controls are run together with each exposed groups to ensure that all samples have been treated in exactly the same way.

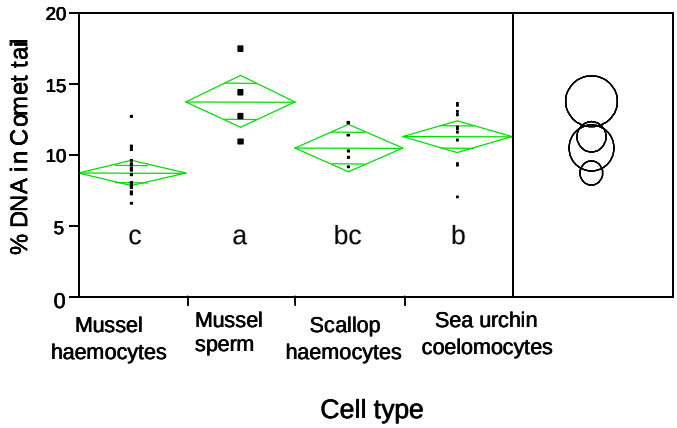


Figure 7. Mean percent DNA in comet tail of the control groups in laboratory exposures preformed at RF-Akvamiljø 2001-2005. The control level for the different cell types was compared with Each pair Student t-test. Groups with different letters are significantly different ($p<0.05$).

## 6.7 Assessment criteria

There are no established assessment criteria for Comet used for fish or invertebrates.

## 6.8 Quality assurance

There has been no international intercalibration for the Comet assay as used in ecotoxicology.

Table 14. Selected results from comet assay analysis performed on mussel *(Mytilus edulis)* haemocytes.

| **Substance** (lab/field) | **Test concentrations** | **Exposure time** | **Response (+/-)** | **Baseline/control** (% DNA in Comet tail) | **Exposed/ Control** | **Reference** |
|---|---|---|---|---|---|---|
| North Sea Oil (lab) | 1000 µg/L + recovery | 2 + 4 weeks | + | 8.9 – 8.5 | 1.6 – 1.3 | Aas *et al.* (2002) Baussant (2004) |
| | 3, 15, 63 µg/L | 1 month | + | 7.7 – 7.3 – 7.9 | 1.4 – 1.9 – 1.7 | |
| | | 7 months | + | 12.6 – 10.3 – 6.5 | 1.3 – 2.6 – 1.3 | |
| Styrene (lab) | 200 µg/L | 10 days | + | 9.5 | 1.8 | Mamaca *et al.* (2005) |
| Bisphenol A (lab) | 59 µg/L | 3 weeks | + | 9.2 | 1.3 | Taban *et al.* (2003) |
| Diallyl phthalate (lab) | 38 µg/L | 3 weeks | - | 8.8 | 0.9 | |
| PBDE-47 (lab) | 0.23 µg/L | 3 weeks | - | 9.3 | 1.0 | |
| Oil spill (field) | Rocknes (Ramsvik, Songalhella) | 1 + 7 months | (+?) | 15.3/15.6 – 6.3/6.5 | [1)] | Børseth *et al.* (2004) |
| Used water based drilling mud (lab) | 0.5, 2, 20 mg/L | 3 weeks | + (0.5, 2 mg/L) | 7.6 – 7.2 – 7.8 | 1.2 - 1.2 – 1.1 | Unpublished results from the PROOF drilling mud project (159183) |
| Barite particles (lab) | 23 mg/L | 3 weeks | + | 10.5 | 1.1 | |
| Metal mix (Pb+Cd+Cu+Zn) (lab) | Sum metals: 62 µg/L | 3 weeks | - | 8.0 | 1.0 | |
| Drilling mud (field; Sleipner) | Reference 20 m, exposed 20 m + 40 m | 5 weeks | + (40 m) | 7.7 - 13.5 | 1.1 - 1.4 | Berland & Sanni (2005) |

[1)] 1 month after the accident both stations had higher DNA damage than average for laboratory controls, but after 7 months the mean level of DNA damage was lower than in average laboratory controls. This may indicate that mussels at both stations were affected after 1 month, and that repair was going on after 7 months. The lack of a good field reference stations makes it difficult to conclude.

Table 15. Overview of results from comet assay analysis preformed on mussel *(Mytilus edulis*) embryo, mussel sperm, scallop (*Pecten maximus, Chlamys islandica*) haemocytes, sea urchin *(Strongylocentrotus droebachiensis)* coelomocytes and sperm and corkwing wrasse blood cells.

| **Species**<br>**Tissue** | **Substance** (lab/field) | **Test concentrations** | **Exposure time** | **Response (+/-)**<br>p<0.05: + | **Baseline/control**<br>(% DNA in Comet tail) | **Exposed/ Control** | **Reference** |
|---|---|---|---|---|---|---|---|
| *Mytilus edulis*<br>Mussel embryo | North Sea Oil (lab) | 63 µg/L (parental + embryo exposure) | 7 months (parents) + 1 day (embryo) | + | 8.4 | 1.8 | Baussant (2004) |
| *M. edulis*<br>Sperm | North Sea Oil (lab) | 1000 µg/L | 2 weeks | + | 14.3 | 2.5 | Aas *et al.* (2002) |
| | Bisphenol A (lab) | 59 µg/L | 4 weeks | + | 12.6 | 1.3 | Taban *et al.* (2003) |
| | Diallyl phthalate (lab) | 38 µg/L | 4 weeks | + | 10.8 | 1.5 | |
| | PBDE-47 (lab) | 0.23 µg/L | 4 weeks | + | 17.4 | 1.4 | |
| Scallop<br>*(Pecten maximus)*<br>Haemocytes | Used water based drilling mud (lab) | 0.5, 2, 20 mg/L | 3 weeks | + (2, 20 mg/L) | 11.3 – 10.2 – 9.1 | 0.9 – 1.1 – 1.2 | Unpublished results from the PROOF drilling mud project (159183) |
| | Barite particles (lab) | 23 mg/L | | + | 9.7 | 1.3 | |
| | Metal mix (Pb+Cd+Cu+Zn) (lab) | Sum metals: 62 µg/L | | - | 12.2 | 1.0 | |
| Iceland scallop *(Chlamys islandica)*<br>Haemocytes | Goliat oil (lab) | 2, 14, 64 µg/L | 4 weeks | + (14, 64 µg/L) | (15.8), 9.3, 10.0 | (0.9), 1.1, 1.9 | Baussant (2004) |
| Sea urchin<br>*(Strongylocentrotus droebachiensis)*<br>Coelomocytes | North Sea Oil (lab) | 1000 µg/L | 2 weeks | + | 12.9 | 1.8 | Bechmann & Taban (2004)<br>Aas *et al.* (2002) |
| | | Recovery after 1000 µg/L | 3 weeks recovery | + | 11.5 | 1.5 | |
| | | 29, 85 µg/L | 1 months | + | 11.7 – 9.3 | 1.4 – 2.0 | |
| | | 4, 29, 85 µg/L | 4 months | + | 11.8 | 1.4 – 1.2 – 1.2 | |
| | | 4, 29, 85 µg/L | 7 months | + | 10.9 – 12.7 – 13.4 | 1.3 - 1.2 - 1.3 | |
| *S.droebachiensis*<br>Sperm | North Sea Oil (lab) | 1000 µg/L | 2 weeks | + | 9.2 | 1.9 | Aas *et al.* (2002) |
| Corkwing wrasse *(Symphodus melops)*<br>Blood cells | Styrene (lab) | 200 µg/L | 10 days | + | 7.0 | 1.6 | Mamaca *et al.* (2005) |
| | Metals (field) | Visnes | - | + | 13.5 | 1.6 | Bjørnstad *et al.* (2002) |

# 7. Micronucleus formation

## 7.1 Introduction

Markers of genotoxic effects are at high priority due to reflection of damage to genetic material of organisms (Moore et al. 2004). Different methods has been developed for the detection of both double- and single-strand breaks of DNA, DNA-adducts, micronuclei formation, chromosome aberrations. The assessment of chemical induced genetic damage has been widely utilized for prediction of the genotoxic, mutagenic and carcinogenic potencies of a range of substances, however these investigations have mainly been restricted to human or mammals (Siu et al. 2004).

With a growing concern over the presence of genotoxins in the aquatic media, there is a rising need to elaborate sensitive methods for the assessment of genetic damage in indigenous marine or freshwater organisms. The application of cytogenetic assays on ecologically relevant species offers the chance to perform early tests on health in relation to exposure to contaminants. During the last decade an increasing attention was paid to the micronucleus test, which is simple to perform, sensitive enough and a fast test to detect genomic alterations due to clastogenic effects and impairments of mitotic spindle caused by aneuploidogenic poisons (Heddle et al. 1983; 1991; MacGregor 1991; Seelbach et al. 1993; Kramer 1998; Zoll-Moreux and Ferrier 1999).

The marine environment becomes a sink for various mutagenic/genotoxic compounds in complex environmental mixtures that can have adverse health effects on indigenous biota (Hose and Brown 1998). Three methodologically different approaches has been used for the genotoxicity assessment in marine environment: analysis of micronuclei (MN) in indigenous organisms *in situ,* assessment of MN in caged organisms in the gradient of contamination and validation of MN assay in laboratory experimental exposure.

## 7.2 Micronucleus analyses *in situ*

Some studies have shown that mutagenic PAHs can be responsible for between 10-20%, or sometimes up to 70-80% of sediment mutagenic activity in marine media (LaRocca et al. 1996; White et al. 1998). There are studies that describe increased environmental genotoxicity in zones affected by an oil spill (Parry et al. 1997; Harvey et al. 1999; Pietrapiana et al. 2002; Barsiene et al. 2004). Significant elevation of micronuclei level in mussels 30 days post-oil spill and persistence of the cytogenetic damage up to 100 days later has been described (Lyons et al. 1997). Elevated levels of the micronuclei were detected in flounder and blue mussels 8 months after oil spill from Būtingė oil terminal in the Baltic Sea (Barsiene et al. 2004; 2005b; 2005c). More frequent cytogenetic damage has been described in molluscs inhabiting the marine port and oil terminal areas in the Baltic Sea (Barsiene and Lovejoy 2000; Barsiene 2002).

In marine and estuarine ecosystems, most often environmental genotoxicity investigations have been performed using bivalves from genus *Mytilus* (Scarpato et al. 1990; Brunetti et al. 1992; Bolognesi et al. 1996; Burgeot et al. 1996; Barsiene 2002; Dailianis et al. 2003;

Izquierdo et al. 2003; 2004; Bolognesi et al. 2004; Kalpaxis et al. 2004; Hagger et al. 2005; Baršienė et al. 2005c; 2005d). In most of the studies the increased levels of MN have been shown in polluted areas. Considering that MN incidences in molluscs reflects the action of clastogenic and aneuploidogenic substances, this parameter was suggested to use as marker of cytogenetic damage in marine monitoring programs (Bolognesi et al. 1996; Izquierdo et al. 2003; Barsiene et al. 2004; Bolognesi et al. 2004; Kalpaxis et al. 2004; Baršienė et al. 2005c; 2005d).

The multiple application of micronuclei analysis in various fish and mollusk species inhabiting different sites of the North Sea, Baltic Sea and areas of the North-Atlantic showed that the technique is highly sensitive tool to monitor the cumulative effects caused by genotoxic compounds distributed in the area of exposure *in situ* (Barsiene and Lovejoy 2000; Barsiene 2002; 2004; 2005a; 2005b)(refer also to Baršienė et al., unpublished data; Schiedek et al., in press; Kopecka et al., in press).

## 7.3 The micronucleus test as laboratory bioassay

The approach to measure genotoxic effects in the aquatic environment was mainly elaborated in freshwater fish and marine mollusk species. Nevertheless in some studies it served as a promising method in marine fish treated with crude oil, produced water from oil platforms, different alkylphenols, PAHs, endocrine disruptors (Gravato and Santos 2002; Maria et al. 2002b; 2003; Baršienė et al. 2005b; 2006c). The treatment of Atlantic cod to different concentrations (0.06, 0.25, 0.5 and 1 ppm) of crude oil from Statfjord B platform showed an elevated level of MN in liver after 3-day treatment and the highest micronuclei induction after 14-day exposure (Baršienė et al. unpublished data - *Biosea programme*). In terms of methodology of crude oil genotoxicity assessment, very important finding of our experimental work was that the efficient recovery occurs after 2 weeks. In overall it means, that genotoxic effects of hazardous crude oil compounds arise early after agent appears in the fish environment and comparatively fast elimination of cytogenetic damage when genotoxic agents are absent in the habitat of the fish. Stepwise Regression analysis of influence of fish weight, liver weight, oil concentration and exposure time on MN formation showed, that the concentration of oil is the most important factor in treatment and recovery groups of whole cod cohort studied (Baršienė et al. unpublished data - *Biosea programme*). Furthermore, crude oil from different platforms, as well as temperature of exposure, has potency to modify the pattern of MN induction in fish (Baršienė et al., unpublished data - *Biosea programme*).

In mussels, cells with micronuclei were found to increase in the gills after treatment with zinc chloride (Majone et al. 1988), benzo[*a*]pyrene and vincristine (Burgeot et al. 1995), mitomycin, colchicine (Majone et al. 1987; 1990), and mercury chloride (Bolognesi et al. 1999). Elevated levels of micronucleated cells have been described in gills or hemolymph of marine molluscs treated with benzo[*a*]pyrene (Burgeot et al. 1995; Venier et al. 1997; Siu et al. 2004), dimethylbenzol[*a*]anthracene (Bolognesi et al. 1996), bisphenol A, diallyl phthalate and tetrabromodifenyl ether-47 (Baršienė et al. 2005b). The highest induction of micronuclei and nuclear buds was observed in mussels and perch after 10-day treatment with 0.5 ppm of crude oil (Baršienė et al. 2006a). Moreover the results of the MN assays showed clear dose- and time-dependent responses to benzo[*a*]pyrene exposure in the bivalve *Perna viridis*. It was

pointed, that the micronuclei test provides a convenient, highly sensitive, non-invasive monitoring tool of genotoxic assessment in the aquatic environment (Siu et al. 2004).

## 7.4 Assessment of MN in caged organisms

Some of the latest biomonitoring programmes involve environmental genotoxicity assessment in caged marine organisms. The micronucleus test was used in mussels *Mytilus galloprovincialis* caged in selected sites along Gulf of Patras (Kalpaxis et al. 2004) and along the Ligurian coast of the Mediterranean Sea (Bolognesi et al. 2004). The approach was approved as reliable tool for coastal biomonitoring. Micronuclei analysis was performed in cod and mussels caged in different zones around oil drilling platforms in the North Sea (Baršienė et al. unpublished data). There was a gradient of micronuclei induction in hemolymph of mussels caged in different distance from the Statfjord B oil platform. A 2-3-fold increase of MN was detected in mussels immersed at 500 and 1000 meters downstream the platform, when compared with mussel group before the caging experiment. Similar pattern of MN induction was found in cod liver cells, whilst the response was at a lower level than in mussels. Thus, the response to genotoxins showed a good discriminatory power in identification of pollution gradient and the approach was suspected as sensitive tool in biomonitoring for the evaluation of potential ecological risk with regard to marine pollution by crude oil or produced water effluents (Hylland et al. 2005). Compariing responses to genotoxic action in caged and indigenous mussels suggests that wild populations are able to accumulate higher content of contaminants in their tissues and express higher level of time-integrated response to genotoxic agents (Bolognesi et al. 2004). This finding confirms our previous studies on environmental mutagenicity using indigenous and caged molluscs (Baršienė & Bučinskienė, 2001).

Overall, the cytogenetic damage serves as a promising method to monitor environmental contamination with oil genotoxic substances because aquatic organisms activate oil and respond to genotoxic metabolites at low, environmentally realistic concentrations. Hazardous effects of different PAHs arise mainly as a result of oxidative biotransformation producing highly DNA-reactive metabolites. These metabolites are recognized as carcinogenic and mutagenic compounds (Torres-Bugarin et al. 1998; Woodhead et al. 1999). Mechanisms of PAH metabolic transformation have been studied and genotoxic potency of metabolites was confirmed in fish (Pacheco and Santos 1997; Pacheco and Santos 2001; Maria et al. 2002a; Maria et al. 2002b; Teles et al. 2003).

## 7.5 Target tissues

### 7.5.1 Fish

Tissue and species-specific response to crude oil was shown in Atlantic cod and turbot. Induction of micronuclei was significantly higher in turbot compared to Atlantic cod. The frequencies of micronuclei in cephalic kidney from all treated groups of turbot and cod were about 2-fold higher than in mature erythrocytes from peripheral blood (Baršienė et al. 2006c). In teleost fish, the main hemopoietic tissue is a cephalic kidney, but fish peripheral blood has been traditionally used as an endpoint in genotoxicity assessment. Since MN can arise after cell division, one disadvantage of the MN test in peripheral blood is the comparatively low response to genotoxic agents. It is not yet known, what proportion of injured erythroblasts

could appear in the peripheral blood circulating system, what extent of DNA repair there is and how long a complete red blood cell turnover in fish would be (Buschini et al. 2004). Treatment with Cytocholasin B, cytokinesis blocker, demonstrated, that in *Tilapia rendalli, Oreochromis niloticus* and *Cyprinus carpio* immature kidney erythrocyte cells start to circulate in peripheral blood after 2-4 days. Nevertheless, the rate of cell proliferation varied between fish species (Grisolia and Cordeiro 2000).

Liver was used as a tissue directly affected by oil metabolic components, which have been described as mutagenic agents in fish (Stegeman et al. 1993). Hepatic micronuclei approach has been considered a sensitive tool for the evaluation of genotoxic effects in contaminated sites (Williams and Metcalfe 1992; Rao et al. 1997; Arcand-Hoy and Metcalfe 2000; Pietrapiana et al. 2002). In Atlantic cod, hepatic micronucleus assay was shown being a more sensitive system for the assessment of oil genotoxicity than in peripheral blood erythrocytes (Baršienė et al. 2005a). Cristaldi et al., (2004) demonstrated selective removal of micronucleated erythrocytes from peripheral blood in spleen. Thus, micronuclei counting in peripheral blood can give the wrong information about fish exposure to genotoxic compounds. On the other hand, the comparison of genotoxicity levels in fish liver and peripheral blood showed that cytogenetic changes in peripheral blood reflect recent exposure as a result of the more rapid turnover of blood cells (Tell-Krakoc et al., 2001).

In 5 of the 9 studies where (freshwater and marine) fish were sampled at clean and contaminated sites, the authors conclude that there were differences in MN (Table 16; Tripathy and Das 1995; Minissi et al. 1996; Rao et al. 1997; Arcand-Hoy and Metcalfe 2000; Pietrapiana et al. 2002). A relevant caging experiment with fish has also been done. Tuvikene et al. did not observe a higher frequency of MN in rainbow trout caged at a PAH contaminated site, although the PAH concentration in the fish was high (Tuvikene et al. 1999).

Exposure of Atlantic cod to crude oil resulted in significantly increased frequency of micronucleated eryhtrocytes and other nuclear abnormalities in head kidney (Aas et al. 2002).

### 7.5.2 Mussels

In the same specimens of mussels, a much higher MN induction (up to 8.85 MN/1000 cells) has been observed in gills compared to haemocytes (Dolcetti and Venier 2002). Lower MN response in mussel haemocytes than in gill cells was also described by Dailianis et al. (2003). Dose-response relationships between contaminants and MN incidences was shown in gill cells of mussels experimentally treated to certain contaminants as well as pollution gradient-response pattern that was found in mussels from Wismar Bay and Lithuanian coast of the Baltic Sea and from southern Norwegian fjords (Barsiene et al. 2004; 2005b; 2005c; 2006b; Schiedek et al, in press).

Three field studies with invertebrates (mussels and snails) have been found where the MN test has been successfully used to separate contaminated and clean sites (Bolognesi et al. 1996; Barsiene and Lovejoy 2000; 2002). Positive results have also been observed in laboratory experiments (Wrisberg and Rhemrev 1992; Bolognesi et al. 1996), and in a mesocosm experiment (Wrisberg and Rhemrev 1992), but the results of laboratory and field studies by

Burgeot et al. indicate that the MN test are not yet applicable for biomonitoring of genotoxic exposure of molluscs (Burgeot et al. 1995; 1996).

### 7.5.3 Other indicator species

The nereid marine polychaete *Platynereis dumerilii,* marine tubeworm *Pomatoceros lamarckii* and sea urchins has been used for the evaluation of environmental genotoxicity. Marine worm *Platynereis dumerilii* served as indicator species in assessment of genotoxins in municipal sewage effluents (Jha et al. 1997), genotoxic, cytotoxic properties of TBT (Hagger et al. 2002), benzo[*a*]pyrene and other genotoxins (Jha et al. 1996). Colchicine-induced effects were analyzed in *Pomatoceros lamarckii* (Wilson et al. 2002). Hose studied both anaphase aberrations and MN in sea urchin *(Strongylocentrotus purpuratus)* embryos exposed to benzo[*a*]pyrene in the laboratory (Hose 1985). MN were present at 1 µg/L benzo[*a*]pyrene and higher (anaphase aberration was more sensitive) (Hose 1985). Saotome et al. have developed a micronucleus assay for use in sea urchin embryos (Saotome et al. 1999) (but Hose used the MN test on sea urchins 14 years earlier). The test chemicals (mitomycin C, vinblastine and 1-beta-D-arabinofuranosylcytosine) induced clear micronuclei dose-dependently in *Clypeaster japonicus* and *Hemicentrotus pulcherrimus* (Saotome et al. 1999). The MN test in sea urchins has been improved to consider osmolarity and salinity in laboratory test conditions (Saotome and Hayashi 2003).

Analysis of interspecies variation in fish, mussel and crab DNA damage caused by genotoxic compounds *in situ* showed species specific susceptibility to environmental genotoxins (Bihari and Fafandel 2004). Thus, the multi-species approach in impact and risk assessment should be linked to harmful effects of pollution in sentinel organisms to their ecological consequences. In the context of ecological genotoxicity or ecosystem health, genotoxicity biomarkers are also being used to link cytogenetic damage through to higher levels, i.e. pathology with reduced reproductive success of marine organisms inhabiting zones affected by incidental or operational oil spills from all activities related to transport or processing of petroleum products.

We have not been able to find any field studies where the MN test has been used on sea urchins, but the results from the *BioSea programme* on micronuclei in oil exposed cod and mussel indicate that the micronucleus assay can be useful for detecting genetic damage caused by oil (at least in the laboratory). The study performed by Pietrapiana et al. (2002) indicates that the micronucleus assay also can be used to detect chromosome damage in fish exposed to oil in the field.

Table 16. Overview of results from biomonitoring studies using cytogenetic assays (tests measuring effects at the chromosome level) on different species of freshwater and marine fish collected in the field; + : significant difference between exposed and control; ÷ : no significant difference between exposed and control; ? : results not clear.

| Test | Species | Type of contamination | Result | Reference |
|---|---|---|---|---|
| Chromosome aberration test | Pacific herring *Clupea pallasi*) | Exxon Valdez oil spill | + | Hose and Brown 1998 |
| | | | + | Hose et al. 1996 |
| | | | + but recovery | Norcross et al. 1996 |
| | | | + **caging exp**. | Kocan et al. 1996 |
| | Winter flounder *Pseudopleuronectes americanus)* | The *North Cape* oil spill | + | Rovero et al. 1999 |
| Micronucleus | *Barbus plebejus*) freshwater fish | Polluted river Tiber), | + | Minissi et al. 1996 |
| | Freshwater fish | Paper mill effluents | + | Tripathy and Das 1995 |
| | Bullheads *Ameiurus nebulosus)* | Hamilton Harbour | + | Rao et al. 1997 |
| | Various fish | The Noril'sk mining and smelting complex | ? | Zakhidov et al. 1996. |
| | Freshwater: *Carassius sp.*, *Zacco platypus* Marine: *Leiognathus nuchalis, Ditrema temmincki* | River, harbour | ? | Hayashi et al. 1998 |
| | **Caged** rainbow trout *Oncorhynchus mykiss)* and roach *Rutilus rutilus*) | The oil shale industry, PAH | ÷ | Tuvikene et al. 1999. |
| | Brown bullheads *Ameiurus nebulosus)* freshwater) | Genotoxic contaminants in the field | +/÷ seasonal differences | Arcand-Hoy and Metcalfe 2000 |
| | *Tilapia rendalli, Oreochromis niloticus* and *Cyprinus carpio* | Sewage treatment plant discharges | ÷ | Grisolia and Starling 2001 |
| | *Lepidorhombus boseii. Merluccius merluccius* and *Mullus barbatus* | The "Haven" oil spill | + species differences | Pietrapiana et al. 2002 |
| | Eel *Anguilla anguilla)* | Heavy metal pollution river) | ÷ | Sanchez-Galan et al. 2001 |

Table 17. Overview of results from biomonitoring studies using micronucleus assessment on different species of aquatic invertebrates collected in the field; + : significant difference between exposed and control; ÷ : no significant difference between exposed and control; ? : results not clear.

| Species | Type of contamination | Result | Reference |
|---|---|---|---|
| *Mytilus edulis* | produced water, Statfjord | + | Hylland et al., 2005 (WCM) |
| Mussel *M. galloprovincialis* | French Mediterranean coast | ÷ | Burgeot et al. 1996. |
| Oyster *C. gigas* | Cadmium and copper | ÷ | Burgeot et al. 1995 |
| Mussel *M. edulis* | Contaminated sediment **mesocosm** | ? | Wrisberg and Rhemrev 1992 |
| 5 species of freshwater bivalves and snails | Contaminated port area | + | Barsiene and Lovejoy 2000 |
| Mussel *M. edulis* | Baltic Sea at Butinge oil terminal | + | Barsiene 2002 |
| Mussel *M. galloprovincialis* | The La Spezia gulf, Ligurian sea | + | Bolognesi et al. 1996 |

## 7.6 Confounding factors

There is limited knowledge about the non-contaminant factors that may affect micronucleus formation.

## 7.7 Links

Micronucleus formation indicates chromosomal breaks, known to result in teratogenesis (effects on offspring) in mammals. There is however limited knowledge of relationships between micronucleus formation and effects on offspring in aquatic organisms.

## 7.8 Background responses

There is not sufficient data to establish the background response for micronucleus formation, but baseline levels appear to be low in most studies.

## 7.9 Assessment criteria

There are no established assessment criteria for micronucleus assessment. Results from the WCM 2003 and 2004 indicate that it should be feasible to establish such criteria, but this requires some more data.

## 7.10 Quality assurance

There has been no international intercalibration for micronucleus assessment as used in ecotoxicology.

# 8. Multixenobiotic response (MXR)

## 8.1 Introduction

For many organisms it is observed that tissue levels of certain contaminants are lower than levels observed in the environment. It has been suggested that this is mediated by the MXR phenomenon, which is similar to the multi drug resistance MDR phenomenon that was first observed in tumour cell lines resistant to anti-cancer drugs. The MXR mechanism acts as an energy dependent pump that translocates a wide variety of structurally and functionally diverse compounds. These tend to be moderately hydrophobic and planar natural products which are often substrates for or metabolites of detoxification enzymes such as cytochromes P450 (CYPs). The protein responsible for this transport function is the transmembrane P-glycoprotein (Pgp), which is found in specialized epithelial tissues such as gut, liver, kidney and blood vessels at the blood brain barrier. Although Pgps have been intensively studied in relation to cancer biology, less in known regarding multixenobiotic resistance in wild populations of organisms, and there is similarly little information on environmentally relevant Pgp substrates.

Immunoblot and immunohistochemical studies of Pgp expression can be performed in a variety of organisms. The most successful and widely used antibody is C219, which identifies all known Pgps. A list of aquatic organisms and the tissues in which C219 reactive proteins have been detected is provided in Bard (2000).

Two methods can be used to measure MXR transport activity: bioaccumulation assays and efflux assays. In the first case, the accumulation of a marked substrate (radioactive or fluorescent e.g. rhodamine dye), is measured with or without the presence of a competitive inhibitor in translucent living cells or in tissue homogenates. In efflux assays, the rate of efflux of a previously accumulated (marked) substrate into the medium is measured in the presence or absence of a competitive inhibitor. The efflux rate is directly proportional to Pgp transport activity. This assay is simple and inexpensive and animals are not injured by the procedure.

## 8.2 Dose-response

### 8.2.1 MXR induction

Most studies have reported induction of multixenobiotic resistance transport activity and elevated Pgp protein levels in

- field populations of pollutant exposed aquatic organisms
- transplantation of organisms to different polluted sites
- laboratory exposure experiments

Reviews of MXR activity and relevant studies has been provided by several authors recently (Bard 2000; Daughton and Ternes 1999; Epel 1998; Galgani 2000; Jha 2004; Livingstone *et al.* 2000; Minier *et al.* 1999; Moore *et al.* 2004; Pain and Parant 2003; van der Oost *et al.* 2003).

Induction of the MXR system has been reported after exposure to several pollutants, among them diesel oil, metal pollution, PAH, alkylphenols, pesticides PCB and polluted sediments (Table 18 and Table 19).

Table 18. Induction of MXR in bivalves.

| Species and tissue | Substance | Concentration | Exposure time (days | Response | Reference |
|---|---|---|---|---|---|
| **Method: Measurement of protein expression** | | | | | |
| *Mytilus edulis* Blood cells | Vincristine | | 19-29 | 1,5-2,5 | Minier and Moore 1996 |
| *Mytilus californianus* Gill tissue | Chlorthal/PCB | 5 ppm | 3 | + | Eufemia and Epel 1998 |
| *Mytilus californianus* Gill tissue | Chlorthal/PCP/DDE | 1-5 ppm | 1-3 | + | Eufemia and Epel 2000 |
| *Mytilus californianus* Gill tissue | $CdCl_2$ $NaAsO_3$ | 0,5 1-5 ppm | 1-3 | + | Eufemia and Epel 2000 |
| *Crassostrea gigas* Gill tissue | Contaminated sites (12 sites | | 30 | 1 site | Ringwood *et al.* 1999 |
| **Method: Reduction of accumulation** | | | | | |
| *Mytilus edulis* Blood cells | Vincristine | 5-10 µg/ml | 19-29 | 35-48% | Minier and Moore 1996 |
| *Mytilus galloprovincialis* Gill tissue | Contaminated site | | 3 | 62% | Kurelec 1995 |
| *Mytilus galloprovincialis* Gill tissue | Rhodamine 123 Diesel 2 oil | | 4 | 45-50% | Smital and Kurelec 1998 |
| *Mytilus californianus* Gill tissue | Chlorthal/PCB | 5 ppm | 3 | >75%/ 50% | Eufemia and Epel 1998 |
| *Mytilus californianus* Gill tissue | Chlorthal/PCP/DDE | 1-5 ppm | 1-3 | >50% | Eufemia and Epel 2000 |
| *Mytilus californianus* Gill tissue | $CdCl_2$ $NaAsO_3$ | 0,5 1-5 ppm | 1-3 | 50% 40-80% | Eufemia and Epel 2000 |
| *Corbicula fluminea* Gill tissue | Diesel-2 oil | | 3 | 43% | Kurelec *et al.* 1996 |
| *Corbicula fluminea* Gill tissue | Sediments from the Rhine river | | 3 | 28% | Kurelec *et al.* 1996 |
| **Method: increase of efflux** | | | | | |
| *Dreissena polymorpha* Whole organism | Contaminated site | | 21 | ×4,5 | Parant and Pain 2001 |

Part of the table is based on Pain & Parant (2003).

Table 19. Induction of MXR in fish.

| Species and tissue | Substance | Concentration | Exposure time days) | Response | Reference |
|---|---|---|---|---|---|
| **Method: Measurement of increased protein expression** | | | | | |
| *Oncorhynchus mykiss* Liver | Dieldrine | 5, 15 ppm | 70 | - | Curtis *et al.* 2000 |
| *Oncorhynchus mykiss* Liver | Prochloraz, Nonylphenol diethoxylate | 10, 100µg/l 100µg/l +33 µg/l | 7-14 | - | Sturm *et al.* 2001 |
| *Fundulus heteroclitus* Liver | Creosot contaminated site vs. unpolluted site | | | × 2,65-3,25 | Cooper *et al.* 1999 |
| *Fundulus Heteroclitus* Liver | 3-methylcholanthrene | 100 ml/kg | *ip* -injections | - | Cooper *et al.* 1999 |
| *Ictalurus punctatus* Intestine and liver | VIN BNF BaP TCB | 20 µg/kg diet 10-50 mg/kg diet 0.5- mg/kg diet 1-10 mg/kg diet | 10 | - | Doi *et al.* 2001 |
| **Method: Reduction of accumulation** | | | | | |
| *Oncorhynchus mykiss* Hepatocytes | Prochloraz, Nonylphenol diethoxylate | 10, 100µg/l 100µg/l +33 µg/l | 7-14 | 50-76% | Sturm *et al.* 2001 |

### 8.2.2 Chemosensitisers

The protective role of the MXR defence system appears to be fragile. As opposed to MXR induction, it has also been demonstrated that many classes of chemicals are capable of competitively inhibit the MXR function Kurelec et al. (1997). These compounds are referred to as chemosensitisers and can be both naturally occurring compounds and pollutants. Chemosensitisers can saturate the MXR system, hence promoting the accumulation of potentially toxic xenobiotics. The relevance of MXR chemosensitisers has been investigated in several studies (see table below) and the results showed that their presence led to the apparition of toxic effects in organisms exposed to toxic compounds below the non-observed effect concentration.

The inhibitors of MXR activity has been described by Smital *et al.* (2004). In this article, it is described that pesticides, fragrances, microbial degradation products and natural inhibitors possess the ability to inhibit the important function the MXR defensive system. Other relevant work is presented in Table 20.

Table 20. Effect of chemosensitisers (inhibition of MXR activity).

| Species and tissue | Factor | Inhibitory effect | Reference |
|---|---|---|---|
| *Dreissena polymorpha* Gill tissue | Sava river (high conc. chemosensitisers) compared to water with low conc. | ×2 number of mutagens produced | (Britvic and Kurelec 1999) |
| *Mytilus edulis* *Lytechinus pictus* Embryo | Extracts of *Egregia* | + | (Eufemia *et al.* 2002) |
| Fibroblasts | Extracts from landfill (household and industrial) | + | (Kurelec *et al.* 1998) |
| *Mytilus californianus* Gill tissue | Musk ketone Musk xylene | + | (Luckenbach *et al.* 2004) |
| *Suberites domuncula* Single cells *Corbicula fluminea* Gill tissue | Extracts of "marine snow" (amorphous aggregates) | + | (Muller *et al.* 1998) |
| *Corbicula fluminea* Gill tissue | Verapamil Staurosporine | + | (Waldmann *et al.* 1995) |

## 8.3 Species sensitivity

In bivalves, results from in the field studies showed that it was possible to detect evidence of MXR activity in quite every organism investigated so far (for review see Pain & Parant, 2003).. The literature indicate that MXR expression in aquatic organisms such as bivalves, worms and sponges can reliably be used as and indicator of contaminant exposure and /or resistance (van der Oost et al., 2003). For fish, examples of interactions have been reported, but further characterization of wild fish populations is required.

## 8.4 Relevance of other factors

The main difficulty of using biomarkers in a monitoring programme is the interference of natural environmental factors with the biological responses. *In situ* biomarker responses can be altered or increased by physical or chemical parameters, hence, making the interpretation rather difficult. It is, therefore, essential to characterise the physical and chemical state of the sampling sites and to study natural variations in biomarker responses in order to differentiate

a pollution-induced stress from the background response and to correct for natural variations (Minier et al., 2002). Seasonal variations of MXR activity in polluted and unpolluted sites have been studied, as well as the effect of salinity, temperature and oxygen supply. The hypothesis that the defence system could be affected by environmental parameters such as temperature or algal proliferation has been proposed and supported by results of laboratory studies (Table 21). In addition, measured MXR activity is variable at the species level and even at the population level (Pain & Parant, 2003).

Table 21. The relevance of non-contaminant factors on MXR in bivalves.

| Species and tissue | Factor | Response | Reference |
|---|---|---|---|
| **Method: Measurement of increased protein expression** | | | |
| *Mytilus edulis*<br>Digestive gland and gill tissue | temperature<br>salinity<br>oxygen supply | -<br>+<br>+ | Luedeking and Koehler 2004 |
| *Mytilus galloprovincialis*<br>Digestive gland | Seasonal variations | ×10 | Minier *et al.* 2000 |
| *Crassostrea virginica*<br>Gill or mantle tissue | Temperature, seasonal variation | ×3 | Keppler and Ringwood 2001 |
| **Method: increase of efflux** | | | |
| *Dreissena polymorpha*<br>Whole organism | Fuel oil 2 | | Pain & Parant, 2003 |

## 8.5 Links

MXR activity could conceivably increase the efflux of components required by the cell and thereby cause cellular damage, but this has not been documented experimentally. Further research is required to clarify the relative importance of induction as compared to chemosensitisation and both in relation to cell and individual health.

## 8.6 Background responses

Baseline responses have not been established for MXR activity for any species yet.

## 8.7 Assessment criteria

The assay still needs to be clearly defined; as yet there is no authoritative protocol for the determination of the various components of the system(s). Although shown to be affected by oil components in earlier work, it is premature to include MXR in offshore monitoring programmes at the moment.

## 8.8 Quality assurance

There has been no quality assurance activity in relation to MXR measurements as yet.

# 9. Peroxisomal proliferation

## 9.1 General introduction

Peroxisomes are ubiquitous single-membrane limited versatile organelles essential for β-oxidation of fatty acids, especially very long-chain fatty acids. Peroxisomes are highly heterogeneous organelles that vary in size, shape and protein composition depending on species, tissue, cell, metabolic state and developmental stage. In general, peroxisomes are single membrane-bounded organelles with a finely granular electron-dense matrix. They have a diameter ranging from 0.1 to 1.5 µm, and are more abundant in tissues active in lipid metabolism in general. Reviews of cell biology of peroxisomes in aquatic organisms and use as biomarker are given by Cancio and Cajaraville (2000) and Cajaraville *et al.* (2003).

Peroxisomes acquired relevance with the discovery that clofibrate, a pharmaceutical drug to lower the serum levels of lipids in patients with hypercholesterolemia, caused a massive proliferation of peroxisomes in rodents (Hess *et al.* 1965). It has later been shown that it is a link between the proliferation process and carcinogenesis in rodents (Reddy *et al.* 1980). Peroxisomal proliferation is usually accompanied by induction of all three enzymes of the peroxisomal β-oxidation pathway, namely acylCoA oxidase (AOX), peroxisomal hydratase-dehydrogenase or multifunctional enzyme (PH), and thiolase (Cajaraville *et al.* 2003). High-fat diets, cold adaptation, vitamin E deficiency, riboflavin deficiency, genetic obesity, diabetes and starvation can also lead to peroxisomal proliferation in rodents (Bentley *et al.* 1993). Disappearance of the peroxisomes proliferators results in a quick return of peroxisomes to basal numbers and enzyme activity levels. The most abundant peroxisomal enzyme is catalase, which is often used as a marker enzyme.

In addition to fibrates and other hypolipidemic drugs, peroxisomal proliferators comprise a heterogeneous group of compounds including phthalates, certain classes of pesticides, steroids, solvents, industrial chemicals, food flavours, therapeutic drugs, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and natural products (Bentley *et al.* 1993; Cancio and Cajaraville 2000). One common feature of these compounds or their metabolic derivatives is a hydrophobic-lipophilic backbone with an acidic function, generally a carboxylic group. As peroxisomal proliferators are non-genotoxic and non-mutagenic compounds, their carcinogenicity has been ascribed to a number of different mechanisms including increased oxidative stress and DNA damage, increased DNA synthesis and mitogenesis, promotion of initiated liver foci and suppression of apoptosis (Cajaraville *et al.* 2003). Although there is a strong correlation between peroxisomal proliferation and hepato-carcinogenesis in rodent, a causal link remains to be established.

## 9.2 Dose-response

Aquatic organisms are exposed to a variety of contaminants in their environment. Some of these are known for their ability to cause peroxisomal proliferation, and increasing evidence suggests that aquatic organisms such as bivalves and fish are responsive to these environmental pollutants (Table 22 and Table 23).

Table 22. Effects on peroxisomal proliferation parameters in fish.

| Species and tissue | Substance | Conc | Exposure time (days) | Response Peroxisomal proliferation | Response Catalase | Reference |
|---|---|---|---|---|---|---|
| *Anguilla anguilla* Liver | Dinitro-o-cresol | 5, 50 250 µl/l | 28 | + | ×1.4 | (Braunbeck and Volkl 1991) |
| *Ictalurus punctatus* Liver | BKME | 10, 20, 40% | 14 | | ×2-3 | (Mather-Mihaich and Di Giulio 1991) |
| *Oncorhynchus mykiss* Liver | Endosulfan + disulfoton | 50 mg/l 1 or 5 µg/l | 18-34 | ×2.7 (transient) | None | (Arnold *et al.* 1995) |
| *Oncorhynchus mykiss* | Ciprofibrate injection | 25, 25, 35 mg/kg | 21 | ×2.3 | ×3.5 | (Yang *et al.* 1990) |
| *Oncorhynchus mykiss* Liver | Atrazine | 10, 20, 40, 80, 160 µg/l | | + | | (Oulmi *et al.* 1995) |
| *Oncorhynchus mykiss* Liver | Linuron | 30 µg/l | | + | | (Oulmi *et al.* 1995) |
| *Sparus aurata* | Dieldrine (injection) | 0.15-1 mg/kg | 2 and 7 | | × 2.7 (7 days) | (Pedrajas *et al.* 1996) |
| *Fundulus heteroclitus* liver | 2,4-dichlorophenoxyacetic acid | 0.01, 0.1 and 1 ppm | 7, 14 or 21 | + | | (Ackers *et al.* 2000) |
| *Brachydanio rerio* Liver | Lindane | 40, 80, 110, 130, 150 µg/l | | | Decrease | (Braunbeck *et al.* 1990) |
| *Anguilla anguilla* Hepatocytes | Dinitro-o-cresol | 5, 50, 250 µg/l | 28 | + | + | (Braunbeck and Volkl 1991) |
| *Cyprinus carpio* liver | PFOS (injection) | 16-864 ng/g | 1 and 5 | - | | (Hoff *et al.* 2003) |
| *Oncorhynchus mykiss* liver Transcriptome responses (microarray) | Pyrene<br><br>Carbon tetrachloride | 25, 100 µg/l<br><br>5,10,15 µl/l | 4 | <br><br>down regulation | suppression | (Krasnov *et al.* 2005) |
| *Danio rerio* liver | Estradiol<br>Dibutylphthalate<br>Methoxychlor<br>4-t-octylphenol<br>ethynylestradiol | 10 µg/l<br>500 µg/l<br>100 µg/l<br>500 µg/l<br>10 µg/l | 7 and 15 | +<br>+<br>+<br>+<br>+ | <br><br>Up. (15d)<br><br>Down (7d) | (Ortiz-Zarragoitia and Cajaraville 2005) |
| *Solea ovata* | B[a]P (injection) | 0.1, 0.5, 1, 10 mg/kg | 7 | + (1mg/kg and higher) | | (Au *et al.* 1999) |

Part of the table is based on Cancio et al. (2001).

Table 23. Effects on peroxisomal proliferation parameters in bivalves.

| Species and tissue | Substance | Concen tration | Exposure time (days) | Response Peroxisomal proliferation | Response Catalase | Reference |
|---|---|---|---|---|---|---|
| *Mytilus edulis* Digestive gland | PAHs or PCBs | | 30 | + | ×2 (PCBs) | (Krishnakumar *et al.* 1997) |
| *Mytilus edulis* Digestive gland | Benzo[a]pyrene<br>Menadione | 50 ppb<br>1 ppm | 2, 6, 19 | | ×1.2 (transient)<br>×1.4 | (Livingstone *et al.* 1990) |
| *Mytilus galloprovincialis* Digestive gland | WAF lubricant oil<br>WAF ural crude oil<br>WAF maya crude oil | 0.6, 6, 40% | 21, 49, 91 | 3.8-5<br>2-3<br>1.4-1.5 | ×2,25<br>×1.9<br>×1.8 | (Cajaraville *et al.* 1997) |
| *Mytilus galloprovincialis* Digestive gland | WAF lubricant oil<br>WAF (injected)<br>Clofibrate<br>Clofibrate (injected) | 5%<br>100 µl 5%<br>2.5 mg/l<br>1,5 mg/100µl | 1, 7, 21 | Decrease<br>×1.45-1.6<br>Decrease<br>×1.12-2.7 | None<br>Decrease<br>None<br>Decrease | (Cancio *et al.* 1998) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | DEHP<br>DEHP (injected)<br>B[a]P<br>B[a]P (injected | 60 µg/l<br>100µg/100µl<br>300µg/l<br>500µg/100µl | | Decrease<br>None<br>None<br>None | None<br>None<br>None<br>None | |
| *Mytilus galloprovincialis*<br>Digestive gland | B[a]P<br>DEHP<br>Cd<br>Mix B[a]P and Cd | | 21 | +<br><br>Reduction<br>- | +<br>+ | (Orbea *et al.* 2002) |
| *Mytilus galloprovincialis*<br>Digestive gland | Different sites (PAH burden) – transplant study | | 29 | ×4 (polluted vs. nonpolluted) | | (Cajaraville *et al.* 2003) |

Part of the table is based on Cancio et al. (2000).

In addition to exposure studies with bivalves, field studies have demonstrated differences in the number and size of peroxisomes of *Mytilus* sp. collected in sites with different concentrations of organic pollutants (Cancio et al., 2000). In a reciprocal transplant experiment with mussels, it was demonstrated that the peroxisome proliferation response may be reversible.

In field studies with fish from sites with different pollutant concentrations have been compared, contradictory results have been obtained with activities of peroxisomal antioxidant enzymes.

## 9.3 Species sensitivity

The first studies of peroxisomes in the aquatic environment was in fish, and in fish there are data indicating that fibrate hypolipidemic drugs, various pesticides, bleached kraft pulp and paper mill effluents (BKMEs), PAHs, phthalates, alkylphenols and estrogens can cause peroxisome proliferation. An overview of these studies has been provided by Cajaraville et al. (2003). Later research has focussed on mussels (*Mytilus* sp) because these filter-feeding bivalves are used worldwide as a bioindicator and sentinel species of environmental pollution in marine and estuarine areas. Peroxisomes in the digestive gland of mussels share several characteristics with vertebrate liver peroxisomes, but they also show some special features.

## 9.4 Confounding factors

The detailed knowledge of seasonal variations in peroxisomal parameters is essential to be able to detect changes induced by contaminants. In addition, peroxisomes may respond differently to pollutants depending on the time of the year. Several studies have investigated this, and some of them are presented in the table below. In addition to seasonal fluctuations and population-specific responses, gender of animals could be another possible confounding factor (see table for details).

Table 24. Overview of factors that may affect peroxisomal proliferation in various organisms.

| Species and tissue | Factor | Response | Reference |
|---|---|---|---|
| *Mytilus galloprovincialis* Digestive gland | Seasonal variation | × 8 (spring vs. winter) | (Cancio *et al.* 1999) |
| *Leuciscus idus melanotus* hepatocytes | Low and high temperatures (14 and 28 °C) | + | (Braunbeck *et al.* 1987) |
| *Mytilus galloprovincialis* Digestive gland | Seasonal variation<br><br>PAH burden (different sites) | + (uncontamin. site) | (Orbea *et al.* 1999) |
| *M. galloprovincialis*<br>*Crassostrea* sp<br>*Carcinus maenas*<br>*Mugil cephalus* | Seasonal variation<br><br>PAH/PCB body burden (different sites) | - (season var. mussel)<br>+ (season var. oyster)<br>+ (season var. crab)<br>+ (season var. fish) | (Orbea *et al.* 2002) |
| *Salmo trutta* | Seasonal variation | + (more pronounced in females) | (Rocha *et al.* 1999) |
| *Fundulus heteroclitus* | Starvation (96 h)<br>gender | Decrease | (Haasch 2002) |
| *Mugil cephalus* hepatocytes | Seasonal variation | Level higher in summer | (Orbea *et al.* 1999) |
| *Danio rerio* | Gender<br>Developmental stage | No significant difference observed | (Ibabe *et al.* 2005) |

## 9.5 Links

The potential deleterious effects on reproduction, development and growth are unknown, providing a challenge for future investigations (Cajaraville et al., 2003). In rodents, peroxisome proliferators are known to adversely affect reproduction and development. This opens a new field of research aiming to establish possible connections between peroxisome proliferation and endocrine disruption (Ortiz-Zarragoitia et al., 2005).

## 9.6 Background responses

There are still only a limited number of laboratories that have sufficient competence to perform the required analyses. Baseline responses have been defined by the University of the Basque Country for mussel, but it is not clear whether the baseline will be similar in the North Sea.

## 9.7 Assessment criteria

Criteria have been established for Spain, but as mentioned above it remains to be clarified whether they can be applied to mussels from other geographical areas.

## 9.8 Quality assurance

Internal quality assurance guidelines exist, e.g. SOP for procedures. No international intercalibration has been performed for the relevant methods.

# 10. Acetyl cholinesterase (AChE) inhibition

## 10.1 Introduction

In vertebrates, acetylcholine (ACh) acts as an excitatory transmitter for voluntary muscle in the somatic nervous system. ACh also serves as both a preganglionic and a postganglionic transmitter in the parasympathetic nervous system. Cholinesterase enzymes (ChE) are responsible for the removal of ACh from the synaptic cleft by hydroxylation. Cholinesterases are usually divided into the acetylcholinesterases (AChEs) and the butylcholinesterases (BChEs). In fish, the tissues containing most AChE are brain and muscle.
Among the AChE inhibitors, the most common are a) organophosphate insecticides (OPs) such as terbufos, trichlorfon and chlorpyrifos b) carbamate insecticides such as carbofuran and carbaryl and c) synthetic pyrethroid compounds. OPs have become one of the most widely used classes of insecticides worldwide because of their relatively non-persistent characteristics in the environment. Although rapidly degraded, the substances lack target specificity and have high acute toxicity toward many nontarget vertebrate and invertebrate species. OPs and carbamates are applied in agricultural and residential uses, and can also be accidentally introduced into the environment through overspray, aerial drift or runoff. Thus, many terrestrial as well as aquatic organisms may be at risk for intoxication caused by exposure to these compounds.

This biomarker could be affected by e.g. biocides used in oil and gas production (and hence released with produced water). It is a sensitive marker for a range of substances that may have neurotoxic effects.

Tissue residues of AChE inhibitors associated with reduced survival appear to be highly variable, ranging from 0.00004 mmol/kg to 29 mmol/kg (Barron *et al.* 2002). It has been suggested that brain AChE inhibition levels of >70% are associated with mortality in most species (Fulton and Key 2001).

## 10.2 Dose-response

Most of the studies involving AChE inhibitors have been performed with insecticides, but some studies have also focussed on the effect of classical pollutants on the activity of AChE (Table 25 and Table 26).

Table 25. Dose-response, background response and sensitivity in fish species (brain tissue if nothing else mentioned).

| species | substance(s) | concentration | exposure time (days) | baseline/control (level/activity) | inhibition % | reference |
|---|---|---|---|---|---|---|
| **Experimental studies** | | | | | | |
| Salmon, *Salmo salar* | Fenitrothion | 0.005-0.213 mg/L | 7 | | 12 – 58 | (Morgan *et al.* 1990) |
| Coho Salmon, *Oncorhynchus kisutch* | Chlorpyrifos | 0.6-2.5 µg/L (nominal) 0.3-1.7 µg/L initial measured) | 4 | Brain: 23.2 ± 0.5 (SE) Muscle: 38.9 ± 1.4 (SE) µmol/min/g | lowest conc: Brain: 23% Muscle: 12% highest conc: Brain: 64% Muscle: 67% | (Sandahl *et al.* 2005) |
| larval rainbow trout (*Oncorhynchus mykiss*) | Diazinon<br><br>Malathion | 250-1000 µg/L<br><br>20-40 µg/L | 1 and 4 | ~13 and ~11.5 µmol/min/g<br><br>~12.5 and ~13 µmol/min/g | lowest conc: ~30% -~21% Highest conc: ~38% - ~30% lowest conc: ~64% - ~72% Highest conc: ~64% - ~41% | (Beauvais *et al.* 2000) |
| *Oncorhynchus mykiss* | Polluted sediments, mainly PAH | 5.8 ng/g d.w. | 14 | | No significant decrease | (Sanchez-Hernandez *et al.* 1998) |
| **Field studies** | | | | | | |
| Menhaden, *Brevoortia tyrannus* | OPs | | | | ~50% comp. to reference site | (Williams and Sova 1966) |
| Flounder muscle | Sites in the Baltic, organic pollutants | | | 94-315 nmol/min/mg | | (Kopecka *et al.* 2004) |
| *Anguilla anguilla* muscle | PAH and organochlorine content in fish | | | Relationship between volatile PAHs and activity of AChE | | (Roche *et al.* 2002) |
| **Cage deployment studies** | | | | | | |
| *Fundulus heteroclitus* | Azinphosmethyl | 0.00-7.00 µg/L | 4 | | 98% at highest conc. (mortality 17%) | Fulton & Key, 2001 |

Table 26. Dose-response, background response and sensitivity in bivalves.

| species | substance(s) | concentration | exposure time (days) | baseline/control (level/activity) | inhibition % | reference |
|---|---|---|---|---|---|---|
| **Experimental studies** | | | | | | |
| *Crassostrea gigas* gills | Dichlorpyros | 0.1-1.0 mg/L | 0-48 h | | 83% (6h) 87% (4h) | (LeBris *et al.* 1995) |
| *Mytilus galloprovincialis* gills | iso-OMPA<br><br>Eserine | $10^{-4}$ M<br><br>$10^{-6}$ M, $10^{-5}$ M and $10^{-4}$ M | 15 min | 24.2 ± 3.5 SEM nmol/min/mg<br><br>24.2 ± 3.5 SEM nmol/min/mg | ~15% ~58%<br><br>~70% and ~80% | (Escartin and Porte 1997) |
| *Mytilus galloprovincialis* | B[a]P in food | 50 mg/kg dry weight mussel | 28 | | Significantly depressed | (Akcha *et al.* 2000) |

| species | substance(s) | concentration | exposure time (days) | baseline/control (level/activity) | inhibition % | reference |
|---|---|---|---|---|---|---|
| gills | | | | | | |
| **Field studies** | | | | | | |
| *Mytilus edulis* gills | Sites in the Baltic, organic pollutants | | | 15-38 nmol/min/mg | | (Kopecka *et al.* 2004) |
| *Mytilus edulis* | Oil spill after "Erika" accident | | 1 year after accident | | Significant reduction | (Bocquene *et al.* 2004) |
| *Mytilus galloprovincialis* hemolymph | Portuguese coast | | | 30-70 nmol/min/mg | Decreased in polluted site vs. unpolluted | (Moreira and Guilhermino 2005) |
| *Mytilus galloprovincialis* Digestive gland and Gills | West coast of France | PAH, PCB and metals in mussels determined | | | Inhibition by unidentified compound | (Romeo *et al.* 2003) |

## 10.3 Species sensitivity

Both fish and mussels have been successfully employed in studies of interactions of OPs with AChE response. However, it seems that much of the work with classical pollutants (PAH, PCB, metals) are done with bivalves in field studies. In a comparative study, Galgani & Bocquené (1990) found that AChE from whole mussel (*Mytilus edulis*) was less sensitive to five OPs than the enzyme from the muscle tissues of the shrimp *Palaemon serratus* and two other fish species.

## 10.4 Relevance of other factors

Seasonal variations of AChE activity in polluted and unpolluted sites have been studied (Table 27). In addition, the effect of fish length and the location of the enzymes in the cells have been studied.

Table 27. Factors that have been found to affect AChE in fish and mussels.

| Species and tissue | Factor | Response | Comment | Reference |
|---|---|---|---|---|
| *Leuciscus cephalus* | Fish length | + | The larger fish had the lower ChE-activities | Flammarion *et al.* 2002 |
| *Crassostrea gigas* | Responsiveness of different ChE to OPs | + | Soluble enzymes insensitive, membrane bound highly sensitive | Bocquene *et al.* 1997 |
| *Sciaenops acellatus* *Fundulus heteroclitus* | Relationship between azinophosmethyl-induced AChE inhibition and mortality | | Inhibition of AChE in the peripheral nervous system may be a better predictor of OP-induced mortality than brain inhibition | VanDolah *et al.* 1997 |
| *Mytilus galloprovincialis* | Seasonal variation (2 year study) | | Variation of AChE seemed less variable with season than other biomarkers studied | Bodin *et al.* 2004 |
| *Mytilus galloprovincialis* hemolymph | Seasonal variation (1 year study) | + | AChE activity showed an apparent seasonal trend in 3 of 4 sites | Moreira and Guilhermino 2005 |
| *Mytilus galloprovincialis* gills and digestive gland | Seasonal variation | + | Strong seasonal variation from June to October of all parameters was noted. | Romeo *et al.* 2003 |

## 10.5 Links

It has been suggested that brain AChE inhibition levels of >70% are associated with mortality in most species (Fulton & Key, 2001). However, some species may tolerate much higher levels (>90%) of brain AChE inhibition. Sublethal effects on stamina have been reported for some estuarine marine fish in association with brain AChE inhibition levels as low as 50% (Fulton & Key, 2001).

Reduced swimming stamina has been shown for e.g. sheepshead minnow (Cripe et al., 1984) Swimming stamina was affected in fish exposed to the organophosphorus insecticide EPN at 4.1 and 2.2 µg/l (reduced by 54% and 43%, respectively).

Brain AChE inhibition and reductions in spontaneous swimming and feeding activity were significantly correlated in Coho Salmon (*Oncorhynchus kisutch*) exposed to chlorpyrifos (Sandahl et al., 2005).

Changes in swimming speed and distance of larval rainbow trout (*Oncorhynchus mykiss*) were significantly correlated with changes in ChE activity after exposure to diazinon and malathion (Beauvais et al., 2000).

## 10.6 Background responses

There is not sufficient data to set baseline responses for AChE for species relevant to the North Sea.

## 10.7 Assessment criteria

AChE is one of the methods with an obvious link to higher levels effects. A fish that swims slowly or is disoriented will have a much lower probability of survival than a healthy fish. Such values could be modelled without too much effort with the right input data. Unfortunately such data are not available for North Sea species.

It is less clear whether AChE inhibition in mussels will affect e.g. clearance rate, which would have direct consequences for fitness.

As indicated above, a percentage inhibition of AChE could be set as a limit, but effects on behaviour or swimming stamina is highly species dependent. There is not sufficient data to set assessment criteria for North Sea species.

## 10.8 Quality assurance

An internationally agreed protocol has been developed for AChE determination (Bocquené & Galgani, 1998). International intercalibrations have been performed within specific projects.

# 11. Vitellogenin (vtg) induction

## 11.1 Introduction

In all egg-laying vertebrates, including fish, most of the material that will eventually constitute the egg is synthesised in the liver of the female. This includes both the precursor-protein for egg yolk, vitellogenin, and precursor proteins for the eggshell (Oppen-Berntsen et al. 1992). Since the proteins are synthesised in the liver they need to be transported with blood to the gonads, which means that at the time of ovarian development the concentration of vitellogenin in the blood plasma of female fish can be phenomenal; up to 70% of the total protein in the plasma can be vitellogenin. Synthesis of vitellogenin is regulated by the steroid estrogenic hormone estradiol. High levels of estradiol mean high output of vitellogenin from the liver. For some reason, the gene for vitellogenin is also available for transcription in male fish, but it is rarely turned on because estradiol-levels are generally low in male fish. There are some fish species in which the male has naturally somewhat higher estradiol concentrations in the blood, which means that they also synthesise vitellogenin (to no use). This has been found for plaice (Scott et al., 2000) and for some wrasse species (Hylland, unpublished).

Early in the 1990s, fish physiologists that studied reproduction in freshwater fish in UK rivers observed male fish with high levels of vitellogenin in the blood and even some individuals with oocytes mixed into their testes. Further studies showed that substances in sewage effluents were responsible, which included natural estrogens (from the female half of the human population), synthetic estrogens (from pharmaceuticals) and industrial or household chemicals.

In the past decade it has been established that most sewage effluents are estrogenic and that many chemicals have estrogenic or anti-estrogenic activity, including substances in produced water effluents. Vitellogenin is close to a perfect biomarker in that the response is well-characterised and limited to substances with estrogenic (or anti-estrogenic) activity. Following exposure to estrogens, the response measured in plasma may increase million-fold compared to controls, although 10-fold or 100-fold increases are generally more commonly seen under environmental conditions (Scott & Hylland, 2002).

## 11.2 Dose-response

As for most of the biomarkers there is most data on freshwater fish. For the purpose of this review the main focus will be on marine fish. All measurements of vitellogenin referred to in this review have been done on plasma. It is possible to analyse for vitellogenin in liver as well, but concentrations are generally much lower than in plasma. Studies on gene expression naturally use liver tissue. Relevant studies are indicated below (Table 28 and Table 29).

Table 28. North Sea relevant field studies on vitellogenin responses (all studies with male fish).

| *species* | *areas* | *exposure time* | *concentration at reference location* | *induction (fold)* | *reference* |
|---|---|---|---|---|---|
| Atlantic cod (Gadus morhua) | caging - Statfjord gradient | 5 wks | 30-50 ng/mL | 5 (trend, ns) | Scott et al., 2006a |
| Atlantic cod (Gadus morhua) | North, Norwegian, Barents sea | field | 100-1000 ng/mL | 5-10 | Scott et al. 2006b |
| Atlantic cod (Gadus morhua) | Skagerrak coastal sites | field | n/a | 4 | Hylland et al.,1996 |
| flounder (Platichthys flesus) | UK estuaries | field | 200 ng/mL | 5-1000 (different estuaries) | Kirby et al. 2004 |
| flounder (Platichthys flesus) | Dutch coastal areas | field | 500 ng/mL | 2-3 | Vethaak et al. 2005 |
| flounder (Platichthys flesus) | North Sea, UK estuaries | field | 100 ng/mL | 10-4 - 10-6 | Allen et al. 1999 |

Table 29. Selected studies on dose-dependency of vitellogenin following exposure to contaminants; all studies with male fish except Hylland (unpublished), which used juvenile fish.

| *species* | *ecosystem* | *substance* | *lowest-highest concs* | *exposure time* | *baseline/ control (level/activity)* | *induction (fold)* | *LOEC* | *reference* |
|---|---|---|---|---|---|---|---|---|
| Atlantic cod (Gadus morhua) | marine | water, 4-NP | 5, 20, 100 µg/L | 1 wk | 100 ng/mL | 15 | 20 mg/kg | Hylland, unpublished |
| Atlantic salmon (Salmo salar) | freshwater | inject, BPA | 1, 5, 25, 125 mg/kg | 2 wk | n/a | >5 | 25 mg/kg | Arukwe et al. 2000 |
| eelpout (Zoarces viviparus) | marine | inject, 4-NP | 10, 100 mg/kg | 25 d | 1-5 mg/mL | 1000 | 10 mg/kg | Christiansen et al. 1998 |
| flounder (Platichthys flesus) | marine | oral, 4-OP | 0.5, 1, 2.5, 5, 7.5, 10, 25 mg/kg | 11 d | 40-80 ng/mL | >500 | 5 mg/kg | Madsen et al. 2003 |
| flounder (Platichthys flesus) | marine | oral, 4-OP | 10, 50, 100 mg/kg | 11 d | 40-80 ng/mL | >10-6 | n/r | Madsen et al. 2003 |
| flounder (Platichthys flesus) | marine | inject, 4-NP | 10, 50, 100, 150, 200 mg/kg | 2 wk | n/a | >5 | 10 mg/kg | Christensen et al. 1999 |

## 11.3 Relevance of other factors

As mentioned above, the vitellogenin response is thought to be limited to estrogenic or anti-estrogenic substances. In contrast to other biomarkers other endogenous or exogenous factors do not appear to have a strong influence. In a series of studies, juvenile cod were tested at NIVAs research facility at Solbergstrand for its suitability as an indicator species for vitellogenin research and monitoring. One of the issues to be resolved was whether there would be a seasonal variation in the sensitivity of juvenile cod to estrogens. There was some seasonal variation in the baseline level of the protein, but no variation in the response following treatment with estrogens (Figure 8). Other studies showed that starvation did not affect the induction and neither did differences in acclimation temperature, salinity or changes in temperature (Hylland & Aspholm, unpublished).

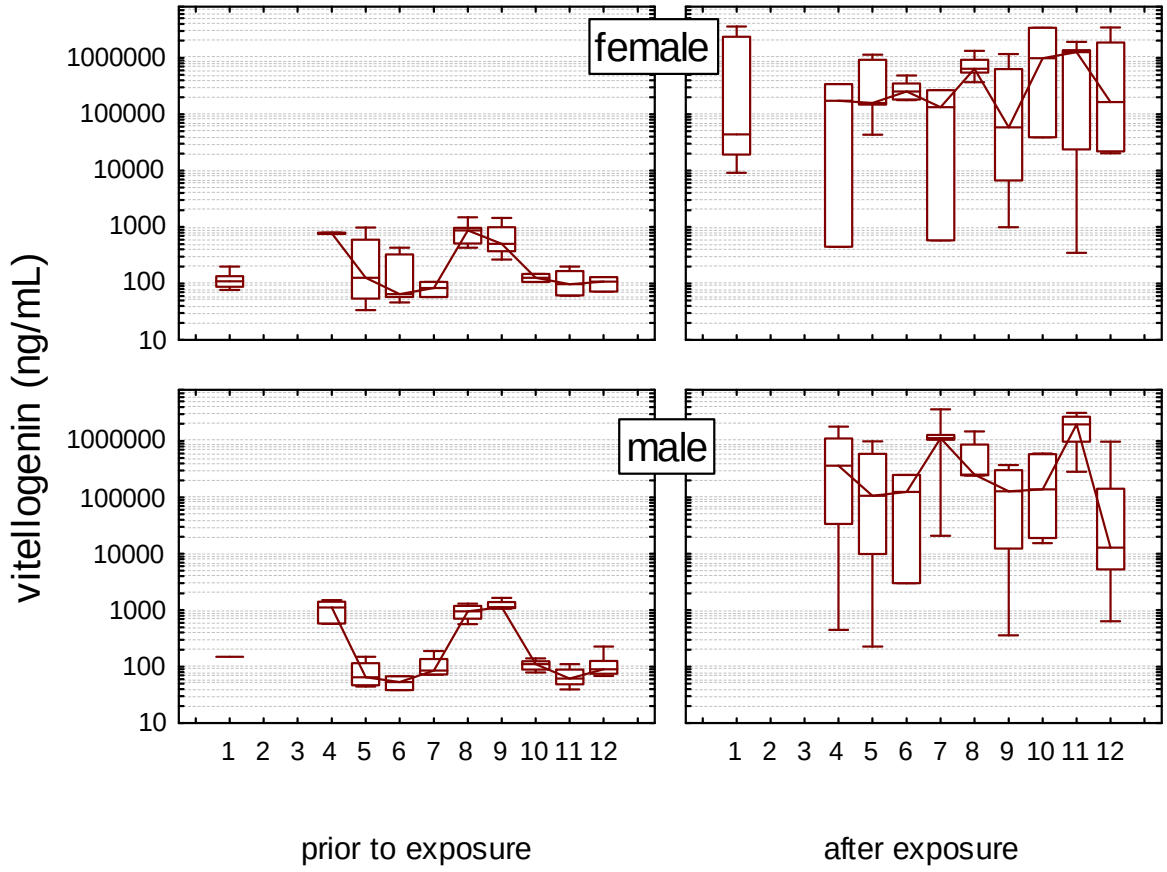


Figure 8. Vitellogenin in juvenile cod exposed for one week to 5 ng/L estradiol (top) or 50 µg/L 4-nonylphenol (bottom) through water; the figure shows median, quartiles and 10/90 percentiles. Note logarithmic y-axis.

## 11.4 Links

Elevated concentrations of plasma vitellogenin in male or juvenile fish (which would be low) indicates the presence of estrogens in the environment. Although exceedingly high concentrations of vitellogenin in the plasma may clog the kidneys of fish and cause mortality due to kidney malfunction and that there is some metabolic cost in producing the protein, it is unlikely that the presence of vitellogenin in itself will have strong negative effects. The presence of estrogens in sufficient quantities to cause induction of vitellogenin may affect larval stages of fish in the environment. Studies on model species such as zebrafish indicate that exposure to estrogens may skew the sex ratio in favour of female fish even at very low concentrations (Andersen et al. 2003, Örn et al. 2003). In fact, the concentrations of model estrogens needed to change the sex ratio was lower than that needed to induce vitellogenin in those studies.

## 11.5 Background responses

The background response of vitellogenin in North Sea species has until now been defined by the quality of the analytical procedures used for analyses of the protein, i.e. RIA and ELISA, since vitellogenin levels in male and juvenile fish are very low for most species (on the order

of 10-100 ng/mL). As indicated above there is a seasonal variability in plasma vitellogenin, even in juveniles (and presumably males); 1 µg vitellogenin/mL plasma appears to be the upper limit for plasma vitellogenin in Atlantic cod from unpolluted areas (Scott et al., in press; Hylland, unpublished observations). The concentration would have to be determined using a quantitative immunoassay (ELISA, RIA).

## 11.6 Assessment criteria

As mentioned above, changed sex ratios have been seen in experimental studies at concentrations below those for which there was an increase in vitellogenin. Sex ratios have only been determined in field studies for species with suitable reproductive physiology, such as eelpout (*Zoarces viviparus*). The high sensitivity of some species to estrogens indicate that some caution should be exercised and that a three-fold increase over the upper limit of the baseline for cod (i.e. 3 µg/mL) would indicate the presence of estrogens in the environment.

## 11.7 Quality assurance

A widely available protocol for the analysis of plasma vitellogenin in fish is available (Scott & Hylland, 2002) and there are commercial kits available for relevant species, e.g. Atlantic cod (http://www.biosense.com). In 2004-2005 an international intercalibration was performed within BEQUALM, the results of which indicated that laboratories that used similar methods (quantitative ELISA) had comparable results.

# 12. Metallothionein (MT) concentration

## 12.1 Introduction

The low-molecular-weight protein metallothionein (MT) is present in most cells in vertebrates and some invertebrates. It is a peculiar protein in that around a third of the protein is made up by the sulphydryl-containing amino acid cysteine, which contributes directly to the binding of 7-12 metal atoms in each MT molecule (Klaassen et al. 1999). Early studies indicated that concentrations of MT would increase following metal exposure (especially to cadmium) and it was then suggested that metal detoxification was the major function of the protein. Later work has shown that MT is involved in the regulation of the intracellular availability of zinc (Zn) and/or copper (Cu), which to some implied a possible role in total gene-regulation (through regulating Zn availability). Metallothionein is induced in cells exposed to free radicals and do indeed appear to protect against damaging effects from e.g. ionising radiation or radical-generating agents (Kagi & Schaffer 1988). The production of MT knock-out mice definitely established that the protein is not essential to survival, at least not in mammals, but will provide protection from toxic metals such as cadmium (Masters et al. 1994).

In addition to Cu and Zn, MT binds non-essential metals such as cadmium (Cd), mercury (Hg) and silver (Ag). The synthesis of the protein is induced by elevated intracellular concentrations of the metals mentioned above. Metallothionein induction is a response to elevated intracellular metal concentrations and the protein has for that reason been suggested as a biomarker for environmental metal contamination (Engel & Roesijadi 1987, Haux & Förlin 1989, George & Olsson 1994).

## 12.2 Analytical aspects

The methods most commonly used to quantify MT in tissues exploit some of the characteristics of this protein: its metal content, the number of sulphydryl groups, its size and/or its heat-stability. In addition, various immunochemical assays using MT-specific antisera have been established in the past 10-15 years (Roesijadi et al. 1988, Hogstrand et al. 1989, Hylland 1999). More recently, metallothionein gene expression (mRNA) has been quantified in fish species using rtPCR (Cheung et al. 2004, Tom et al. 2004). Similar methods have also been developed for mussels (Dondero et al. 2005). Quantification of the protein, and not mRNA, should theoretically be most useful for monitoring purposes for two reasons. Firstly, the physiological and toxicological significance of a response will depend upon the concentration of MT, and not MT mRNA, present in the cell. Secondly, the half-life of MT mRNA is much shorter than the half-life of MT and the time-span of a measurable response thus shorter. On the other hand, recent studies do suggest that expression studies (rtPCR) may also be relevant for monitoring (see George et al. 2004).

The existing methods for quantification of MT in both fish and invertebrates (mussels) may conveniently be divided into four categories: (1) quantification by metal-content, (2) metal-substitution, (3) quantification of sulphydryl groups and (4) immunoassays. Quantification of sulphydryl groups is either done using a spectrophotometric method (viarengo2000) or electrochemically using a polarograph (Olafson & Olsson 1991).

## 12.3 Metallothionein in mussels

As for many other biomarkers, mussels have been favourite invertebrate species for metallothionein studies (Noël-Lambot 1976, Bebianno & Langston 1991, Cajaraville et al. 2000, Mourgaud et al. 2002). Metallothionein in mussels have been suggested for monitoring and there have been international intercalibration exercises (Viarengo et al. 2000). Recently, rtPCR techniques were developed to quantify the two isoforms in mussels, MT10 and MT20 (Dondero et al. 2005). As for fish, different quantification methods have been used and a recent study has showed that they give entirely different results (Zorita et al. 2005). Metallothionein was determined in mussels during the BECPELAG workshop with the most widely used method (polarography), but there were no differences between locations (Erk & Raspor, in press). The general impression is that metal-derived increases in mussel MT is of a much smaller magnitude than increases that can be seen in fish, although (Viarengo et al. 1999) contends that mussel MT is more suited for monitoring than fish MT due to its closer link to external metal stress. The ICES working group on biological effects of contaminants (WGBEC) has not recommended MT in mussels for monitoring, but has included it with "promising methods" (WGBEC, 2005).

## 12.4 Dose-response in fish

Although MT is found in detectable amounts in most tissues, there is limited data on organs other than the liver. As for most other biomarkers, there is a wealth of studies in freshwater fish, but less for marine species. As mentioned above, it is not really feasible to compare studies with different methods.

Table 30. Selected studies of metallothionein in marine fish species; MT measured with pulse polarography in all studies except Sulaiman et al., who used the Cd-saturation assay.

| *species* | *tissue* | *lowest-highest concs* | *exposure time* | *baseline/control (level/activity)* | *induction (fold)* | *reference* |
|---|---|---|---|---|---|---|
| Atlantic cod (Gadus morhua) | liver | field | field | 4-7 µg/mg protein | none | Holth, 2004 |
| Atlantic cod (Gadus morhua) | liver | caging – 4 locations | 3 mo | 1 µg/mg protein | none | Beyer et al., 1997 |
| Atlantic cod (Gadus morhua) | liver | reference locations | field | 6.5-16 µg/mg protein | none | Ruus et al., 2003 |
| dab (Limanda limanda) | liver | reference locations | field | 7.2-13 µg/mg protein | none | Ruus et al., 2003 |
| flounder (Platichthys flesus) | gill | caging – 4 locations | 3 mo | 0.8 µg/mg protein | none | Beyer et al., 1996 |
| flounder (Platichthys flesus) | kidney | caging – 4 locations | 3 mo | 0.4 µg/mg protein | none | Beyer et al., 1996 |
| flounder (Platichthys flesus) | liver | field | field season | 7-16 (female), 5-12 (male) µg/mg protein | none | Nissen-Lie, 1997 |
| flounder (Platichthys flesus) | liver | field | field | 6-11 µg/mg protein | none | Holth, 2004 |
| flounder (Platichthys flesus) | liver | field | field gradient | 20 µg/g ww | 19 | Sulaiman et al., 1991 |
| flounder (Platichthys flesus) | liver | caging – 4 locations | 3 mo | 1 µg/mg protein | none | Beyer et al., 1996 |
| flounder (Platichthys flesus) | liver | reference locations | field | 8.4-15 µg/mg protein | none | Ruus et al., 2003 |
| plaice (Pleuronectes platessa) | liver | reference locations | field | 6.5-14 µg/mg protein | none | Ruus et al., 2003 |

## 12.5 Species sensitivity

There is limited knowledge of how metallothionein in tissues of different fish or invertebrate species respond to metal stress. For the marine species relevant in the North Sea area there appear to be surprisingly little difference in baseline hepatic MT levels. Both design and analytical protocols differ between studies and it is rarely feasible to compare species directly.

## 12.6 Relevance of other factors

There are species differences in how endogenous and exogenous factors other than metal stress affect MT in fish tissues, as indicated in Ruus et al. (2003). In a comprehensive multiple regression analysis for Atlantic cod (including more than 600 individual fish sampled at 8 sites over 5 years), both organic contaminants and essential metals came up as highly significant, in addition to size and a site-related factor that was not reflected in tissue-levels of metals or organochlorines. Similar regression models for flatfish included essential metals, but also tissue concentrations of organic contaminants and size. All samples were collected at the same time of the year (September-October) and water temperatures were surprisingly similar (within 2-3°C) at the different locations sampled through a five-year period.

The relevance of various factors that affect hepatic MT in flounder was assessed by Nissen-Lie (1997) in a seasonal study over 1 year. As for cod, hepatic concentrations of the essential metals copper and zinc appeared to drive (or be driven by?) hepatic concentrations of MT. Metallothionein in the liver varied through the year in both male and female fish, apparently related to the demand for Zn in gonad development and maturation. As for cod, results from Lacorn et al. (2001) for dab similar identify season as an important factor for hepatic MT, but also suggest that water temperature is relevant. The latter can not easily be distinguished from season, so no clear conclusions can be drawn. The main conclusions in Rotchell et al. (2001) were that age and season are important for variations in hepatic MT in flounder.

The results in Hylland et al. (1996) indicated that starvation had less influence on MT than on other biomarkers in flounder fed cadmium-contaminated polychaetes. There was also surprisingly little effect of co-exposure of cadmium with BaP or PCB in that study. Corresponding results were found in the study by Sandvik et al. (1997), but fish treated with BaP prior to Cd appeared to have a lower capacity to synthesise MT than fish who only received Cd or PCB prior to Cd. There are unfortunately no similar studies with Atlantic cod or any other gadiid.

## 12.7 Links

As mentioned in the introduction to this section, metallothionein appears to be a household protein in many organisms (at least in mammals) with a range of functions, but none really essential. It is not clear whether this is the case for fish. Fish have higher levels of metals in the liver than most mammals and some fish species have phenomenally high concentrations of metals under normal conditions (Hogstrand et al., 1996; Hogstrand & Haux, 1996; Thompson et al., 2003). Data for flounder and cod from unpolluted to moderately polluted areas support a role for metallothionein in trace metal (zinc and possibly copper) metabolism in marine fish, but non-essential metals such as cadmium do not appear to have a strong effect (in contrast to the situation in some freshwater ecosystems). Metallothionein is however one of the genes most clearly expressed in rtPCR studies and the protein appears to have a function in a general stress response, as much or more related to organic contaminants as to metals. There are however no clear links from metallothionein induction to individual health or reproduction. Some freshwater studies have shown reduced growth at metal-levels which also induce MT (e.g. Roch et al., 1984), but such levels are rarely if ever encountered in marine ecosystems.

## 12.8 Background responses and assessment criteria

As long as an appropriate species is used, sampling is performed during the appropriate time period and individuals selected on the basis of sex and size, absolute assessment criteria may be used for hepatic metallothionein. The main dataset used in this context has been derived from the JAMP monitoring programme (Ruus et al., 2003), but supplemented with other studies, e.g. Nissen-Lie (1997) for flounder.

## 12.9 Quality assurance

Metallothionein was included in BEQUALM and intercalibration between 14 laboratories run on liver samples from cod. The results clearly showed some of the inherent problems of the analytical procedures. Metal-saturation assays and polarographic analyses produced remarkably similar results, whereas immunochemical assays (ELISA) and the colorimetric assay produced results that deviated from the overall median

# 13. δ-aminolevulinic acid dehydratase (ALA-D) inhibition

## 13.1 Introduction

Many metals are in fact essential to all organisms, e.g. Cu, Zn, Fe, Mn and Mo, but there are also metals for which no biological function is known such as Hg, Cd, Au, Ag and Pb. Non-essential metals generally have much higher toxicity than the essential metals. Uptake, storage and excretion are also less well controlled for the non-essential than for essential metals.

One of the most important toxic mechanisms of non-essential metals is interaction with and inhibition of enzymes, especially enzymes with metal co-factors. Δ-aminolevulinic acid dehydratase (ALA-D, E.C. 4.2.1.24) is such an enzyme, which has zinc as a co-factor (Granick et al., 1972). This enzyme catalyses a step in the synthesis pathway for heme and is found in bacteria, plants and invertebrates as well as in vertebrates. Heme is incorporated in macromolecules such as hemoglobin and cytochromes. In mammals and birds, inhibition of ALA-D may lead to anaemia since it is one of the rate-limiting enzymes in heme (and hence hemoglobin) synthesis. This does not appear to be the case for fish (Larsson et al. 1985). The reason for ecotoxicological interest in ALA-D is its inhibition by Pb, even at very low exposure levels (Hodson et al. 1984, Haux & Förlin 1988).

ALA-D has been used to investigate effects of metals (mainly lead) in rainbow trout (Hodson 1976, Hodson et al. 1977, Hodson et al. 1978, Addison et al. 1990, Sordyl & Osterland 1990), longear sunfish (Dwyer et al. 1988), perch (Haux et al. 1985), salmon (Johansson-Sjöbeck & Larsson 1979), flounder (Johansson-Sjöbeck & Larsson 1978), carp (Nakagawa et al. 1995, Nakagawa et al. 1998), various catostomid species (Schmitt et al. 1984), Atlantic cod (Ruus et al. 2003) and grey mullet (Krajnovic-Ozretic & Ozretic 1980). As will be apparent from this list, the method has predominantly been used with freshwater species.

## 13.2 Analytical considerations

There has been a tradition to express results for ALA-D in terms of blood cell volume (e.g. hematocrit), but more recent recommendations suggest to use protein (Hylland, 1999). As for other methods, it is not possible to compare different studies because values have not been reported in the same format.

## 13.3 ALA-D in invertebrates

As mentioned above, ALA-D is also present in invertebrates and it is possible to adapt the existing protocol for fish. There is limited knowledge of effects in invertebrates, although ALA-D would be assumed to be sensitive to metals in invertebrates as well.

## 13.4 Dose-response and species sensitivity

ALA-D activity can be determined in liver, kidney, spleen and red blood cells in teleosts (see (Hodson et al. 1984). The sensitivity, timing and specificity of ALA-D inhibition in response to lead exposure have been reviewed (Hodson et al. 1984). For rainbow trout, red blood cell

ALA-D activity may inhibited following exposure to less than 5 µg/L of lead through water (Haux et al. 1986) and blood lead concentration of 300 µg/L (Hodson et al. 1982), although other studies indicate a lower sensitivity (Burden et al. 1998). In rainbow trout, ALA-D inhibition is evident 1-2 days following exposure to lead and there is a clear dose-response relationship (Hodson et al. 1982). The half-life of the response will depend on the amount of lead accumulated in other tissues (in equilibrium with blood), but will generally be of the order of months. No dose-response studies have been done with marine species. It is however likely that responses will be similar to those observed for rainbow trout taking into account that lead is generally less bioavailable in marine systems than in freshwater.

ALA-D activity in red blood cells of Atlantic cod (*Gadus morhua*), flounder (*Platichthys flesus*) and dab (*Limanda limanda*) has been used in the Norwegian national monitoring programme for 5 years (1997-2001; Ruus et al. 2003). In that programme, ALA-D was generally found to be inhibited in blood of fish from areas with known urban impact and in one area with a known metal impact (Ruus et al. 2003). In fact, results for ALA-D in that study indicated that there could be lead toxicity in individual cod with hepatic lead levels below the detection limit for routine analyses. Despite its specificity, ALA-D has not been widely used in monitoring. It has been included in OSPAR's list of recommended methods to monitor biological effects of metals (JAMP, 1998). In addition to the above, Holth (2004) investigated ALA-D in Atlantic cod and flounder in southern Norway. He found clear inhibition of the enzyme in both species sampled in the inner Oslofjord compared to individuals collected from the outer Oslofjord. Individuals from both species collected in the outer Oslofjord had values in the range of baseline values (see below).

## 13.5 Background responses

From the analyses done under Norwegian JAMP, typical values from reference areas are: Atlantic cod 15-21 ng PBG/min/mg protein, plaice 13-21 ng PBG/min/mg protein, flounder 13-21 ng PBG/min/mg protein and dab 10-20 ng PBG/min/mg protein.

## 13.6 Links

As mentioned above, inhibition of ALA-D will lead to anaemia in birds and humans. Initial studies with very high inhibition of the enzyme in fish did not find such effect in freshwater fish and it is thought that the enzyme is not rate-limiting for heme synthesis in fish (and hence inhibition will not necessarily lead to less heme produced). There is thus no clear link to higher level effects in fish.

## 13.7 Other factors

Lead is generally found in the environment as the inorganic form, but the alkylated form may be present. Alkyllead does not affect ALA-D strongly and there may thus be a situation where there is little inhibition, but high blood lead levels (Hodson et al. 1984). Data on the influence of metals other than Pb are contradictory (Jackim 1973, Dwyer et al. 1988, Sordyl & Osterland 1990). There is some evidence that very high levels of Cd may inhibit the enzyme in flounder (Johansson-Sjöbeck & Larsson 1978). Zinc may ameliorate inhibition by lead, and appears to affect fish red blood cell ALA-D *in vivo* (Schmitt et al. 1984, Schmitt et al. 1993, Schmitt et al. 2005). The influence has been found to be weak and variable in rainbow trout (Hodson et al. 1984).

## 13.8 Quality control

There exists no commercially available standard reference material for fish ALA-D. Bovine ALA-D is available commercially and may be used to test the assay. The pH of the assay needs to be established for each fish species (and will differ from the pH optimum of bovine ALA-D). Suitable internal reference materials (blood supernatants from relevant fish species) should be prepared by each laboratory undertaking ALA-D analyses. This material should be aliquotted and stored at -80°C for use with each batch of analyses. Control charts should be prepared during the analyses.

ALA-D was one of the methods included in the EU-funded project BEQUALM (Biological effects quality assurance in marine monitoring programmes; project PL97-3587). The low number of laboratories that participated in the exercise (5) precludes general conclusions on the use of the method. The exercise did show that homogenous test samples for intercalibration purposes can be prepared from red blood cells diluted in buffer.

# 14. Concluding remarks

A range of biomarkers relevant for monitoring offshore Norway have been reviewed. Histological biomarkers, including lysosomal stability, were not included.

The review shows that a range of methods should be considered for inclusion in the water column monitoring programme offshore (PAH bile metabolites, GST, DNA adducts, the Comet assay, micronucleus formation, AChE, vtg, ALA-D), some need more research (peroxisomal proliferation, MXR) and two have either been found to be not very useful (CYP1A activity) or not very relevant (metallothionein) (Table 31).

Table 31. Overview of methods included and their characteristics; relevant species are gadiids and/or blue mussels; all factors shown to affect the biomarker in question are indicated under "confounding factors", the most important are shown in bold.

| biomarker | datasets available for relevant species | link to population effects | confounding factors | assessment criteria | quality assurance | consider for WCM |
|---|---|---|---|---|---|---|
| PAH bile metabolites | extensive | indicates exposure | **recent feeding** sex, size/age | yes | QUASIMEME | yes |
| cytochrome P4501A (CYP1A) | extensive | contaminant-related, but no clear link | **sex env temperature season, age** | yes | BEQUALM | as protein, not activity |
| glutathione *S*-transferase (GST) | limited | contaminant-related, but no clear link | **sex, season** starvation | no | no | yes |
| DNA adducts | good | correlative link to cancer and population effects | **sex season** (mussel) | yes | BEQUALM | yes |
| Comet assay | limited | possibly correlative link to cancer and population effects | sex? **sample handling age, starvation, adaptation** | no | no | yes |
| micronucleus formation (MN) | limited | expected from mammalian studies, but not shown | unknown | no | no | yes |
| acetyl cholinesterase (AChE) | very limited | contaminant-related, but no clear link | other contaminants? | no | other | yes |
| peroxisomal proliferation | limited | uncertain | reproductive status sex | no | no | research |
| multixenobiotic resistance (MXR) | limited | uncertain | unknown | no | no | research |
| metallothionein (MT) | good | presumably contaminant-related | other contaminants sex season | yes | BEQUALM | no |
| vitellogenin (vtg) | good | indicates level of xenoestrogens that may have population effects | (only use male or juvenile fish) season | yes | BEQUALM | yes |
| delta-aminolevulinic acid dehydratase (ALA-D) | good | contaminant-related, but no clear link | other metals? | yes | BEQUALM | if metals |

From the range of biomarkers included in this review there are some for which links to population effects is largely unknown or not established (peroxisomal proliferation, MXR,

MT), one that indicates exposure to a specific class of contaminants (PAH metabolites), three for which there is some evidence in the literature for links with putative population effects (CYP1A, GST, ALA-D), three for which links could be expected from studies with other species or life stages (micronucleus formation, Comet assay, vitellogenin) and one for which there is correlative evidence of such links (DNA adducts).

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