Mariculture Committee

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REPORT OF THE

SPECIAL MEETING ON THE USE OF LIVER PATHOLOGY OF FLATFISH FOR MONITORING BIOLOGICAL EFFECTS OF CONTAMINANTS

Weymouth, UK
22–25 October 1996

International Council for the Exploration of the Sea

Conseil International pour l'Exploration de la Mer

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1. Opening of the meeting and general introduction

The Special Meeting was held at the MAFF, Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth Laboratory, Weymouth, England from 22 - 25 October 1996 under the joint Chairmanship of S.W. Feist, MAFF CEFAS, Weymouth and T. Lang, BFA für Fischerei, Cuxhaven, Germany, with the assistance of A. Köhler, BAH, Hamburg, Germany. 18 scientists from 9 ICES Member Countries attended the Special Meeting (Annex 1a).

S. W. Feist opened the meeting by introducing the Head of CEFAS, Weymouth Laboratory, B. Hill, who formally welcomed all participants to Weymouth and wished the meeting all success for a successful outcome. He stressed the importance of the topic area, welcoming the progress which he has noted to be occurring from the broad approach progressively towards specific studies, even to the extent of focusing on a single organ as was the case with the current Special Meeting. S.W. Feist also expressed his thanks for the excellent level of participation and in particular, for the attendance of specialists from the USA.

T. Lang outlined the background to the ICES Special Meeting and tabled a general introduction summary paper (Annex 1b). He informed the participants that the idea to hold the Special Meeting had been developed by the ICES Working Group of Pathology and Diseases of Marine Organisms (WGPDMO) and was adopted by ICES at the 1995 Annual Science Conference with ICES Council Resolution/1995 2:31. The task to organise and plan details of the meeting was given to the ICES Sub-group on Statistical Analysis of Fish Disease Data in Marine Fish Stocks and the ICES Working Group on Biological Effects of Contaminants (WGBEC) was invited to participate in the planning and nominated A. Köhler as its representative.

Following successful ICES efforts regarding the development and implementation of standard methodologies for sampling, diagnosis and recording of grossly visible diseases of marine fish, it was appropriate that the attention of ICES should turn to a similar consideration of liver pathologies. It was stressed that the liver had been particularly identified by environmental monitoring groups as a target organ which, due to its central role in metabolism and detoxification and the well-documented contaminant-associated biochemical and cellular/subcellular changes, is particularly appropriate for research/monitoring studies on biological effects of contaminants.

R. Stagg, as Chairman of the ICES Working Group on Biological Effects of Contaminants, drew attention to the report of the 1995 ICES/OSPAR Workshop on Biological Effects Techniques held in Aberdeen, UK, and particularly to biological effects techniques recommended by the Workshop for contaminant-specific (PAH) and general monitoring of biological effects of contaminants (Annex 1c), which besides other techniques also include liver (histo)pathology (other recommendations do exist for TBT- and metal-specific biological effects monitoring). This recommendation has been adopted by the Oslo and Paris Commissions (OSPARCOM) as part of the OSPAR Joint Assessment and Monitoring Programme (JAMP). It was indicated that the next phase of the adoption of this

programme is its progression at a meeting of the OSPAR *Ad Hoc* Working Group on Monitoring to be held in Stockholm in November 1996, when guidelines for biological effects monitoring will be developed and integrated with existing chemical monitoring guidelines. The occurrence of the present Special Meeting was therefore particularly timely as the conclusions would be a valuable assistance in taking forward techniques in liver pathology monitoring.

2. Adoption of the agenda

The objectives of the Special Meeting were detailed (Annex 1 b) and related to the proposed programme of the Special Meeting. The agenda (Annex 2) was accepted by the participants. A comment was made on the emphasis on biochemical techniques in the agenda and it was indicated that due weight would be given to the balance as indicated in the agenda.

3. Appointment of rapporteurs

Rapporteurs as detailed in Annex 3 were agreed.

4. Current status of studies on liver pathology

In order to obtain an overview on on-going activities in ICES Member Countries, four key note contributions (4.1, 4.2, 4.3, 4.5) were given by participants adressing relevant aspects. In addition, national reports from 9 ICES Member Countries were presented the summary of which is provided in section 4.4. A summary of the current status highlighting gaps in information is given in section 4.6.

4.1 Histological, cellular/subcellular and biochemical techniques and their field application

A. Köhler presented an overview on techniques with the potential to link histopathological changes (in fixed/frozen tissue samples) to metabolic/biochemical changes in cells and tissues and the validation of their potential in field studies (a summary paper and technical guidelines are provided in Annex 4.1 and Annexes 5.2 a and 5.2 b, respectively).

Subcellular/cellular techniques available and established for the analysis of liver sections considered in the presentation were:

- lysosomal stability test
- enzyme-histochemical identification of glucose-6-phosphatase-dehydrogenase (G6PDH) in foci of cellular alteration
- immuno-histochemical identification of proliferating cell nuclear antigen (PCNA) in neoplastic and pre-neoplastic liver lesions
- immuno-histochemical identification of cytochrome P4501A1 (CYP1A) in healthy tissue and neoplastic/pre-neoplastic liver lesions
- the immuno-histochemical identification of multidrug/ multixenobiotic resistance (MDR/MXR)
- histopathological and electron microscopic identification and classification of liver neoplasms and putative pre-neoplastic lesions

These techniques can either be used as biomarkers for early biological effects of contaminant exposure or in conjunction with histopathology in order to elucidate mechanistic processes involved in the development of lesions. It was emphasized that, for monitoring purposes, there is a need for robust early warning biomarkers of contaminant exposure and cellular/subcellular injury. However, besides biochemical biomarkers (e.g. EROD and DNA adduct measurement), the incorporation of histopathogical/histochemical studies like those listed above is crucial in order to allow an interpretation of biochemical results obtained from measurements in homogenised tissues.

Since neoplasia is generally regarded as one of the most relevant endpoints of exposure to carcinogenic contaminants, the early histological/histochemical detection and quantification of altered cells with the putative potential to develop into malignant carcinomas were considered relevant for monitoring. During the discussion, it was agreed that, as in the mammalian liver, the basophilic foci found in H&E-sections is a relevant cellular phenotype during carcinogenesis in the liver of flatfish whilst the role of other foci of cellular alteration (e.g. clear cell and eosinophilic foci and intermediate stages) still needs some clarification. Similar to studies in mammalian models there is no general agreement on the phenotypic sequence in the early cellular and focal stages during tumor development in fish, and it was noted that pronounced differences in the patterns of tumour histogenesis between fish species have been demonstrated. There was consensus that the use of enzyme altered foci (G6PDH) as biomarkers facilitates the identification of early pre-neoplastic lesions prior to their appearance in H&E stained histological section.

Furthermore, it was pointed out that biological effects of xenobiotics are influenced by natural exogenous environmental habitat factors (e.g. temperature, salinity, oxygen contents) as well as by endogenous physiological factors (e.g. age, sex, reproduction). This has to be taken into account in any biological effects studies and, therefore, appropriate measurements have to be included in monitoring programmes. Interpretation of data may also be complicated by the fact that factors of cell protection counteract those of cell injury.

It was emphasised that, in contrast to classical epidemiology related to diseases occurring with clinical signs, the new approach of cellular and molecular epidemiology provides early biomarkers of the susceptibility of cells for tumour development which will certainly be of high relevance for future risk assessment based on monitoring programmes in the marine and terrestrial environment as well as for human cancer epidemiology.

4.2 European epidemiological studies

A.D. Vethaak presented an overview of European studies on liver pathology which have been conducted in relation to pollution monitoring (a summary of his presentation is given in Annex 4.2). Objectives have been either to monitor biological effects of contaminants or to establish cause-effects relationships.

It was stressed that epidemiological and histopathological studies are not necessarily always compatible. In field studies, where there is no opportunity to control variables, it is difficult to identify cause-effect relationships and although such relationships can be addressed in experimental situations (with mesocosm studies intermediate), there is a loss of relevance to natural conditions. A contrast was noted between the success of linking tumours with classes of pollutants in some North American studies with those in European waters where no such relationship has been established. In the latter, the causes of, and factors influencing the variability in occurrence of tumours are clearly highly complex and at present it can only be concluded that pollution probably has a contributory role in causing liver tumours.

ICES has conducted two practical sea-going workshops in 1984 and 1988 in order to develop and intercalibrate standardised methodologies for the monitoring of grossly visible fish disease (including macroscopic liver nodules/tumours in dab and flounder) leading to the publication of the ICES Training Guide for the Identification of Common Diseases and Parasites of Fish in the North Atlantic in 1996 (Bucke *et al.* 1996, ICES TIMES No. 19). The experiences and conclusions of the ICES Bremerhaven Workshop on Biological Effects of Contaminants in the North Sea in 1991 and of the BMB/ICES Sea-going Workshop on Diseases and Parasites in the Baltic Sea in 1994 are also of relevance to this field. Furthermore, ICES has established a fish disease databank as part of its

Environmental Databank which includes long-term data on externally visible fish diseases and liver neoplasms in dab and flounder submitted by ICES Member Countries.

A.D. Vethaak made the following main conclusions from his review of the European work already conducted on liver pathology of flatfish:

- On the whole, field surveys have produced only limited evidence for a role of pollutants such as PAHs and PCBs in the causation of liver tumours in flatfish inhabiting European waters.
- However, supporting causal evidence has been provided by a mesocosm study.
- The aetiology of liver tumour in North Sea dab and flounders appears to be complex.
- Recording of grossly visible liver lesions (nodules > 2 mm) is a cost-effective method of collecting data on the occurrence of liver neoplasms in flatfish, but the identity of all lesions observed should be confirmed histologically.
- Clarification of sveral aspects of the epidemiology of liver tumours is required before their full potential as a monitoring tool can be assessed.
- Experimental studies are needed to investigate the chemical agents responsible for the onset of pre-neoplastic lesions and their progression into neoplasms.

During discussions, the need to use experimental studies was stressed when cause-effect relationships were being investigated. The possibility that tumours may be initiated in areas far apart from the site they are recognised should be considered, particularly for species such as flounder which migrates from fresh to sea water and vice versa. In addition, the lack of success in linking liver tumours with pollution in the North Sea compared to the North American situation could be related to the fact that the contaminant levels found in the North American studies were a factor of 10 to 50 times higher than in the North Sea.

4.3 US perspective

M. Myers presented an overview of studies on liver pathology in the United States (a summary is given in Annex 4.3). Multiple studies carried out in the US since the 1970's have established the utility of routine histopathology of liver in flatfish species to detect adverse biological effects of contaminants such as PAHs, PCBs, DDTs and other pesticides. The success of these studies is directly proportional to their degree of multidiscliplinarity with the highest level of success demonstrated in studies linking exposure measures (bile fluorescent aromatic comounds (FACs) as well as sediment and tissue chemistry) to early reference measures (e.g. CYP1A and DNA adducts in liver) and thence to biomarkers of chronic changes such as pre-neoplastic and neoplastic liver lesions. Although pathologic responses, biochemical responses and chemical risk factors differ among the species examined, the relationships identified have provided strong evidence for the involvement of environmental contaminants in the aetiology of these hepatic lesions.

During discussion, it was indicated that no evidence of recovery from liver tumours was detected within three months in US studies, but that no long-term studies had been conducted. The increased significance of the interaction of several compounds compared with effects of single chemicals was stressed. Although cause-effect relationships are therefore difficult to determine in the field, these studies can give an indication where to focus experimental investigations. There was no indication of a correlation between liver pathology and stock density, but it was recognised that high fishing intensity could decrease the prevalence of liver pathologies by removing the larger, more affected fish. The inability of European field studies to suggest cause-effect relationships in comparison to the US studies was attributed to the more enclosed waterways with higher contamination levels in the US. Risk factors linked to neoplasia vary between different species of fish.

4.4 Reports on national activities in ICES Member Countries.

Belgium

Since 1980, two annual fish disease monitoring surveys have been carried out in spring (April) and autumn (October). Four areas, the Belgian Shelf, the Flambourough, the Oyster Ground and the Deepwater Channel were included. During the first 10 years of the project, liver lesions were reported as "liver anomalies" but since 1990, the recording of liver nodules has been conducted according to the ICES standard protocol. A marked decrease in liver lesions in dab (*Limanda limanda*), plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*) has been observed since 1993.

Canada

A written report of current activities was submitted by C Couillard, Institut Maurice-Lamontagne, DFO, Quebec, Mont-Joli, Canada. In the last 5 years, three fish disease monitoring programmes including liver pathology have been conducted. One project studies lesions in American eel (*Anguilla rostrata*) from the St. Lawrence River drainage area and the St. Lawrence Estuary. Basophilic liver nodules were observed most frequently at the end of the migration season where the eel were found more contaminated with organochlorine compounds than in the beginning of this period. A second project investigates diseases in mummichog (*Fundulus heteroclitus*) living downstream from a bleached kraft mill in the Miramichi Estuary. These investigations include histopathological investigation of liver, spleen and gonads. The third project involves white sucker (*Catostomus commersoni*) living up- and downstream from a bleaching kraft pulp mill in the St. Maurice River. Fish caught at a distance of 10 and 95 km downstream the pulp mill exhibited higher density of pigmented macrophage aggregates relative to age in liver, spleen and kidney than found in fish living upstream.

Denmark

No fish disease studies including liver pathology are conducted at present.

England/Wales

The Ministry of Agriculture, Fisheries and Food (MAFF), CEFAS, Weymouth, undertakes regular monitoring of marine fish diseases in several North Sea and Irish Sea areas as part of its biological effects monitoring programme. Emphasis is given to those areas which have been recognised as having consistently higher levels of external disease conditions when compared to 'reference areas' and to those close to or at National Monitoring Programme (NMP) sampling sites. Regular monitoring sites in the North Sea include stations off the north east coast of England, off Flamborough, Dogger Bank, off Humber, sole pit and Smith's Knoll. Reference stations at Rye Bay are also routinely visited. Irish Sea stations include Liverpool Bay, Morecambe Bay and Dundrum Bay off the Irish coast which is used as a reference site for the Irish Sea. In addition to these areas, two stations at the north and the south of Cardigan Bay are also monitored.

Techniques applied follow standard ICES guidelines for disease recording in dab (*Limanda limanda*), flounder (*Platichthys flesus*) and cod (*Gadus morhua*). The recording of hepatic pathology in flatfish species has been incorporated for many years and increasing emphasis is being given to the study of hepatic pathology in other flatfish species such as plaice (*Pleuronectes platessa*) and Dover sole (*Solea solea*) wherever sufficient numbers are caught.

Research activities at CEFAS' Burnham-on-Crouch laboratory within the field of contaminant induced liver lesions pursue the link between PAH exposure, induction of enzymatic systems such as EROD as indicators of cytochrome P450 activity, metabolites of PAH in bile and determination of PAH adducts in DNA as early indicators of genetic damage. The DNA adduct work has been in collaboration with the University of Swansea. Other biomarker studies for genotoxic damage have been undertaken at the University of Birmingham in liaison with the Plymouth Marine Laboratory.

The application of these techniques are only just begining to be applied to long-term carcinogenicity studies. Other activities of the Plymouth Marine Laboratory are covered in section 5.2 of this report.

Experiments in progress at CEFAS Weymouth investigate the effect of contaminants, especially PAHs, on marine flatfish. Initial trials have used turbot (*Scophthalmus maximus*) as experimental animals and further studies will use dab and possibly other species as available.

Estonia

Since 1994, fish disease investigations including liver histopathology in flounder have been conducted at two sites in the vicinity of Tallin habour. These studies are combined with analysis of 12 different PAHs and the analysis of the activity of CYP1A, AHH, APND, ALA and HEM synthetases in liver as well as with the measurement of flourescent aromatic compounds (FAC) in bile. Different kinds of liver lesions were observed in 50 % of the fish and were more frequent in urban areas as compared to reference sites.

Finland

A fish disease monitoring programme with flounder as target species was carried out from 1987 to 1991. On average, 3 % of the examined flounder larger than 20 cm were affected by liver nodules and, in fish larger than 30 cm, the prevalence was 10.1 %. Female fish were significantly more affected than males. The present activities focus on efforts to investigate the aetiology of the pre-neoplastic/neoplastic liver lesions previously recorded. Livers from all fish are examined by means of light microscopy using histopathological and immuno-histochemical methods, and for the activity of EROD, CYP1A, for DNA adducts and PAH contamination. Two areas with known disease status and one reference area are included in the present investigations.

Germany

The German fish disease monitoring programme in the North Sea (two cruises per year in January and during the period May-July) and the Baltic Sea (one cruise per year in December) started at the end of the 1970's and is carried out by the Bundesforschungsanstalt für Fischerei, Cuxhaven. Sampling sites cover large areas in the North Sea and adjacent areas (e.g. Irish Sea, English Chanel) and the south-western Baltic Sea from German to Polish waters. The detection of liver lesions has been included in the monitoring programme, which originally was focused only on externally visible diseases and parasites, since 1988 (North Sea) and 1991 (Baltic Sea) and has been carried out according to standard methodologies recommended by ICES. Major target species for liver pathology are common dab (North Sea) and European flounder (Baltic Sea). EROD measurements in both species have been incorporated in the programme in 1991. General temporal trends in the North Sea reveal a steady decrease in the prevalence of liver nodules > 2mm in diameter. For larger nodules (> 5 mm) there is no indication of such a trend.

Additional German research projects related to contaminant-associated fish liver pathology have been carried out by different German institutes.

- *Fish Diseases in the Wadden Sea* (1987-1990) was a field study involving disease investigation of flounder and other species in the German Wadden Sea. Investigation of flounder livers included histopathology, lysosomal membrane stability, proliferation, hypertrophy, accumulation of neutral lipids, EROD activity and contaminants (organochlorines and metals).
- *Fish Diseases in the North Sea* (1991 -1992) studied diseases of flounder and dab in North Sea estuaries, coastal areas and a northerly and a southerly transect through the North Sea. The liver examinations included histopathology, lysosomal membrane stability, accumulation of neutral lipids, EROD and contaminants (organochlorines and metals).

- *Stresstox* (on-going 1996-1997) investigates biological effects of contaminants in dab in the North Sea, Channel and the Baltic Sea area. It includes both field and laboratory studies. The liver examinations involve histopathology, apoptosis, heat shock proteins and contaminant analyses.

Besides these projects a number of field and experimental studies on contaminant-induced liver changes in flatfish species have been performed or are underway (e.g. DNA damage measured by DNA-unwinding assay, Comet-assay, DNA-adduct formation; enzymatic induction (MFO) measured biochemically or immunohistochemically and immunohistochemical assessment of enzytme alternations (ATPase, GGTase, G6PDH) and cell proliferation status (PCNA) in precursor lesions involved in the histogenesis of liver neoplasia.

The Netherlands

Several institutes in the Netherlands (e.g. the National Institute for Coastal and Marine Management (RIKZ), the National Institute for Public Health and the Environment (RIVM), department of Pathology of the Faculty of Veterinary Medicine of the Utrecht University and the National Institute for Fisheries Research (RIVO)) are involved in the so called "Fish disease project". This project is set up with an integrated study design which combines field, mesocosm and laboratory research. Monitoring external diseases and liver nodules by RIKZ and RIVO in dab and flounder are continued on an annual basis following the ICES standard protocol. General trends depicted indicate an overall decreasing prevalence of both external lesions and liver neoplasms. Since 1996, PAH related effect monitoring has been including measurement of PAH sediment concentrations, EROD activity, biliary PAH metabolites, DNA adducts and liver histopathology.

The 3 year mesocosm experiment on the isle of Texel has produced convincing evidence that chemical contaminants (PAHs) in the contaminated harbour sediment are capable of inducing liver tumours and related pathology in flounder at similar concentrations occuring in the field.

Laboratory experiments are carried out with flounder, a species relevant to the Dutch situation. Flounder is kept under controlled laboratory conditions and exposed to several xenobiotic substances like 2,3,7,8 tetrachloro-dibenzo-*p*-dioxin (TCDD), bis(tri-*n*-butyltin)oxide (TBTO), 3,3',4,4',5 penta-chlorobiphenyl (PCB 126), benzo[a]pyrene (BaP) and dimethyl-benz[a]anthracene (DMBA). The effects of the xenobiotics are studied by investigating clinical parameters (e.g. behaviour, length, weight and hepatosomatic index) and performing pathology (both gross and histopathology) using histochemistry and immunohistochemical techniques (PCNA, Cyt. P450 1A1, immunoglobulin). Because several of the used xenobiotics have immunotoxic effects apart from carcinogenic effects, special attention is paid to the immune system.

Scotland

As part of a long-term monitoring study on diseases of common dab, *Limanda limanda*, three sampling areas off the east of Scotland have been annually investigated since 1988 for liver anomalies; namely St Abb's Head and Bell Rock Sewage dump sites (with the immediately adjacent areas across the main current directions as local reference areas) and a distant reference area east of Orkney. In order to reduce as far as practically possible the data variations due to observer, biological and physical factors, these investigations were highly focused in terms of sampling dates, areas, staff and methods of capture. ICES standardised methods of sampling, screening and diagnosis were followed throughout. It was usually necessary to use multiple hauls on the same position to obtain the necessary minimum number of 50 fish from each position on each sampling occasion. Identical trawl tracks were followed on each sampling occasion in an area to eliminate local spatial variations. Livers were removed from all fish above 24 cm in length and both the ventral and dorsal surfaces examined for evidence of anomalies. Livers with parasitic infections and nodules less than 2mm in diameter were rejected and all remaining suspect lesions subjected to histopathological laboratory analysis. Any lesions showing histological changes with clearly identifiable non-tumour aetiologies (or early

stages of such lesions) were also excluded from the recorded data. The level of liver tumours in Scottish waters is low and consequently, it is not possible to detect any pattern.

Biochemical biomarker investigations carried out in Scotland are detailed in section 5.3.

Sweden

Studies using biomarkers in wild fish, as well as in fish kept under experimental conditions, have been carried out in Sweden since the 1970's. Three regular monitoring programmes have been conducted.

- Integrated monitoring of fish populations is part of the Swedish marine environmental monitoring programme which includes annual monitoring of a range of parameters in fish, from biochemical biomarkers to population densities, together with analysis of tissue contaminant concentrations. The aim of this programme is to identify trends in the effects of anthropogenic contamination from point sources within relatively unpolluted areas. Three areas were chosen for study, one on the Swedish west coast (monitored since 1988) and two on the east coast (monitored since 1989). The study organisms are perch (*Perca fluviatilis*) and viviparous blenny (*Zoarces viviparus*), and the biomarker measured in the livers of these fish is EROD activity. This programme is being run by Stockholm University in cooperation with Göteborg University, and The Swedish Museum of Natural History.
- Studies on the effects of a modified bleaching process on the health status of fish living in coastal water polluted with bleached pulp mill effluents has involved sampling outside the Norrsundet pulp mill on the Swedish east coast in 1985, 1988, 1990, 1993 and 1995. Several pollution-related physiological and biochemical parameters in perch have shown a clear decrease over the period of the study. This correlates well with the improvement of the bleaching process at the mill, which has resulted in a clear reduction of contaminants in the effluent. The majority of the biomarkers being analysed do not presently differ from levels found in reference areas. However, levels of EROD activity are still significantly higher in the immediate vicinity of the mill by comparison with surrounding areas. This programme is being run by Göteborg University.
- Investigations of disease in flounder at four different localities in the G^teborg and Bohus counties of Sweden have been carried out annually since 1991. A range of diseases and conditions, including pre-neoplastic and neoplastic liver lesions and organosomatic indices, have been studied. Large variations have been found in the prevalence of liver nodules > 2 mm in diameter, both between sampling localities and between years at each locality. In general, the prevalence has been found to vary between 1 % and 5 % in cohorts of fish within the length interval 25-35 cm. This programme is being run by the National Board of Fisheries.

A couple of "stand-alone studies" has been carried out at different Swedish institutions, e.g. enzymological and histopathological biomarkers in roundnose grenadier (*Coryphaenoides rupestris*) exposed to anthropogenic contaminants from non-point sources in the Skagerrak and DNA adducts and histopathological lesions in perch and northern pike (*Esox lucius*) along a PAH concentration gradient outside a aluminium smeltery at the Swedish east-coast.

USA

The national activities in the United States are described under section 4.3 and a summary document is provided in Annex 4.3

4.5 Experimental studies on hepatic carcinogenesis in fish.

M. Okihiro presented an account of experimental hepatocarcinogenesis in Medaka (*Oryzias latipes*) in the US (a summary paper is provided in Annex 4.5). Two studies were described:

- a medium-term study using brief (1 day) aqueous exposure to log concentrations of diethylnitrosamine (DEN)
- a long-term study using brief (2 day) and prolonged (5 week) DEN exposure.

DEN induced a wide range of lesions and a medaka specific classification scheme was developed using 7 classes of foci of cellular alteration (FCA) and 6 classes of neoplasms. Basophilic, eosinophilic and clear cell foci were the most common FCA observed. Basophilic foci and adenomas were strongly correlated with sexual maturation of female medaka, while eosinophilic foci and adenomas were associated with males. Prolonged (5 wk) exposure to DEN resulted in rapid progression to hepatocellular (ave # days PE< 60) and mixed (ave # days PE < 100) carcinomas. Metastatic lesions were observed in 19 of 61 carcinomas and transcoelomic was the most common route of spread. Carcinogenesis in medaka exposed to DEN is similar to rodent models of hepatocarcinogenesis and both differentiated (hepatocytes and biliary epithelial cells) and stem (bile preductular epithelial cells, BPDECs) cells appear to be targets for carcinogenic initiation.

Possible reasons for the increased frequency of neoplasms, including spontaneous neoplasms, in female medaka were considered in discussion. Ovarian oestrogen seems to be a tumour promoting factor, but the consequences of the greater liver size and therefore the number of target cells available, could not be excluded. Basophilic foci were the most important lesions induced and these were promoted over other types of foci. In these experimental studies, the status of clear cell foci in relation to tumour formation was not clear.

4.6 Summary review of the current status of studies on flatfish liver pathology.

A.D. Vethaak summarised the conclusions of the ICES Special Meeting of the current status of studies on flatfish liver pathology as follows:

- Field studies on liver nodules of flounder and dab using the ICES standard protocol have continued in the North Sea and have more recently also included the Baltic Sea. These have shown that spatial patterns are difficult to interpret but that temporal patterns indicate generally decreasing prevalences in most areas although there are still some areas with high levels. In general, chemical data to support these pathological findings are largely absent.
- Some studies have included histological early markers of liver pathology which give better insights into cause-effect relationships.
- Conclusive causal evidence for tumours is largely lacking for relevant species in field studies but mesocosm studies have produced significant results. However, laboratory studies are necessary to identify specific compounds and underlying mechanisms and it is encouraging that several studies are now underway using flounder and dab.
- There is no general consensus on the diagnosis of liver lesions and tumours. Different species of fish may show different lesions.
- The most promising sensitive early biomarkers identified include CYP1A, DNA adducts, PCNA, and G6PDH.
- There are good prospects for the inclusion of early markers of liver pathology into biological effects monitoring programmes. It is recognised that North Sea data available at present are more difficult to interpret than US data.

The discussion ranged over several areas:

It was recognised that biological effects monitoring should be supported whenever possible by chemical monitoring and by experimental work to gain better insight into the causes and mechanisms of liver pathology. The design of experiments (e.g. long-/short-term exposure, susceptibility of target species used, use of parallel studies on known susceptible species such as medaka).

For monitoring purposes and associated standardisation of applied methodologies, there is a clear requirement for guidelines specifying which histopathological features are most relevant, which should be excluded from monitoring studies, for agreed histopathological classification criteria, detection guidelines, quality assurance procedures and species relevant for monitoring.

There is need to clearly define the objectives of studies as the nature and structure of investigations may differ (and may possible be incompatible) in different types of studies:

- broadly based "alarm bell" studies aimed at identifying areas affected by contaminants by measuring more general biological effects of contaminants including indicators of environmental stress ("top down" studies)
- monitoring of the distribution and effects of specific chemicals/groups of chemicals ("bottom up" studies), such as PAHs, TBT (e.g. under the OSAPAR Joint Assessment and Monitoring Programme, JAMP)
- mechanistic studies elucidating causal mechanisms involved in the stepwise development of liver lesions.

The possibility of the development of a "decision tree" (possibly in co-operation with the ICES Working Group on Biological Effects of Contaminants) was considered useful for future risk assessment studies with the objective of helping to define the nature of studies in the spectrum between broad overview investigations, through studies on specific contaminants to in depth mechanistic studies.

5. Assessment of techniques for the determination of liver pathology

In this section, relevant biological effects techniques are considered under a more practical point of view in the light of their advantages, disadvantages and limitations for monitoring purposes. Furthermore, the integration of chemical and biological effects monitoring is addressed.

5.1 Histopathology and electron microscopy

D. Bucke presented an overview of general procedures necessary to produce interpretable specimens from fish for the purposes of routine histopathology, electron microscopy, histochemistry from frozen sections, and immunohistochemistry (see Annex 5.1 a). Initial topics covered included factors involved in target species selection, capture methods, post-mortem (necropsy) technique, and macroscopic examination. For comparison purposes, a working document prepared by M. Myers providing information on guidelines used in the USA for field studies was circulated (Annex 5.1 a).

It was stressed at several points in the presentation and discussion that optimal material and data from histologic specimens can only be obtained from fresh or recently sacrificed fish.

Also covered were basic methods and concepts of histological technique, including

- optimal fixatives to be used (10 % neutral buffered formalin, Bouin's, and as suggested in discussion by M. Myers, Dietrich's fixative), their advantages and disadvantages
- optimal tissue:fixative volume ratio (1:20)
- optimal thickness of tissues (<5mm, preferably ~3mm)
- the need for relatively standardised schedules for tissue processing (dehydration, clearing, paraffin infiltration and embedment)
- advantages/disadvantages of embedding media (paraffin, glycol methacrylate)
- tissue sectioning and optimal section thickness (~5 microns)

- preferred routine methods for section staining (various hematoxylin and eosin schedules)
- preparation of sections from frozen tissues for histochemistry
- procedures for electron microscopy

D. Bucke stressed the need for well-trained technicians to produce interpretable sections for all of these applications, as well as the need for high quality optical microscopes and a relatively standardised procedure for examination of tissue sections.

In his summary, the advantages, disadvantages, and limitations of histology were presented. Advantages include

- the ability to observe and characterise *in situ* changes in tissue and cells
- the ability to use specific immunocytochemical markers to characterise lesions
- the relatively permanent nature of histological specimens allowing archiving of material for comparative purposes or use of novel techniques.

Disadvantages and limitations include

- the high level of skill and training needed to produce and interpret specimens
- the lack of automation for examination and diagnosis of tissues
- the relatively subjective nature of histopathologic diagnosis
- the fact that only a proportion of the total tissue can be examined histologically
- the typical lack of specificity in pathological response to specific contaminants.

5.2 Cellular/subcellular biomarkers

A presentation of this field was provided by M. Moore from Plymouth Marine Laboratory (PML). A number of different techniques are today available for the determination of morphologic subcellular processes. The strength of these techniques is the possibility of mechanistically linking early induced biochemical processes in the cell with cellular processes on a higher biological organisation level in the organism.

Different fluorescent molecular probes are available for describing molecular processes and subcellular structures such as EROD, endoplasmic reticulum, lysosomes etc. The fluorescence is then determined with confocal laser scanning microscopy (CLSM). The probes are, however, to be used for vital staining, and the applicability for this technique is primarily for scientific purposes. For monitoring purposes the techniques has to meet the challenge of determining the mixture of complete effluents and should follow the following criteria:

They should be:
- inexpensive
- rapid
- sensitive
- precise
- easily learnt
- readily interpretable

A test which fulfills these criteria is the lysosomal membrane stability test. The lysosomes are involved in adaptive processes and pathological reactions and accumulate both metals and hydrophobic/ lipophilic pollutants.

The reactions of lysosomes to injury are:

- change in contents
- change in fusion events
- change in membrane stability

The consequences of these reactions is cell dysfunction. Lysosomes are present in all nucleated cells. Thus the test can, at least theoretically, be applied for most organism carrying lysosomal rich tissue. At PML the test has been performed in fish, crustaceans and molluscs and was first set up for fish using liver as a lysosome rich tissue. However, because many areas do not have stationary fish species, the major effort at PLM has now been to set up the test for molluscs and crustaceans. Techniques have recently been developed for the in vitro determination of lysosomal damage following exposure of molluscan and crustacean blood cells to a variety of environmental contaminants in both laboratory and field studies.

Results were presented from a field study using mussels for the lysosomal membrane stability test in a gradient outside Venice, Italy. The study showed a clear correlation with the contamination of anthropogenic compounds in the area. The method as a prognostic biomarker of contaminant exposure and effect, is given in a paper presented at the 1996 ICES Annual Science Conference (Lowe, D.M., Moore, M.N.: Lysosomal pathology as a biomarker of pollution impact in the marine environment. ICES C.M. 1996/E:13) which was made available at the Special Meeting.

During discussion, the question arose about the specificity of lysosomal activity to the impact of contaminants. It was stressed by M. Moore that the test is robust, that it is pollution-specific, and that it is prognostic for histopathological responses. The question also arose whether it has been tested on fish white blood cells. So far it has not, but M.Moore considered the test also applicable for blood cells.

Technical guidelines for studies on cellular/subcellular biomarkers used for cytochemical studies of the liver of teleost fish are provided in Annexes 5.2 a and 5.2 b.

5.3 Biochemical biomarkers

R. Stagg presented an overview of biochemical marker techniques which are made on whole or homogenised tissues, including protein or enzyme measurements, measures of DNA damage, and measures of contaminant metabolites (see Annex 5.3 a). Proteins or enzymes to be considered that are inducible by contaminant exposure include cytochrome P4501A1 (CYP1A), metallothionein, glutathione-S-transferase, UDP-glucuronyl transferase, and vitellogenin. Reduced activity or expression due to enzyme inhibition has been shown for acetylcholinesterase, ATPase, ALA-D. These methods can be used as general methods, or in studies designed to assess response to particular contaminants such as PAHs and PCBs. Biochemical methods for assessment of DNA damage discussed include the ^{32}P-postlabelling method, which measures the covalent binding of reactive intermediates of contaminants to DNA as bulky hydrophobic adducts. Damage to specific genes can be assessed by comparing sequence of wild type and mutated alleles using amplified sequences of specific genes using polymerase chain reaction (PCR). Specific genes and their expression products commonly assayed in liver include the ras-oncogene and p53 tumour suppressor gene. A test for nonspecific damage touched upon was alkaline unwinding in the COMET assay. Exposure and metabolism of specific chemical classes (i.e., PAHs) can be measured as hydroxylated PAH metabolites in the bile of fish. Also mentioned were various blood and tissue metabolites to indicate biological effects, such as the produced free radicals, bilirubin, haemoglobin and alkaline phosphatase.

Advantages of protein biochemical measures, using the example of the EROD surrogate for CYP1A induction, include their sensitivity, specificity for particular contaminant classes (i.e., PAHs, chlorinated hydrocarbons and other chemicals binding to the Ah receptor), rapidity of induction (within several days for EROD), low cost for analysis, and accepted quality assurance procedures. Using the example of EROD, disadvantages discussed were the problems of synergistic or antagonistic effects caused by exposure to complex mixtures of contaminants, and variability due to phenotypic and genetic differences, sex, maturation stage, ecological niche, season and especially temperature. During the discussion the significant issue of temperature-related and diurnal variability in EROD were discussed at length with respect to its use in biomonitoring studies. R. Stagg stated also that EROD is commonly preferred over the aryl hydrocarbon hydroxylase (AHH) surrogate for CYP1A induction because of its relative convenience and lower cost.

R. Stagg presented results from several field studies carried out by his laboratory in which these biochemical biomarkers were efectively used alone or in conjunction with fish histopathology, such as in farmed salmon and flatfish after the Braer oil spill at the Shetland Islands, dab in the vicinity of oil platforms in the North Sea, and investigations of the pigmented salmon syndrome. In the discussion it was stressed that the isolated use of biochemical biomarkers without involving other techniques to measure contaminant-associated liver changes might lead to misinterpretation of results. For example, the detection of low EROD activities generally is considered an indicator of low exposure to enzyme-inducing contaminants (such as carcinogenic substances) but can in fact be due to the opposite since EROD activity may also be low in highly exposed fish with tumourous liver tissue due to cellular changes associated with neoplasia leading to an inhibition of the P4501A system. Therefore, EROD measurements (and the use of other biochemical biomarkers) should ideally be combined with histopathology in order to facilitate the interpretation of data.

In a separate contribution, C. Malmström presented information on the measurement of DNA adducts in fish and the applicability of this technique for biological effects monitoring purposes (see Annex 5.3 b). The limitations in the use of hydrophobic DNA adducts in biomonitoring studies are mainly related to the time consuming procedure and high costs involved. However, they have been proven in numerous studies to be a useful tool, specifically when looking for the genotoxicity of PAHs, and are therefore considered promising for future monitoring purposes.

5.4 Integration of chemical and biological effects monitoring

M. Waldock presented an overview of strategies used in designing integrated chemical and biological effects monitoring (see Annex 5.4). He presented Holdgate's dichotomy of factor monitoring (factors that may induce changes in target organisms, such as chemicals) and target monitoring programmes that measure changes in distribution, abundance, performance or health of living organisms, and the pros and cons of each approach. In general, chemists tend to prefer factor monitoring, while biologists prefer target monitoring.

Disadvantages of factor monitoring lie in the fact that environmental quality objectives and standards are typically set according to concentrations of individual chemicals not to be exceeded, with various assumptions made (biologically active concentrations of compounds, bioavailability, no effect of environmental factors on effects of chemicals, representativeness of sampling programs). Advantages are that these chemical monitoring programmes give data on spatial distribution, trends that give a basis for management of chemical inputs.

Advantages of target monitoring (e.g., oyster embryo assay) are increased relevance to biological systems, the fact that organisms integrate effects of contaminants over time, and causal factors need not be known. The main drawback revolves around this issue of the lack of understanding of specific causality, so that management of contaminant input is problematical. However, biomarkers help to bridge the gap between factor and target monitoring by providing biologically relevant surrogates for

chemicals (factors). Certain of these surrogates are more specific for exposure to particular chemicals (e.g., DNA adducts measuring exposure to genotoxic PAHs) than others (e.g. lysosomal stability). Fish disease monitoring may be used as compliment factor and target monitoring of environmental quality. Examples of linked sets of determinants of contaminants and effects in relevant environmental matrices (hepatic CYP1A, DNA adducts, PAH metabolites in bile, liver pathology) as proposed for inclusion in the Joint Assessment and Monitoring Programme (JAMP) of OSPAR were presented. Risk factor models for liver diseases possibly induced by PCBs are strengthened by linking liver CYP1A to PCBs in tissues (liver) and sediments.

M. Waldock presented the opinion that there are very few appropriate and accepted targets (e.g., fish diseases) for biological effects monitoring, and identifying causality is very problematic when using these targets, so that bioassays have been used in UK and European programmes (using the Toxicity Integrative Evaluation approach, TIE) along with benthic ecology and fish disease. Bioassays have recently been developed to identify causal agents by testing fractions of sediment extracts, followed by methodical retesting of subfractions of these extracts, eventually leading to isolation and identification of the toxic components, which can then be monitored in the environment. This approach has been used successfully in the yeast cell assay (with the oestrogen gene spliced into the yeast's genome) to identify oestrogens and xenoestrogens in environmental samples. His strong opinion was that if liver disease is used in a target monitoring programme, one must measure everything or nothing, and use some additional mechanism for determining causality and the genotoxicological properties of each compound measured in order to effectively use the data in risk assessment.

M. Waldock presented data from the U.K.'s National Monitoring Programme, as an example of a factor monitoring programme linked somewhat with target monitoring, and included examples of poor correlations shown between contaminants in sediment and tissue (PCBs). He also discussed his contract work with the yeast cell assay to determine oestrogen-mimetic behaviour of environmental contaminants.

He summarised his presentation by saying that it is necessary to perform both target and factor monitoring, the proportion of which depends on the purpose of the monitoring programme.

In the discussion, the need for incorporation of biological effects measurement into environmental monitoring programmes was again emphasised. In the context, the strategy for incorporating biological effects in an integrated monitoring programme comprising biological and chemical components developed by ICES (1995 Report of the ICES Advisory Committe on the Marine Environment, ICES Coop. Res. Rep. 212, 1995) was highlighted.

Other points raised relevant to the topic of this workshop included the opinion that it has to be recognised that chemicals other than PAHs may be responsible for toxicity and liver lesions in fish, and that these factors need to be measured. It was furthermore noted that it might be appropriate to include contaminant analysis in stomach contents of target fish species since this could give better information on bioavailability of contaminants than levels in water, sediments or even fish tissue.

6. Practical workshop: demonstration of techniques

A major part of the Special Meeting was dedicated to practical work aiming at demonstrating different techniques in use to measure liver pathology at different levels of biological organisation and at evaluating the usefulness of these techniques for monitoring purposes. Main emphasis was given to considerations related to histopathology, e.g. defining diagnostic criteria for common histopathological liver lesions.

6.1 Histopathology

A subgroup was formed including S. Bogovski, D. Bucke, S.W. Feist, G. Grinwis, A. Köhler-Günther, M. Myers, M. Okihiro, and A.D.Vethaak. This subgroup defined the pathological lesions found in flounder, dab and winter flounder that should be registered in fish examined in monitoring programmes (see Annex 8.3).

These lesions were then rated on a scale of 1-3 according to their relation to toxicant exposure, with 1 being of highest importance. Slides and sections demonstrating different histopathological lesions provided by various participants were read and reviewed according to the diagnostic criteria defined in the list of significant contaminant-related lesions.

6.2 Glucose-6-phosphatase dehydrogenase

This test which detects early enzyme altered foci (G6PDH/PGDH) during carcinogenesis was demonstrated by A. Köhler-Günther. The test detects earlier foci than can be detected with standard H&E stained paraffin- or methacrylate- embedded material. All steps from cryosectioning to the analysis in light microscopy were demonstrated. A protocol for the method is enclosed (see Annex 5.2 a).

6.3 Proliferating cell nuclear antigen

Slides and sections stained immuno-cytochemically for proliferating cell nuclear antigen (PCNA) were demonstrated during the practical workshop and a protocol for the method was discussed (see Annex 5.2 b).

6.4 Immunhistochemical demonstration of cytochrome P4501A1

Slides prepared prior to the Special Meeting and stained immunocytochemically for cytochrome P4501A1 were examined by members of the Special Meeting. Methods are detailed in Annex 5.2 b.

6.5 Lysosomal membrane stability

A test using living cell of *Mytilus edulis* was demonstrated by M. Moore using the molecular probe neutral red in mussel blood.

The test includes the following main steps:

- with a syringe bleed 0.5 ml of mussel blood from the posterior abductor muscle
- add a saline and put a drop of the mix to a microscopical slide, leave in humidity chamber for 30 min.
- add the neutral red working solution to the slide
- check with regularly time intervals in a light microscope how long time the dye remains in the lysosomes

The method is simple to perform and needs little experience by the performer. It was stressed that the sampling of mussels should not be made during the spawning season.

A. Köhler-Günther demonstrated another lysosomal stability test performed in cryosections of frozen fish liver tissue. This test has already been recommended for inclusion in the biological effects monitoring component of the OSPAR JAMP as early biomarker for toxicity (a protocol of methods is provided in Annex 5.2 a).

The advantage of this approach is the possibility of long-term storage of frozen tissue samples as well as of prepared sections. The test included the following steps:

- cutting of serial cryosections (10 µm)
- destabilisation the lysosomal membrane in citrate buffer for defined time intervals
- incubation in substrate containing media for specific lysosomal enzymes
- incubation of sections with diazonium salt (Fast Blue) for colour reaction
- fixation in 4 % Bakers formalin
- mounting in gelatine
- determination of time needed for lysosomal destabilisation as maximum staining intensity

7. Recommendation of techniques for monitoring purposes

Over the past years, a broad range of biological effects techniques measuring pathological liver changes of fish have been developed and have already been assessed by ICES in the light of their usefulness for international monitoring purposes such as the OSPAR Joint Assessment and Monitoring Programme (JAMP) (see section 1 and Annex 1 c). Particularly the ICES Working Group on Biological Effects of Contaminants (WGBEC) covered this issue throughout previous years.

Ideally, techniques used for monitoring purposes should fulfil the following basic requirements:

- techniques should be selected according to the objectives of the monitoring programme (e.g. whether it is contaminant-specific or more general)
- they should be indicators at different levels of biological organisation (e.g. molecule, cell, organ, individual, population, community) in order to provide a more complete overview on the extent of effects
- they should include early warning techniques for detecting acute effects and techniques detecting more chronic exposure
- they should be established methods practically tested in field studies
- background data (e.g. on spatial and temporal aspects) from field studies should be available
- there should be sufficient information on the contaminant-response mechanisms inolved
- they should be robust and cost-effective
- for international programmes or for comparisons between laboratories, quality assurance requirements should be met (see section 8.4 and Annex 8.4)
- they should be subject to further research activities in order to improve them and provide additional data to be incorporated in the data analysis

A major objective of the ICES Special Meeting was to identify and assess biological effects techniques which can be used in monitoring programmes to detect contaminant-induced liver pathology. The participants agreed that particularly liver neoplasms and other lesions related to their histogenesis (e.g. putative pre-neoplastic lesions such as foci of cellular alteration) have been shown in multiple research/monitoring studies to be useful biomarkers of contaminant exposure effects, particularly in flatfish and other bottom-dwelling fish species living in contact with contaminated sediments and that they, therefore, should be given priority in monitoring programmes.

Liver lesions found in exposed wild fish are comparable to neoplasms and related lesions induced by experimental exposure of fish and mammals to chemical carcinogens and other toxicants. In this way, these lesions act as perhaps one of the most direct and integrative biomarkers of contaminant exposure, as an adverse health effect and injury. However, since there is evidence (mainly from US

studies) that also certain non-neoplastic liver lesions of wild fish are good markers of contaminant exposure, the participants of the Special Meeting felt that these lesions should also be included in monitoring programmes.

The histopathological classification of liver lesions is still largely based on existing criteria from rodent and fish experimental carcinogenesis. In section 8.3 and Annex 8.3 a new classification scheme elaborated at the Special Meeting is provided in which common pathological liver conditions of flatfish (both neoplastic and non-neoplastic) useful as biomarkers for monitoring programmes are listed together with a grading system indicating the relative importance of the lesions as marker of contaminant exposure/effects. These lesions should be recorded by routine histopathology on the basis of formalin-fixed, paraffin-embedded, and H&E stained liver specimens. General methodologies applied should be according to guidelines provided in report sections 5.1 (Annex 5.1), 8.2 (Annex 8.1), and 8.4 (Annex 8.4).

It was emphasized that, besides histopathology, the examination of livers for macroscopic liver nodules and subsequent histological confirmation should be continued according to ICES standard operating procedures (see section 8.2). This technique is well-established within ICES Member Countries carrying out fish disease surveys and has been applied sucessfully for years.

There was general consensus amongst the participants that recommended histochemical and immuno-histochemical procedures should be used as an adjunct to macroscopic examination and routine histopathology in H&E-stained paraffin-embedded sections, according to the monitoring objectives, resources, preference and monitoring/research interests of the laboratories involved.

In the table, techniques and parameters to be measured are listed which have been identified by the particpants of the Special Meeting as useful tools for monitoring liver pathology. Particular emphasis was given to those techniques which can be applied either macroscopically or using paraffin or frozen liver sections. However, it was noted that many techniques recommended do not fulfil all the requirements listed above. Nevertheless, some were considered useful for monitoring since they fulfil most of them.

It was emphasized that, besides the techniques listed in the table, there are a number of other techniques/parameters which can be applied using liver tissue sections and which are promising for future monitoring programmes (e.g. multi drug resistance (MDR), oncogenes, apoptosis etc.). However, these techniques were considered to be at present only research tools since they do not yet fulfil basic criteria which have to be met for immediate inclusion in monitoring programmes.

The participants recommended that, according to the objectives of the monitoring, biological effects techniques should be selected and applied on the basis of a decision-tree-type model. For general monitoring, this should comprise biomarkers of general toxicity (e.g. liver lysosomal stability) as primary techniques, followed by more specific biomarkers of early change (e.g. general and specific degnerative changes, foci of enzymatic/cellular alteration, PCNA, routine histopathology) and endpoint biomarkers of progressive change (e.g. benign and malignant tumours, histopathology). For contaminant-specific monitoring, such as monitoring effects of PAHs, biomarkers of exposure (e.g. CYP1A) could be followed by biomarkers of early toxicity (DNA adducts), biomarkers of cellular changes indicative of carcinogenesis (foci of cellular alteration) and endpoint biomarkers (benign/malignant liver tumours). All techniques should be integrated with chemical monitoring detecting the most relevant anthropogenic contaminants.

Biological effects techniques for measurement of fish liver pathology recommended for monitoring purposes

Effects measure	Technique	Status	Contaminant response
macroscopic liver changes			
• nodules > 2 mm in diameter	macroscopic, subsequent histological confirmation, paraffin sections, H&E	**A**	neoplasia: probably specific, carcinogens (1) non-neoplastic: probably unspecific
liver histopathology			
• general necrotic/degenerative changes	paraffin sections, H&E staining	**B**	probably unspecific (2)
• unique degenerative changes	same	**B**	specific, PAHs, PCBs, DDTs (1)
• storage conditions	same	**B**	probably unspecific (3)
• inflammatory changes	same	**B**	probably unspecific (3)
• non-neoplastic proliferative lesions	same	**B**	probably unspecific (3)
• vascular abnormalities	same	**B**	probably unspecific (3)
• foci of cellular alteration	same	**B**	probably specific (1)
• benign tumours	same	**B**	probably specific (1)
• malignant tumours	same	**B**	probably specific (1)
liver histochemistry			
• lysosomal membrane stability	cryo sections, neutral red	**B**	general toxicity
• enzyme-altered foci (G6PDH)	cryo sections,	**B**	specific
liver immuno-histochemistry			
• proliferating cell nuclear antigen (PCNA)	paraffin sections, antibodies	**B**	regeneration, proliferation (3)
• CYP1A	paraffin sections, antibodies	**B**	polar contaminants, PAHs, PCBs
liver biochemistry			
• CYP1A (EROD)	photometrically	**B**	polar contaminants, PAHs, PCBs
• DNA adducts	^{32}P-postlabelling	**B**	genotoxicity (PAHs, others)

Status A: Quality assurance in place
B: development and intercalibration needed
(1-3) : relative importance as biomarker for exposure/toxicity, 1 highest importance

8. Standardisation of monitoring techniques

R. Stagg outlined the guidelines for standardisation of biological effects monitoring techniques already developed by the ICES Working Group on Biological Effects of Contaminants (WGBEC) and at the 1995 ICES/OSPAR Workshop on Biological Effects Techniques.

He informed the participants of the EU COST programme which provides funding for the coordination of existing research activities in a particular area of science. The activities to be

coordinated are funded nationally, but the COST system can support coordination activities to maximise the benefit to the participating countries. Within the WGBEC, a proposal has been developed (coordinated by I. Davis, SOAEFD Marine Laboratory, Aberdeen, UK) and discussed in order to apply for funding to be used for standardisation and quality assurance of biological effects monitoring techniques. The participants agreed that R. Stagg should explore ways to incorporate a programme for standardization of liver pathology according to the outcome of the ICES Special Meeting in such a proposal.

R. Stagg emphasized the need for quality assurance (QA) of biological effects techniques used for monitoring programmes which can be defined as the total management scheme required to ensure the consistent delivery of quality controlled information. He gave an overview of the main elements of QA to be adressed:

Generic requirements (for QA)

- Provide training (workshops, development)
- Within laboratory calibration/standards
- Interlaboratory calibration
- Definition of limits
- Action when limits exceeded

Specific methods to be used

- which techniques should be included
- the necessary elements of an intercalibration programme
- the intercalibration standards required
- the sampling requirements
- suitable sample collection, preparation and preservation procedures
- training requirements
- acceptable performance limits
- action to be taken if limits are breached
- the requirements of good laboratory practice
- appropriate species to be used

It was decided in plenum to create 3 subgroups which should adress relevant issues related to standardisation of monitoring of various aspects of liver pathology. The conclusions of the subgroups and the plenary discussions are reflected in the following report sections.

8.1 Field sampling

Information on this issue is contained in section 8.2

8.2 Processing of samples

A subgroup consisting of D. Bucke, D. Bruno, D. Declerck, E. Lindesjöö, and S. Mellergaard discussed agenda items 8.1 and 8.2 together and developed a scheme providing information on requirements for field sampling and tissue processing for histopathology/histochemistry (see Annex 8.1).

D. Bucke informed the participants of various former ICES activities related to the development and standardisation of methodologies for fish disease surveys (including externally visible diseases and liver nodules/tumours) the results of which have been published in the ICES Cooperative Research

Report Series (Dethlefsen *et al.* 1986, Anonymous 1989) and the ICES TIMES Series (Bucke *et al.* 1996).

In the discussion of the scheme, the following issues were raised:

- It was recommended that ideally all relevant measurements should be carried out on the same fish. It was recognised, though, that the time course for each parameter is different, but this multiple approach is still desirable. However, all sampling strategies applied should be according to the objectives of the monitoring programme, e.g.whether it is contaminant-specific ('bottom up study') or more general ('top down study').
- The number of fish sampled for histopathological studies of the liver should be according to defined statistical requirements which are based on the objectives of the programme (spatial, temporal monitoring).
- It was emphasized that the age of all fish sampled for any biomarker measurement should be recorded. However, the reading of otoliths is time consuming and exceeds the capacities of many laboratories. As a compromise, it is recommended to take all fish from a standardised size range, preferably from a mid-size group where variation in age is less pronounced as in the largest size groups.
- The size range of fish recommended in the scheme (see Annex 8.1) is mainly based on the availibility of sufficient numbers of fish in the North Sea and Baltic Sea and might be adapted to other conditions.
- It was emphasized that sex and stage of sexual maturity (grossly or, more reliably, histologically) of fish examined should be recorded since both factors are known to potentially influence liver histopathological features and biochemical biomarkers recommended for monitoring (e.g. EROD).
- The recommended routine histopathological monitoring of liver lesions in 50 specimens is not meant to replace the standard methodology recommended by ICES and implemented in many national fish disease monitoring programmes in ICES Member Countries. The latter methodology is based on the examination of 50 fish (dab $\geq$ 25 cm; flounder 25-30 cm) per haul/site for the occurrence of macroscopic liver nodules > 2 mm in diameter and on subsequent histological confirmation of gross findings. The random routine histopathological monitoring should instead be done in addition to the gross examination and later histological confirmation.

8.3 Diagnostic criteria for histopathology

A subgroup consisting of A.Köhler-Günther, D.Vethaak, M.Myers, M.Okihiro, S.Bogovski, G. Grinwis and S.Feist was given the task to develop a classification scheme for diagnostic criteria of common histopathological liver lesions which might be useful indicators for monitoring purposes and to define fish species in the ICES area for which these criteria can be applied (Annex 8.3).

The original idea was that only those lesions should be considered which are easy to recognize and the classification criteria of which are clearly defined and, therefore, are most suitable for monitoring purposes. However, the subgroup decided to include all common histopathological liver lesion, either neoplastic or non-neoplastic, since all of them have shown to be good indicators of contaminant-induced pathological changes. Out of these, lesions could be selected for monitoring, if necessary, which are most likely to be indicative of contaminant effects. In order to facilitate a selection, the subgroup classified the lesions according to their relative importance into 3 grades as shown in Annex 8.3.

In the discussion, concern was expressed regarding possible problems to intercalibrate and standardise diagnosis of such a wide range of histopathological lesions which would be crucial in order to achieve quality assurance of data. It was emphasized that there is a clear need for continuous

training and intercalibration exercises and workshops to establish standardised criteria (see also report section 8.4 and Annex 8.4).

8.4 Quality assurance requirements

A subgroup consisting of R. Stagg, C. Malmström, A. McVicar and T. Lang discussed quality assurance requirements including suitable intercalibration programmes related to the monitoring of liver pathology. It considered the following elements as essential for an intercalibration programme (details are given in Annex 8.4):

- Good laboratory practise (GLP)
- Reference material
- Regulation of analytical quality control
- Training requirements
- Performance limits
- Action to be taken if there is poor agreement

The participants agreed that for quality assurance reasons it would be essential to establish a reference laboratory (or reference laboratories) the tasks of which would be to organise intercalibration exercises and workshops, to identify suitable reference material and distribute it to participating laboratories, and to compile results of intercalibration activities.

In the discussion it was again emphasized that the establishment of standard operating procedures for diagnosis of histopathological liver lesions is difficult and that, therefore, the continous intercalibration of diagnosis between laboratories and within laboratories is essential.

9. Any other business

The participants of the ICES Special Meeting discussed different possibilities to publish the results of the Special Meeting and agreed on the following options:

- Technical aspects shall be published in the ICES TIMES Series based on a manuscript already prepared by . Köhler-Günther (co-authors: Köhler, Feist, Lang)
- Proceedings of the Special Meeting shall be published in the ICES Journal of marine Science (co-authors: all participants)
- A colour atlas of histopathological liver lesions shall be published in a well-known and widely distributed journal, possibly in conjunction with an atlas already in preparation (by S. MacLean) in the USA (co-authors: contributors, possibly edited by S. MacLean and selected participants of the ICES Special Meeting)-

The participants further discussed possibilities to establish a network for future exchange of information between participants and coordination of future research/monitoring activities. However, although the idea was considered promising, no detailed suggestions were made.

10. General Conclusions

- The participants of the ICES Special Meeting on the Use of Liver Pathology for Monitoring Biological Effects of Contaminants agreed that the meeting constituted an important step forward in the identification and standardisation of suitable techniques for monitoring biological effects of contaminants.

- Biological effect monitoring programmes should be designed according to accepted epidemiological principles and methods and should include histological and cellular/subcellular biomarkers. If a logical decision tree approach is used, these biomarkers have high potential for risk assessment.

- Future research should focus on developing more sensitive biomarkers of contaminant exposure and effects in order to elucidate the underlying mechanisms involved in liver pathogenesis/carcinogenesis

- Future joint studies between ICES Member Countries and other countries (such as PICES Member Countries) working in the area of contaminant-associated liver pathology of marine fish species should be encouraged by organising symposia, workshops and joint research activities.

11. Recommendations

Based on the results of the discussions and practical workshops, the participants of the ICES Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects recommended that

- ICES Member Countries should be encouraged to incorporate histological liver lesions and biomarkers, particularly those recommeded in the report of the ICES Special Meeting, in national monitoring programmes on biological effects of contaminants. Methodologies should be according to procedures and diagnostic criteria detailed in the report.

- monitoring should be combined with research activities in order to evaluate and, as appropriate, implement new promising biological effects techniques and to elucidate underlying mechanisms involved in the development of pathological liver changes.

- the ICES Working Group on Pathology and Diseases (WGPDMO) and the ICES Working Group on Biological Effects of Contaminants (WGBEC) review the results of these studies on a regular basis, possibly at joint special topics meetings.

- data obtained from studies on liver pathology and related biomarkers are submitted to ICES and that the ICES WGPDMO together with the ICES secretariat explore ways to incorporate the data into the ICES Environmental Databank for subsequent statistical analysis.

- the MAFF CEFAS, Weymouth, UK acts as reference laboratory for liver histopathology quality assurance procedures involving the preparation and distribution of reference materials and the organisation of intercalibration exercises and workshops as appropriate.

- the technical aspects detailed in the report are published in the ICES TIMES Series under the co-authorship of A. Köhler-Günther, S.W. Feist and T. Lang taking into consideration a draft manuscript already prepared by A. Köhler-Günther.

- the proceedings of the ICES Special Meeting are published in the ICES Journal of Marine Science with all participants as co-authors in order to guarantee a more widespread distribution of the findings and conclusions of the meeting in the scientific community

- a colour atlas on liver histopathological changes of selected marine flatfish species from European and US waters is published, partly based on the liver lesions classified at the ICES Special Meeting

- international environmental monitoring organisations consider the results of the ICES Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants and incorporate recommended biological effects techniques and related quality assurance procedures into monitoring programmes as appropriate.

12. Approval of the report and closing of the meeting

Those sections of the report which were available at the end of the Special Meeting were approved by the participants and it was decided that a completed version of the draft report prepared by the co-convenors would be distributed to all participants as soon as possible for review. As deadline for submission of comments and suggestions for changes to S.W. Feist the 31 December 1996 was suggested.

The co-convenors of the Special Meeting thanked the participants for their contributions and enthusiasm and expressed their gratitude to the head and the staff members of the CEFAS Weymouth Laboratory for their tremendous support throughout the meeting and closed the meeting on Friday at 18.00.

ICES Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants

Annex 1 a

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ICES Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants

Annex 1b

General Introduction (T. Lang)

Since the 1970's, there is a growing body of literature on the occurrence of pathological liver changes in wild fish associated with the exposure to anthropogenic environmental contaminants. From the beginning of relevant studies, mainly neoplastic and putative pre-neoplastic changes have received most attention and the results of a number of field studies involving mainly wild marine flatfish species have indicated that elevated prevalences of these changes may represent a specific biological indicator of the exposure of target species to carcinogenic contaminants. These findings were further supported by experimental and mesocosm studies which demonstrated that histopathological lesions identical to those occurring under *in situ* conditions can be induced by single or combinations of xenobiotics. In addition, biochemical and molecular studies in both wild and experimental fish exposed to xenobiotics has demonstrated the occurrence of parts of the multi-step process of carcinogenesis established before from mammalian cancer research.

Due to the increasing evidence of a cause-effect relationship between environmental carcinogenic contaminants and the occurrence of neoplastic and putative pre-neoplastic lesions, the examination for neoplastic liver lesions has been incorporated in the majority of fish disease surveys carried out by ICES Member Countries in order to assess and monitor biological effects of contaminants and results are reported to the ICES Envronmental Databank on an annual basis for subsequent statistical analysis.

First guidelines for the standardization of methodologies for fish disease surveys were elaborated by the *ICES Working Group on Pathology and Diseases of Marine Organisms* (WGPDMO) and included the recommendation to record prevalences of macroscopic liver nodules larger than 2 mm in diameter in flatfish species (North Sea: the common dab, *Limanda limanda*; Baltic Sea: the flounder, *Platichthys flesus*) as indicator of neoplastic lesions. Most of the on-going fish disease monitoring programmes in the ICES area, particularly those carried out by member countries bordering the North Sea, have been performed according to these guidelines. In realizing that macroscopic examination alone might lead to an over-estimation of the prevalence of neoplastic changes, the WGPDMO further emphasized that macroscopic examination should be supplemented by histological confirmation of detected lesions in order to distinguish between neoplastic and non-neoplastic changes. In order to ensure comparability of results provided by different researchers, the WGPDMO recommended that samples of liver nodules collected by ICES Member Countries should be sent to a reference laboratory (MAFF CEFAS, Weymouth, UK) for histological confirmation. However, due to the increasing workload identified it has been envisaged that standardized and intercalibrated methodologies and diagnostic classification criteria should be developed for identification of neoplastic liver lesions to be applied by ICES Member Countries.

In connection with the revision of the formerly chemically orientated OSPAR Joint Monitoring Programme (JMP) into the new OSPAR Joint Assessment and Monitoring Programme (JAMP) which will include a substantial biological effects monitoring component, ICES has been requested by OSPARCOM to provide advice on biological effects techniques suitable for incorporation in the JAMP and on associated quality assurrance procedures. These issues have been mainly dealt with by the *ICES Working Group on Biological Effects of Contaminants* (WGBEC) which developed recommendations including a concept for an integrated chemical and biological monitoring strategy and the identification of suitable techniques for biological effects monitoring. These recommendations have formed the basis for scientific advice provided to OSPARCOM by the *ICES Advisory Committee on the Marine Environment* (ACME).

During the 1995 *OSPAR/ICES Workshop on Biological Effects Monitoring Techniques*, Aberdeen, UK, programmes were proposed for different aspects of monitoring including the identification of biological effects techniques related to liver pathology and suitable for specific and general biological effects monitoring (EROD, DNA adducts, PAH metabolites, lysosomal stability, macro- and microscopic liver pathology). Furthermore,

the clear need for the development and implementation of quality assurance (QA) procedures prior to the application of recommended techniques was emphasized.

Although the work carried out by ICES Working Groups and the results of the OSPAR/ICES Workshop are considered a major step forward, there is still a the lack of standardization and intercalibration of methodologies applied by ICES Member Countries for the measurement of different aspects of liver pathology (biochemical, cellular/subcellular and histopathological) and recommended for monitoring purposes. Therefore, ICES decided to organize a ***Special Meeting on The Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants*** (ICES C.Res. 1995/2:31: *A Special Meeting on The Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants (Co-Convenors: Dr. S.W. Feist, UK and Dr. T. Lang, Germany) will be held in Weymouth, UK from 22-25 October 1996.).* Since the main emphasis of the meeting is placed on pathological aspects, the planning and organization of the meeting was carried out by the ICES WGPDMO and the *ICES Sub-group on Statistical Analysis of Fish Disease Prevalence Data in Marine Fish Stocks.* However, since other biological effects techniques related to liver pathology will also be adressed, the ICES WGBEC was invited to participate in the planning of the meeting and nominated Dr. A. Köhler-Günther as representative.

The main objective of the meeting is to bring together specialists involved in research/monitoring programmes on contaminant-associated liver pathology of flatfish in order to

- *provide an overview of on-going activities in ICES Member Countries*
- *discuss and evaluate the suitability and applicability of different techniques used to measure liver pathology of fishes for monitoring purposes*
- *elaborate standardized guidelines and quality assurance procedures for field sampling, processing and interpretation of liver pathology for monitoring programmes*

In order to provide a sound basis for discussions on the usefulness and standardization of techniques for monitoring purposes, the clear intention of the meeting is to include as much practical work as possible. Participants were therefore asked to demonstrate selected techniques in use and to provide microscopical slides displaying different types of liver lesions for standardization and intercalibration of classification criteria for contaminant-associated histopathological liver changes (non-neoplastic, putative pre-neoplastic, neoplastic). Although it was decided that the main emphasis should be given to studies in flatfish species due to their importance in monitoring programmes, also other fish species will be considered as appropriate for comparative purposes.

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Annex 1 c

Techniques useful for PAH-specific and general biological effects monitoring purposes involving liver pathology of fish as identified by the 1995 ICES/OSPAR Workshop on Biological Effects Techniques, Aberdeen, UK

Effect measure	Status	Contaminant response
A. PAH-specific biological effects monitoring		
• P4501A (EROD)	B	Planar molecules, PAH, PCB
• DNA adducts	B	Carcinogenic contaminants
• PAH metabolites	B	PAH specific
• Liver pathology	B	unspecific
B. General biological effects monitoring		
Bioassay		
• Whole sediment	B	general toxicity
• Pore-water	B	general toxicity
• Water-column	B	general toxicity
Biomarkers		
• P4501A (EROD)	B	Planar molecules, PAH, PCB
• Lysosomal stability	B	Organic contaminants
• Liver pathology	B	General, but can be diagnostic
• Liver nodules	A	Depending on the type of lesion, Cancer inducing chemicals
Population/community responses		
• External fish diseases	A	Not specific to contaminants
• Fish reproductive success	B	Not specific to contaminants
• Macrobenthic fauna	A	Not specific to contaminants
A: Quality assurance in place **B**: Requires interlaboratory comparison and finalisation of QA procedures		

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Annex 2

Agenda

1. Opening of the meeting and general introduction

2. Adoption of the agenda

3. Appointment of rapporteurs

4. Current status of studies on liver pathology
   - 4.1 Histological, cellular/subcellular and biochemical techniques and their field application
   - 4.2 European epidemiological studies
   - 4.3 US perspective
   - 4.4 Reports on national activities in ICES Member Countries
   - 4.5 Experimental studies on hepatic carcinogenesis in fish
   - 4.5 Summary review of the current status of studies on flatfish liver pathology

5. Assessment of techniques for the determination of liver pathology
   - 5.1 Histopathology and electron microscopy
   - 5.2 Cellular/subcellular biomarkers
   - 5.3 Biochemical biomarkers
   - 5.4 Integration of chemical and biological effects monitoring

6. Practical workshop: demonstrations of techniques
   - 6.1 Histopathology
   - 6.2 Glucose-6-phosphatase dehydrogenase
   - 6.3 Proliferating cell nuclear antigen
   - 6.4 Immunohistochemical demonstration of cytochrome P4501A1
   - 6.5 Lysosomal membrane stability

7. Recommendation of techniques for monitoring purposes

8. Standardisation of monitoring techniques
   - 8.1 Field sampling
   - 8.2 Processing of samples
   - 8.3 Diagnostic criteria for histopathology
   - 8.4 Quality assurance requirements

9. Any other business

10. General conclusions

11. Recommendations

12. Approval of the report and closing of the meeting

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Annex 3

Rapporteurs

Tuesday, 22 October:	Alasdair McVicar + Stig Mellergaard
Wednesday, 23 October:	Mark Myers + Eric Lindesjöö
Thursday, 24 October:	Ron Stagg + David Bruno
Friday, 25 October:	Dick Vethaak + David Bucke

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Annex 4.1

Histological, cellular / subcellular and biochemical techniques and their field application

by

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An overview is presented on techniques with the potential to link histopathological changes (fixed/frozen material) to metabolic/biochemical changes (frozen material) in cells and tissues and the validation of their potential in field studies. The need to perform histopathogical studies in order to be able to interpret biochemical results obtained from homogenised tissues (eg EROD) is documented.

The deleterious effects of pollutants which are integratively reflected in the histopathology of the liver are influenced by natural exogenous physical factors (e.g. temperature) of the habitat as well as endogenous physiological factors (eg sex, reproduction).These variables influencing the biomarkers indicating contaminant exposure and biomarkers indicating injurious effects can be minimised by proper sampling strategies when setting up a monitoring program.

Responses for cell protection (lysosomal degradation, Cyp450, GST, MDR, DNA repair) counteract with those of cell injury (eg lysosomal destabilisation, oxiradicals, gene mutation) in order to keep up cellular homoeostasis. Acute high and/or long time chronic low contaminant exposure lead to the breakdown of homeostatis resulting in liver pathologies.

Characteristic liver lesions of flounder and dab representing early liver injury and steps during carcinogenesis of various cell types of the liver (hepatocytes, bile duct epithelial cell, endothelial cell of blood vessels) are shown. Parallel cytochemical tests in identical individual livers evidenced that decreased lysosomal membrane stability sensitively reflected the onset and progression of liver anomalies. Correlations of field data indicated that decreased lysosomal membrane stability coincides with increased contaminant concentrations (eg PCB 52, PCB 138, PCB 118, HCB, y-HCH) in flounder as well as in dab caught in various estuaries (Tyne, Tees, Thames, Schelde, Weser Elbe, Eider) and on North Sea transects.The lysosomal membrane stability test in frozen material is already recommended earlier for the biological effects monitoring.

Neoplasia is generally regarded as one of the most relevant endpoints of toxicant and carcinogen exposure. Therefore, the very early detection and quantification of altered cells with the putative potential to develop malignant carcinomas are relevant. Baseline studies in serial frozen tissue sections allowing the precise localisation of suspicious cell alterations were performed for G6PDH (glucose-6-phosphate-dehydrogenase), Cyp P450 (cytochrome P450 Ial) and MDR/MXR (multidrug/ multixenobiotic resistance).

Baseline studies on the kinetic parameters of G6PDH, the key enzyme of the pentose phosphate cycle producing NADPH and pentoses, -relevant in biotransformation and cell proliferation, showed that altered enzyme activities of G6PDH indicate the presence of early putative preneoplastic foci prior to their appearance in H&E stained fixed and frozen sections of identical specimens. Kinetic parameters and flux rates of G6PDH indicate significantly increased metabolic activities in basophilic cells persisting in foci, adenoma and carcinoma in the carcinogenesis of flounder liver. With the aid of PCNA (proliferating cell nuclear antigen) immunocytochemical labeling in serial sections, early foci can be distinguished from focal satellites of invasively growing carcinomas by a significantly increased nuclear labeling index. Parallel immuno histochemical studies in serial sections of identical frozen livers showed a decrease of Cyp450, increased MDR and increased activities of tumor-associated benz-aldehyde dehydrogenase in various neoplastic lesions types which have been identified by increased G6PDH activity and affirmed in H&E stained sections.

It is emphasised that cellular and molecular epidemiology, in contrast to classical epidemiology, offers biomarkers of the susceptibility of cells for cancer development instead of only demonstrating pronounced tumour developments.

These biomarkers are e.g.

- enhanced activation or inefficient detoxification resulting in reaction of the carcinogen with DNA or protein leaving a characteristic fingerprint (DNA adducts)
- low levels of oxiradical scavenging enzymes (antioxidants) so that the carcinogen converts a benign gene in a cancer causing one (gene mutation, eg k-ras; n-ras, P53)
- inefficient repair of DNA leads to a toxicant resistent cell type, free from normal restraint in growth and differentiation (abnormal cell growth)
- inhibited immune recognition of malignant cells lead to the growth of histologically detectable cell clones (foci, adenoma, carcinoma)

These techniques will have a high relevance in future risk assessment studies in monitoring in the marine and terrestrial environment as well as in human epidemiology.

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Annex 4.2

Epidemiological studies of liver tumours and related lesions in European flatfish: an overview

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Field studies

Field studies of liver diseases in (flat) fish have been carried out in the North Sea since the early 1980s, with the first reports of the existence of epizootics of liver tumours in flatfish coming from Dutch coastal waters in 1984. Since that time, routine histological studies of flounder (*Platichthys flesus*) and dab (*Limanda limanda*) have resulted in the identification of a spectrum of hepatic lesions including neoplasms (hepatocellular adenoma/carcinoma) and pre-neoplastic focal lesions (foci of cellular alteration) as well as non-neoplastic storage disorders and degenerative or necrotic lesions. The epidemiological studies carried out in this area can be placed in three categories: surveys of grossly visible liver nodules (assumed to correspond to tumours), surveys of histological liver lesions, and surveys combining gross examination and histology. The objectives of all these studies have been to monitor for biological effects of contaminants or to establish cause-and-effect relationships. Surveys of grossly visible liver nodules became popular because the nodules were easily diagnosed and an assessment of their prevalence could easily be included in surveys of external diseases, the latter often being part of general fish stock assessment cruises. Surveys of gross pathology and histopathological studies were therefore not necessarily compatible, the latter requiring more specialised methods for sampling and analysis.

It has been shown that epidemiological surveys involving the recording of gross liver anomalies represent a cost-effective method of collecting data in the occurrence of hepatic neoplasms in flatfish. This approach permits the examination of large numbers of fish, and thus a precise estimate of prevalence can be obtained. There is, however, a problem with the approach in that old fish (in which most nodules are found) tend to be scarce at some North Sea sites. Even where they are relatively common, prevalences can sometimes be low (less than 2 %). For the purpose of carrying out spatial and temporal comparisons, therefore, it may be more useful to concentrate on histological markers (such as foci of cellular alteration) that precede tumour formation; these lesions occur at higher prevalences and in younger fish than do neoplasms.

Recently, several multidisciplinary studies focusing on liver disease in flounder and dab in relation to chemical pollution have been carried out in the North Sea and, to a lesser extent, the Baltic Sea. There has been an increasing tendency to measure other environmental factors and contaminant concentrations as well as histological and biochemical markers of exposure to pollution. These studies have produced valuable insights into cause-and-effect relationships. Good examples include the Bremerhaven workshop organized by ICES and IOC in 1990, and Dutch surveys belonging to the Integrated Monitoring Program (INP-MIVE) in the southern North Sea in 1992. Despite these efforts, however, field surveys have on the whole produced only limited evidence for a role of contaminants such as PAHs and PCBs in the causation of liver tumours and related lesions in dab and flounder inhabiting European waters. The factors influencing the occurrence of such lesions appear to be numerous and highly complex. Other than contaminants, important suggested risk factors for liver neoplasms include large size, old age, gender (with females being at higher risk), capture site, season (with risk being higher in winter), high salinity, spawning activities and malnutrition. Because of this complexity, it is likely that biological effects monitoring using liver tumours of histological liver lesions is best targeted at detecting temporal (rather than spatial) trends in this area.

In addition to dab and flounder in the North Sea and flounder in the Baltic Sea, epizootic occurrences of liver tumours in fish have been reported for ruffe (*Gymnocephalus cernua*) and Atlantic hagfish (*Myxine glutinosa*) living along the Swedish west coast. Incidental cases have also been reported in other species, including sole (*Solea solea*), plaice *(Pleuronectes platessa*) and gurnard (*Trigla* sp.). No cases of liver tumours have been reported from the Mediterranean Sea, where there has been a notable lack of epidemiological studies.

Experimental and Mesocosm studies

In field surveys, where there is no opportunity to control any of the variables under study, it is difficult if not impossible to draw definite conclusions about cause-and-effect relationships. Such relationships can only be adequately addressed through the medium of experimental work - with mesocosm studies occupying an intermediate position - but there is a risk that the results may no longer be relevant to the situation in the field. So far there have been no experimental laboratory studies on liver tumour induction in dab and flounder. Recently, evidence for a chemical aetiology of liver tumours in flounder has been produced by a large-scale mesocosm study carried out in the Netherlands. The experiment showed that chronic exposure to contaminated sediments induced liver neoplasms and putative preneoplastic lesions (foci of cellular alterations) at contaminant levels comparable to those found in the natural environment. The pollutants most likely to have been involved were PAHs and perhaps PCBs, but the influence of other unknown chemicals could not be ruled out. Another contaminant-related liver lesions identified during the course of this study, was hydrophobic vacuolisation of biliary epithelial cells, but data on the occurrence of this condition in fish inhabiting European waters is still very limited.

Comparison with American studies

The findings of studies in the North Sea and the Baltic Sea provide a contrast with those of American studies. The extensive field and experimental studies carried out by North American scientists since the 1960s have been successful in linking the occurrence of tumours in marine fish with specific classes of pollutants such as PAHs. The comparative lack of success on this side of the Atlantic may be due to the complexity of the ecosystems in combination with relatively low environmental concentrations of pollutants. It is noteworthy that most studies in the USA have been carried out in partially enclosed water bodies, with contaminant levels routinely many times higher than those encountered in the North Sea. Another significant difference between European and North American studies is the observation that the principal types of lesion affecting th species studied are different. Dab and flounder seem to be affected mostly with liver tumours of hepatocellular origin (hepatocellular adenoma/carcinoma), whereas the predominant tumours in North American flatfish are of biliary epithelial origin (cholangioma/cholangiocarcinoma). The apparent success of the long-term multidisciplinary studies carried out by American scientists has acted as an impetus for similar work in Europe, as described above. Routine histopathology has been incorporated into these more recent studies, together with an assessment of biochemical responses and chemical risk factors.

Activities within ICES

ICES has conducted two practical sea-going workshops, in 1984 and 1988, with the aim of developing standardized methodologies for identifying and recording a range of grossly visible disease signs, including liver nodules in dab and flounder. These workshops led to the publication, in 1996, of the ICES traning guide for the identification of common parasites and diseases of fish in the North Atlantic. Experience gained during the ICES Bremerhaven workshop on biological effects of contaminants in 1990, and the BMB/ICES sea-going workshop on diseases and parasites in the Baltic Sea in 1994, have also made important contributions to this field. Furthermore, ICES has established a fish disease data bank as part of its environmental data bank. The information held includes long-term data from ICES member countries on the occurrence of liver neoplasms in dab and flounder. Recommendations for the recording and diagnosis of liver nodules (greater than 2 millimetre in diameter) in flounder and dab are given in the ICES training guide. These recommendations include the need to obtain histological confirmation of all nodules recorded (using the MAFF Fish Diseases Laboratory in Weymouth (England) as a reference laboratory), and the importance of separating true neoplams (hepatocellular adenoma/carcinoma) from other non-neoplastic lesions. It is hoped that the adoption of standardized methodologies will be applied as a criterion for accepting data for inclusion in the ICES environmental data bank.

Gaps in knowledge and future perspectives

The study of the epidemiology of liver lesions is a promising tool for monitoring the exposure of fish in the North Sea to potential toxicants and carcinogens. However, several areas require further clarification. Firstly, there is a need to identify all the risk factors that contribute significantly to the occurrence of liver neoplasms and related lesions. Secondly, there is a need to identify pre-clinical markers that provide a reliable indication of exposure to toxicants and carcinogens. Thirdly, a knowledge of the migratory behaviour and population ecology of the species affected is a prerequisite for any attempt to interpret prevalences of diseases at specific locations, yet it is currently inadequate even for common species such as dab and flounder.

Monitoring programmes should continue to include examination of livers for visible nodules (with subsequent histological verification), in accordance with ICES standard procedures. However, a higher priority should be given in these programmes to pathological changes that precede tumour formation, including histological and cellular or subcellular biomarkers.

Furthermore, experimental laboratory studies are required in order to elucidate specific cause-and-effect relationships and to better understand the processes of hepatotoxicity and liver tumour formation in dab and flounder.

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Annex 4.3

Toxicopathic hepatic lesions and other biomarkers of chemical contaminant exposure and effects in marine bottomfish species from the Northeast und Pacific Coasts, U.S.A.

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Relationships between toxicopathic hepatic lesions and levels of chemical contaminants in sediments, stomach contents, liver, and bile have been evaluated in English sole (*Pleuronectes vetulus*), starry flounder (*Platichthys stellatus*), and white croaker (*Genyonemus lineatus*) as sentinel species from 27 sites on the Pacific Coast, and in winter flounder (*Pleuronectes americanus*) from 22 sites on the Northeast Coast of the USA in a national biomonitoring study conducted from 1984-1988 as part of the National Benthic Surveillance Project, National Status and Trends Program. Prevalences of and relative risks for hepatocellular and biliary neoplams, preneoplastic foci of cellular alteration (clear cell, eosinophilic, and basophilic foci), non-neoplastic proliferative lesions (primarily hepatocellular regeneration and oval cell proliferation), unique or specific degenerative/necrotic lesions (hepatocellular nuclear pleomorphism and megalocytic hepatosis), and hydropic vacuolation of cholangiocytes and hepatocytes were significantly higher in fish at contaminated sites in Puget Sound, the Los Angeles area, and San Francisco and San Diego Bays on the Pacific Coast, and in Boston Harbor, Raritan Bay, and certain urban sites in Long Island Sound on the Northeast Coast. In logistic regression analyses, exposure to polycyclic aromatic hydrocarbons (PAHs), PCBs, DDTs, chlordanes and dieldrin were significant risk factors for all lesions in Pacific Coast species, with PAH exposure being the most common significant risk factor. In winter flounder, exposure to PAHs, DDTs, or chlordanes were significant risk factors only for hydropic vacuolation, non-neoplastic proliferative and nonspecific necrotic lesions, and less commonly for neoplasms and foci of cellular alteration. Risk of lesion occurrence generally increased with fish age, but sex was rarely a risk factor for hepatic disease.

Related studies in subadult English sole from Puget Sound also showed significant and near-significant correlations between prevalences of earlier-occurring hepatic lesions (foci of cellular alteration, nonneoplastic proliferative lesions, and nuclear pleomorphism/megalocytic hepatosis) and measures of contaminant bioaccumulation (liver PCB levels) and biochemical response (biliary fluorescent aromatic compounds, hepatic induction of cytochrome P4501A [CYP1A] as estimated by aryl hydrocarbon hydrocylase [AHH] activity, and concentrations of hydrophobic DNA adducts in liver). In more recent studies on adult English sole, rock sole (*Lepidopsetta bilineata*), and starry flounder from a creosote-contaminated site in Puget Sound undergoing habitat remediation by capping of contaminated sediments, concentrations of hepatic DNA adducts measured in individual fish were shown to be significant risk factors for the earlier-occurring lesions of hepatocellular nuclear pleomorphism and megalocytic hepatosis in individuals of all of three of these flatfish species, and for preneoplastic focal lesions only in English sole. In logistic regression analyses of these data, each additional nmole adducts/mol bases increased the risk of lesion occurrence by 1.007-1.05 times, depending on species and lesions type.

Studies by our laboratory investigating the applicability of the "resistance to cytotoxicity" paradigm for chemically induced hepatocarcinogenesis in vertebrates to liver neoplasia in wild English sole, have utilized immunohistochemical localization of the Phase I detoxication / activation enzyme cytochrome P4501A (CYP1A) and measurement of concentrations of hydrophobic DNA adducts in liver neoplasm as compared to matched samples of adjacent non-neoplastic liver tissue from the same fish. We have universally shown dramatic reductions in CYP1A expression in hepatic neoplasms and in the vast majority of preneoplastic foci of cellular alteration in English sole, as well as marked and significant reductions (~ 70 %) in DNA adduct concentations in hepatocellular neoplasms as compared to non-neoplastic liver tissue in the same individual

fish. These findings provide additional evidence supporting a PAH-associated etiology for hepatic neoplasia in English sole, and strengthen our hypothesis that hepatocytes composing certain foci of cellular alteration and especially those composing hepatocellular neoplasms possess a phenotype that is resistant to the cytotoxicity of genotoxic toxicants, by virtue of a reduced capacity to metabolize parent PAHs to their toxic and carcinogenic intermediates via CYP1A, and a consequent reduction in the formation of covalent, hydrophobic DNA adducts from these reactive intermediates.

Other studies have recently been completed utilizing segmented regression techniques to determine threshold levels of contaminants such as PAHs in sediments associated with certain toxicopathic hepatic lesions in indigenous bottomfish, such as English sole. Results indicate that threshold levels for earlier occurring hepatic lesions (e.g., megalocytic hepatosis, foci of cellular alteration) and other biological responses are in ~ 200 to 1000 ppb _PAHs. These thresholds are substantially below sediment quality standards set by the State of Washington on the basis of acute effects (i.e., mortality) bioassays done on non-indigenous fish and invertebrate species. Our results are currently being strongly considered in the re-evaluation of sediment quality standards in Puget Sound and elsewhere.

Although pathologic responses, biochemical responses, and risk factors of chemical exposure differ among the species examined in our multiple field studies over the last 20 years, these relationships provide strong evidence for environmental contaminants as etiologic agents for hepatic lesions in several marine bottomfish species, and clearly indicate the utility of these lesions as biomarkers of contaminant-induced effects in wild fish. Consequently, toxicopathic liver lesions are becoming increasingly utilized as biomarkers of contaminant effects not only in national and regional biomonitoring programs, but also as indicators of injury to fisheries resources within the injury assessment phase of the legal process of assessing damage to fishery resources.

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Annex 4.5

Experimental Hepatocarcinogenesis in Medaka (*Oryzias latipes*)

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Introduction: Hepatic carcinogenesis in medaka (*Oryzias latipes*), a small freshwater aquarium fish species with known susceptibility to mammalian carcinogens, was investigated in two studies; 1) a medium-term "Range Finding Study" and 2) a long-term "Metastasis Study." Both studies used the carcinogen diethylnitrosamine (DEN). The goals of the range finding study were to: 1) examine early preneoplastic foci of cellular alteration (FCA); 2) develop a classification scheme for FCA and neoplasms; and 3) determine a subcarcinogenic dose (and duration) for DEN using aqueous bath exposure. The metastasis study was intended to assess malignant potential of hepatic neoplasms in medaka with specific regard to metastasis to distant (extra-hepatic) sites.

Materials and Methods: In the range finding study, six groups of 1000 fish each were used: three were larval (3 wk old) and three were adult (3 mo old). Each group consisted of five subgroups (200 fish each), with one control (0 ppm) and four exposed to log concentrations of DEN (5, 50, 158, 500 ppm nominal). Thirty to 60 fish were sampled 16 and 24 wks post-exposure (PE), fixed in 10% formalin, and routinely paraffin processed. Hematoxylin and eosin (HE) stained sections of whole fish (2/fish) were evaluated for sex (mature females defined as those with ≥ 3 yolked oocytes), hepatic FCA and neoplasms. In the metastasis study, four groups of fish were used: two larval and two adult. In both larval (2 wk old) groups, 450 fish were exposed to either 300 or 500 ppm DEN for 48 hrs. The two adult groups consisted of either 3 mo old fish (n = 424) or 6 mo old fish (n = 145) exposed to 50 ppm for 5 wks. Metastasis study fish were maintained in grow-out aquaria for as long as possible and were only sampled when either moribund or dead. Sampled fish were fixed in either Bouin's or 10% formalin and routinely paraffin processed. HE sections of whole fish were evaluated for hepatic neoplasms and metastases.

Results: A classification scheme, specific for DEN-exposed medaka, was developed including 7 major classes of FCA and 6 classes of hepatic neoplasms. FCA included basophilic foci (2 subclasses, granular and two tone), eosinophilic foci (3 subclasses, granular, hypertrophic, and clear), clear cell (2 subclasses, small and large), amphophilic foci, hyalin inclusion foci, fatty foci, and mixed cell foci. Neoplasms included: hepatocellular adenoma, hepatocellular carcinoma, cholangioma, cholangiocarcinoma, mixed (hepato-cholangiocellular) carcinoma, and spindle cell tumor (possibly a tumor of Ito cell origin).

In the range finding study, large numbers of FCA were observed in both larval and adult exposed fish, and total numbers of foci were dose responsive. Basophilic, eosinophilic, and clear cell foci were consistently the most commonly observed FCA. Prevalence and average number of basophilic, clear cell, and total foci were significantly higher in mature females when compared to males and immature females. In contrast, males (and to a lesser extent immature females) had higher prevalence and higher average number of eosinophilic foci. Differences with respect to gender were observed in both larval and adult exposures, and were seen with the majority of DEN concentrations. There were also marked differences in number and prevalence of FCA between different larval exposure groups, and between sampling time points. Average number of basophilic foci, among female fish in the first larval exposure group (ML1) exposed to 500 ppm DEN, decreased from 4.28/fish (wk 16) to 0.38/fish (wk 24). In contrast, average number of basophilic foci in the third larval exposure group (ML3) exposed to 500 ppm DEN increased from 0.48/fish (wk 16) to 5.6/fish (wk 24). Significantly, percentage of mature females in ML1 decreased from wk 16 (8.5%) to wk 24 (3.5%), while

increasing dramatically in ML3, going from 0% mature females (wk 16) to 52.5% mature females in wk 24. Decreasing numbers of basophilic foci and decreased percentage of mature females in the ML1 wk 24 sample were correlated with decreasing water temperature, while the opposite trend was observed with ML3. Hepatocellular adenomas were also subclassified according to tinctorial staining characteristics with 80% (51/64) of adenomas being basophilic. Similar to foci, the majority (34/51) of basophilic adenomas were found in mature females while most (8/11) eosinophilic adenomas were from male fish.

A total of 423 fish, with 106 neoplasms, were examined during the metastasis study. There were 61 malignant neoplasms, including 37 hepatocellular carcinomas, 13 cholangiocarcinomas, and 11 mixed carcinomas. Intra- and extra-hepatic invasion (invasion of adjacent hepatic parenchyma as well as extension through the capsule into the intestine, pancreas, pericardial sac, and kidney) were relatively common, but vascular invasion was rare. Metastases were observed with 19 of 61 carcinomas with the transcoelomic route (extension through the capsule, fragmentation and translocation of tumor emboli in the peritoneal cavity) being most common (16/19). Rare metastases to spleen and kidney (presumably via the vascular route) were also seen. The majority of neoplasms in larval exposed metastasis study fish were slow growing (average number of PE days > 288 for hepatocellular adenoma and > 307 days for hepatocellular carcinoma). In adults exposed to DEN for 5 wks, no hepatocellular adenomas were observed and time to tumor was much faster, with average number of PE days < 60 for hepatocellular carcinoma and <100 for mixed carcinoma. There were a higher number of mixed carcinomas present in adult exposed fish (compared to larval exposed fish), and adult exposed fish had a unique early (average number of PE days < 15), small (< 500 microns) lesion "nodular proliferation" which was distinct from FCA. Nodular proliferation was characterized by megalocytosis, distinct margins (not continuous with adjacent parenchyma), and marked loss of tubular architecture.

Discussion: Evaluation of results from both studies indicates that carcinogenesis in medaka liver, following brief (1-2 d) DEN exposure, is similar to rodent models of hepatocarcinogenesis, with FCA progressing in step-wise fashion to adenoma and carcinoma (which is consistent with the dedifferentiation theory of cancer). Basophilic foci and adenomas were strongly correlated with sexual maturation in female medaka and presumed increased levels of endogenous estrogens. Endogenous estrogen levels were thought to be influenced strongly by water temperature. [J. Cooke, another member of Dr. Hinton's laboratory, has shown that exogenous estrogen in the diet will preferentially promote basophilic foci in male and female medaka exposed to DEN.] Conversely, eosinophilic foci and adenomas were associated with male fish. The metastasis study demonstrated that hepatic neoplasms in medaka do have malignant potential and can invade and metastasize. The presence of mixed (hepato-biliary) carcinomas and early "nodular proliferation" (possibly "microcarcinomas") may also indicate that bipolar stem cells are target cells for carcinogenic initiation (consistent with the stem cell theory of cancer). [Other studies conducted by Dr. Okihiro using partial hepatectomy and bile duct ligation in medaka and rainbow trout have shown that a bipolar stem cell probably exists in fish liver and that that cell is the bile preductular epithelial cell (BPDEC), which may be the equivalent of the "oval cell" in mammalian liver.]

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Annex 5.1 a

Techniques for the preparation of tissue for histopathology and electron-microscopy: advantages, disadvantages and limitations

David Bucke and Stephen Feist

Introduction

The fish liver has long been recognised as the best target organ for demonstrating histopathological changes resulting from exposure to marine pollutants (Myers et al, 1991). It might be argued that the more recently introduced technologies, including the use of DNA adducts and molecular probes, are more specific, and have superseded traditional histology technique. However, despite the high profile of modern technology, histology is still an essential pathological science, whether it be for field monitoring or controlled experimental research (Bucke, 1994). With even the most basic histologic approach, lesions indicating the position, type and extent of tissue damage can be observed. Furthermore, subcellular changes can be identified with the aid of the electron microscope. Therefore, because of the fundamental benefits of histology, biochemists, immunologists and histologists have adapted and merged their methodologies to go a long way towards specificity of interpreting in situ tissue damage.

This paper deals with the techniques for routine paraffin-wax or plastic embedding procedures for light microscopy, and methods for the preparation of materials for electron microscopy.

Selection of Target Species, Sampling and Post-mortem techniques

Standardised methodologies for the identification and reporting of common diseases of fish in the North Atlantic were detailed in a training guide prepared for ICES (Bucke et al, 1996). This guide was the result of experience gained from 2 ICES sea-going workshops on the Methodology of Fish Disease Surveys (Dethlefsen et al, 1986; ICES, 1989).

Macroscopic examination

For liver, the organ should first be examined in situ for size, colour and any gross lesions (see ICES Training Guide). This detailed examination of the dissected liver, should be made before the organ is sliced for placing in the chosen fixatives, or other preservation procedures.

Histologic technique (See Bucke, 1989; Bucke, 1994 for more details)

There has always been considerable mystic about histology, possibly because the results were mostly empirical, unlike biochemical reactions on tissue samples. As long as the final preparation is of a quality such that a diagnosis can be made by the pathologist, and likewise, when a second opinion is required, other pathologists can see the same morphological structure under the microscope.

The advice to prospective histologists is to experiment with different fixatives, tissue processing and staining methods. Histology is best learned by experience. Therefore, the following techniques are guidelines.

Fixation and fixatives

Fixation must be carried out as soon after death of the fish as possible. If not, autolysis followed by putrefaction will occur, and histopathological features will not be recognised.

Different fixatives have advantages and disadvantages. For this paper, only 2 fixatives will be considered (10% neutral buffered formalin and Bouin's Solution).

10% neutral-buffered formalin is widely used, and providing the slice of liver is no more than 5-6mm thickness, there is at least 20 times fixative to tissue, and the fixation time is not less than 24 hours, then good fixation should be accomplished. The container for the fixed samples should be of plastic, have wide mouth with a well fitting screw-cap. The fixed liver can remain in the fixative for many years. Most staining methods can be used with this fixative.

Disadvantages: fixation is slow, there is a certain amount of shrinkage, and there are harmful vapours from formaldehyde.

All work should be done in a fume-cupboard, and as a general rule, rubber gloves should worn when working with fixed tissues.

Bouin's solution has the advantage that fixation is rapid, there is very little distortion and shrinkage, and tissue staining is intense.

Disadvantages: poor penetration properties, therefore, only small (3-4mm thickness) pieces of liver should be used; the Feulgen reaction for DNA is inhibited; frozen sections cannot be obtained; tissues must be changed from the Bouin's solution after 24 hours and placed in 70% alcohol, where they can be stored until required for further processing.

Processing livers for embedding

Traditionally, tissues are dehydrated, cleared and embedded in paraffin-wax for final blocking out as wax blocks. Nowadays, automatic tissue processors of one form or another are used for this purpose. Individual institutes will have their own preferences for clearing solutions and types of paraffin-wax. Sections can be cut at 4-5 microns from paraffin-wax blocks.

Increasingly plastic embedding media are used for making the blocks. These have the advantage that thinner (1-2 micron), and better detailed sections can be obtained.

Disadvantages: a special microtome knife may be needed to section the hard plastic.

Section-cutting

It should be fairly easy to obtain good sections from livers embedded in wax or plastic. However, some practice is necessary in order to eliminate artefacts

Staining

The haematoxylin and eosin (H&E) method to demonstrate general tissue morphology is the first technique to apply to liver sections. The actual protocol will vary between institutes. Some pathologists combine the PAS method with the H&E.

Frozen tissues for histochemistry

The objective of taking frozen tissues is to retain soluble materials such as enzymes or certain lipids in situ. The exact location of enzymes, lipids or antigens can be visualised at cellular and sub-cellular levels when treated with specific histochemical reagents or labelled antibodies. Histochemistry, further provides a useful tool for the quantification of data. This is because many of the staining reactions produce highly contrasting colours, and with the aid of an image analysis system, images in contrast can be quantified to provide results which can be statistically analysed. In the past, such analyses depended on direct judgement of the pathologist.

The best way of freezing tissues without losing soluble substances from cells is by "snap freezing". It is vital to freeze tissues rapidly, if the process is too slow, ice crystals form in the tissue, greatly distorting cellular morphology.

Liquid nitrogen is used for low temperature freezing (-198°C). The tissue cannot be directly placed in liquid nitrogen, because on contact with a warm object it boils and a layer of gas forms, this insulates the object causing slow and uneven freezing. Standard techniques avoid this by using a quenching solution such as hexane. These are volatile solutions with a high freezing point that provide a buffer between the nitrogen and tissue. The hexane is cooled in the nitrogen until it starts to go slushy, the tissue is then placed into the slush where it is rapidly frozen. Alternatively, we have had good results by sealing tissues in small plastic vials which are placed direct into the nitrogen.

The recommended technique is as follows:-

a) Slice the tissue into blocks no more than 5 mm thick;
b) Place the tissue blocks in the plastic vial and seal the cap;
c) Slowly immerse the vial in the liquid nitrogen and leave it for 5 minutes
d) Store the vial at -80°C or at a lower temperature.

Thin sections of frozen tissues are cut in a cryostat, in which the temperature can be adjusted to suit the particular tissue or histochemical method. (See van Noorden & Frederiks, 1992)

Procedures for Electron-microscopy

For electron microscopy, prompt fixation is even more essential, since degenerative changes occur at the sub-cellular level within a few minutes after the cessation of the blood supply. A variety of fixatives and buffers can be used, with gluteraldehyde being, perhaps, the commonest. Recommended for routine use is a 2.5 % solution of gluteraldehyde in 0.1M sodium cacodylate buffer (pH 7.1), as a primary fixative. Details on numerous other fixatives for electron microscopy can be found in Glauert (1975). Since the penetration of gluteraldehyde into tissues is relatively slow when compared to formalin, it is important to take only small pieces of tissue, no more than 1 mm thick. The fixed tissues will then need to be further trimmed to 1 mm^3 for the next procedure.

Special care has to be taken in the removal and dissection of samples in order to avoid undue contamination with blood or other non-target tissues. Trimming of the tissues must be on a clean surface. Glass, ceramic tile or a sheet of dental wax can be used, but cork or wood are potential sources of contamination, difficult to clean and not recommended. To ensure prompt fixation, trimming can be undertaken in a few drops of cold fixative. Samples are then transferred to glass vials with fresh fixative and kept cool at +4°C. Primary fixation should be complete within 1-2 hours, depending on the size and number of samples. Tissues can be stored in fixative for a few days without undue detrimental effects, but ideally, samples should be processed without delay. The primary fixative is removed and the samples thoroughly washed in 2 or 3 changes of the buffer solution in order to remove any trace of gluteraldehyde. Secondary fixation using 1% osmium tetroxide (in the same buffer) is used to completely fix tissue components, e.g. lipids which are not adequately fixed by gluteraldehyde alone. Because it is highly toxic, special care in the preparation and use of osmium tetroxide must be taken, and all procedures should be carried out in a fume cupboard. Fixation will be complete after approximately one hour, by which time the samples will have reacted with the osmium and turned black. The fixative should then be removed and the tissues, as before, washed with 2-3 changes of the buffer. The samples can then be dehydrated in a graduated ascending series of alcohols and processed to resin blocks using standard procedures (Glauert 1975; Bancroft & Stevens 1977).

Microscopy

It is necessary to have access to good optical microscopes. At least one microscope should be dedicated as a research tool, and should be fitted with a range of objectives (x 4 to x 100) for bright field illumination. This microscope should have phase and ultra-violet light facilities attached, plus an automatic 35 mm2 camera system.

Before examining a slide under the microscope, always check by eye the reference number, the staining technique and the orientation of the tissue on the slide. Make sure the coverslip is on firm, and there are no air-bubbles trapped between the coverslip and slide. Check the section for preservation artefacts and staining condition. When all these points have been checked, start examining the section for changes, first at low magnification, and subsequently at the highest dry objective magnification. Only use the oil immersion objective when all examinations under lower magnifications have been completed.

When taking colour photographs of microscope images, make sure that a daylight (blue) filter is used. For black and white photographs, it is recommended to use a green filter for contrast enhancement.

Summary - examples of advantages, disadvantages and limitations

Advantages

1. Histology allows in situ changes to be observed and characterised.

2. Increasingly, specific immunocytological markers can be applied to histological samples, including paraffin embedded material.

3. Archive material can frequently be examined for comparative purposes or using newly developed techniques.

Disadvantages and limitations

1. Procedures are not all fully automated and several require high levels of practical skills.

2. Interpretation is often left to the individual pathologist. Opinions may vary.

3. Sections can only ever present a proportion of the total pathology that may be present (with exceptions).

4. Pathological responses cannot be attributed to specific contaminants.

References

Bancroft, J.D. & Stevens, A. (1977) Theory and practice of histological techniques. Churchill Livingstone, Edinburgh, London, New York. 436 pp.

Bucke, D. (1989) Histology. In "Methods for the microbiological examination of fish and shellfish". Eds. B. Austin and D.A. Austin, Ellis Horwood Ltd. Chichester, UK. Ch.5, 69-97.

Bucke, D. (1994) Methodologies for demonstrating pathological changes in flounder (Platichthys flesus(L.)). The Baltic Marine Biologists Publication No. 15, 131-143.

Bucke, D., Vethaak, D., Lang, T.& Mellergaard, S. (1996) Common diseases and parasites of fish in the North Atlantic: Training guide for identification. ICES Techniques in Marine Environmental Sciences No. 19, 27 pp.

Dethlesen, V., Egidius, E. and McVicar, A. (eds.) (1986) Methodology of fish disease surveys; Report of a Sea-going Workshop held on RV "Anton Dohrn" 3-12 January 1984. ICES Cooperative Research Report, No. 140. 33pp.

Glaurt, A.M. (1975) Fixation, Dehydration and Embedding of Biological Specimens. North Holland Publishing Co. Amsterdam. 207 pp.

ICES. (1989) Methodology of Fish Disease Surveys; Report of an ICES Sea-going Workshop held on U/F "Argos" 16-23 April 1988. ICES Cooperative Research Report, No. 166. 43 pp.

Murchelano, R.A. & Wolke, R.E. (1991) Neoplasms and Nonneoplastic Liver Lesions in Winter Flounder, *Pseudopleuronectes americanus*, from Boston Harbor, Massachusetts. Environmental Health Perspectives 90,17-26.

Myers, M.S., Landahl, J.T. Krahn, M.M, and McCain, B.B. (1991) Relationships between Hepatic Neoplasms and Related Lesions and Exposure to Toxic Chemicals in Marine Fish from the U.S. West Coast. Environmental Health Perspectives. 90, 7-15.

Noorden, van C.J.F. and Frederiks W.M. (1992) Enzyme Histochemistry: A laboratory manual of current methods. Oxford Science Publications, Oxford, UK, 116 pp.

ICES Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants

Annex 5.1 b

GENERAL GUIDELINES, VARIABLES, METHODS TO CONSIDER FOR FIELD STUDIES USING HISTOPATHOLOGICAL LESIONS IN NATIVE FISH AS BIOMARKERS OF EXPOSURE AND EFFECTS OF ENVIRONMENTAL CONTAMINANTS

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A. Target species selection: Select preferably bottom-dwelling, bottom-feeding species in frequent or continuous contact with bottom sediments, where contaminant levels are usually highest due to adsorption to particulates. Species should have a broad geographic distribution among sites to be sampled. Prior information on susceptibility to contaminant exposure in pilot field or lab studies is preferable. Species should not be highly migratory, should reside most of life in area of capture. The highly territorial mummichog (*Fundulus heteroclitus*) is perhaps the best sentinel fish species around (home range of 30-40m). Significant migration between contaminated and uncontaminated sites can seriously confound interpretation of lesion prevalence data, especially in the absence of chemical markers of chronic exposure (e.g., hepatic PCBs). Problems of migration of the target species in interpretation of the lesion data can be minimized by the concurrent collection and analysis of liver tissue for bioaccumulated compounds such as PCBs and DDTs, and xenobiotic-DNA adducts which act as dosimeters of exposure to genotoxic compounds such as PAHs, and are reliable indicators of chronic exposure to these compounds. Moreover, DNA adducts have been recently shown to be significant risk factors for lesions occurring early in the histogenesis of neoplasia in English sole, especially hepatocellular nuclear pleomorphism, megalocytic hepatosis, and putatively preneoplastic foci of cellular alteration.

B. Fish age and sex: Try to sample a range of ages, and similar sex ratios among the sites, or at least be able to adjust for the effects of age and sex on lesion probabilities. This is especially important for lesions (i.e. liver neoplasms and preneoplastic focal lesions) that have a higher probability of occurrence in older animals. If it is not possible to collect a homogeneous age distribution among the sites, will need to present lesion data by age class or age-adjust the data by statistical methods such as stepwise logistic regression. Always collect anatomical structures such as otoliths, interopercular bones, or scales from each specimen examined histologically, so age can be individually determined. Fish length is generally not suitable as an estimate of age because of the wide variability in growth rates, especially if the geographic area of the survey is very large. The sex and state of sexual maturation should be noted for each fish, as these can influence the histologic appearance of the liver, and may influence the probability of disease prevalence. Lesion prevalence data should also be adjusted for potential effects of sex; a good example of this is in the European flounder, where hepatic neoplasms are more prevalent in females.

C. Sample size: This is a very important factor in any histopathological survey, and required sample size will vary somewhat according to several factors, and according to the objectives of the study. If one is simply interested in determining if a pathological condition is present within a population, one must consider population size, prevalence of the condition in the population and level of desired statistical confidence. Generally, the higher the prevalence of a condition in a population, the lower the required sample size needed to detect this condition. Tables are available in statistical texts and in specific guidelines for this type of study (Tetra Tech, Inc., 1987) to approximate the required sample size. Notwithstanding these guidelines, certain parameters will guide the researcher in selecting the proper sample size will have to be established by conducting smaller pilot studies (e.g. to determine approximate prevalence of a condition, such as hepatic neoplasms or other toxicopathic lesions). The requirement of conducting pilot studies prior to commitment of large amounts of money and manpower cannot be overstressed for these types of studies.

If one is interested in statistically determining whether the prevalence of a condition at multiple test (polluted) sites differs significantly from that in a reference (unpolluted site), the parameters for selection of sample size are somewhat different. Typically, the statistical method for making this comparison of prevalences (expressed as % affected) is the chi-square test, or a modification thereof, the G-statistic. Generally, the smaller the detectable difference in prevalence between two sites, the larger the required sample size needed to statistically verify this difference. For example, if the prevalence of hepatic neoplasms at the reference site is 0%, and the prevalence at a test site is 25%, fewer samples will be needed to verify this difference than if the difference between the sites were, say, only 5%. Again, statistical tables are available to make this sample size determination (Tetra Tech, Inc., 1987) based on a predetermined statistical power and confidence level. Generally, the minimum detectable prevalence at a test site decreases with increasing sample size. However, in our experience, if one can locate reference sites that have a demonstrable prevalence for a condition that approaches 0%, a general guideline of 30-60 specimens per site will probably provide the researcher with the necessary statistical power to detect differences in lesion prevalences among the study sites, provided the prevalence of the same condition in a test area is high enough (10-20%). The issue of required sample size for this type of study can be very problematic, and the required sample will vary quite broadly, depending on the goals of the study and the results obtained. In the final analysis there is no absolutely recommended optimal sample size, and considerable fine-tuning of this factor will have to be done for any particular study.

D. Concurrent collection of sediments, fish tissues, for chemical analyses, other biomarkers of contaminant effects: documentation of potential and actual exposure, biochemical response to exposure: To document potential and actual exposure to contaminants, we recommend, at a minimum, collection of sediments for analysis of PAHs, chlorinated hydrocarbons (CHs), and trace metals. To document actual exposure, liver tissue should be collected from individuals for analysis of CHs, stomach contents for broad spectrum analysis, and bile for measurement of PAH metabolites as fluorescent aromatic compounds (FACs). If necessary because of tissue weight limitations in particular assays, or because of financial constraints, tissue samples from individuals can be composited later in the laboratory. Collection of liver tissue from individual fish for CYP1A quantitation (as AHH or EROD activity) and xenobiotic-DNA adducts adds immeasurably to the investigators' ability to link exposure to biochemical response and thence to histopathological responses, or other morphological measures such as lysosomal stability, in individual fish.

E. Sampling season, seasonal variation: Not much information is available regarding the seasonal effects on hepatic lesion prevalence in other species, but we do know in Puget Sound English sole, that no seasonal differences are seen in the prevalences of the important toxicopathic lesion types, including megalocytic hepatosis, foci of cellular alteration or hepatic neoplasms. However, seasonal migrations such as occur during spawning can dramatically affect the distribution of diseased fish within a geographical region, and therefore large-scale sampling should not be conducted during the actual spawning season for any species, for this and a number of other obvious ecological reasons. It is recommended that sampling for all sites be done within the same season, when the species is on its primary resident feeding grounds and not during periods of annual spawning migration.

F. Location of sampling sites and characterization:
When sediment contaminant levels are already known, one should select sampling sites along a gradient of chemical contamination in order to make the case more convincing when one is testing the hypothesis of showing higher lesion prevalences in more contaminated areas. Included within this selection scheme should be, obviously, severely polluted sites (if present), and a relatively uncontaminated or "reference" site. In practice, the former is relatively easy these days to locate, whereas the latter is more difficult to find. In a preliminary pilot study, one could design a "worst case" scenario in which the chosen test site was severely contaminated, with a relatively pristine control site used for comparison of the chosen endpoints. If the hypothesis held up in this pilot study, then for the definitive study one would choose multiple sampling sites representing a broad gradient of contaminant exposure.

G. Method of fish capture: A technique that minimizes stress on the fish, and that does not select, in a biased fashion, for either diseased or healthy fish should be utilized. In our experience, capture by otter trawl with 5-10 minute tows is an optimal method. In no case should a capture technique be used that kills the fish prior to necropsy, such as gill netting, etc. In cases where a broad size range of fish is to be sampled, proper random subsampling techniques should be employed. If fish cannot be necropsied immediately, they must be held alive in a flow-through sea/freshwater system. If at all possible, dead fish should never be included for collection of histopathological specimens or those for enzyme, lysosomal stability, or DNA adduct analyses.

II. Tissue sampling and collection of biological data:
Before necropsy, each fish should be weighed (g) and measured (mm), examined for external abnormalities, and killed by severing the spinal column. The abdominal cavity is then opened, the liver excised whole (taking care not to puncture the gall bladder that contains proteolytic enzymes) and if possible, weighed. Accurate weighing of samples such as liver or gonad is not always possible to do in the conditions often encountered at sea. The liver should be described as to gross features (nodules, color, texture, etc.). If visible anomalies are present, a section through the entire depth of each affected area including adjacent normal-appearing tissue should be collected and properly identified so that the gross features of an anomaly can later be related to the microscopic diagnoses. In the absence of visible anomalies, a section from the central portion of the liver along the longitudinal axis to include the anterior and posterior regions of the liver in its entire depth should be routinely and consistently collected. To ensure proper fixation and avoid tissue autolysis, each tissue section should not exceed 3mm in thickness, and all tissues should be collected within 5 minutes of the death of the fish.

I. Tissue fixation and processing: Tissues should be fixed immediately after excision from the freshly dead fish (<5 minutes after sacrifice) in a volume $\geq$20 times the volume of the tissue collected. We collect specimens in tissue cassettes that are then placed into 2 liter jugs of fixative, 30 cassettes per jug. Fixation time should be at least 48 hr., with storage of tissues preferably in a coldroom, but never at above 20^0 C.

The choice of fixative is often based on the researcher's preference, but as long as the proper procedures are followed for each type of fixative, any of the following fixatives should be adequate: 10% neutral-buffered formalin, Dietrich's, Carnoy's, Zenker's, Helly's, Davidson's, Bouin's. We prefer Dietrich's for our field studies because of its inherent decalcifying properties (acetic acid), ease of handling and good nuclear fixation. Probably the best fixative for fish tissue is Bouin's, but it is not preferred for field studies where the picric acid cannot be rinsed out in a timely fashion, and where rough seas can make an unhealthy mess of things, quickly. For our lab studies, especially those involving use of small fish species such as medaka, Bouin's is the preferred and superior fixative.

Because relatively large sections of liver tissue are collected from each fish in our field studies, fixation and subsequent embedment in glycol methacrylate medium is generally impractical due to the inherent size limitation for tissue blocks. However, for small fish species in lab studies where full longitudinal sections are taken, glycol methacrylate embedment is the preferred method. For specimens collected in our field studies, we embed in Paraplast-extra with an automated Shandon tissue processor using tissue cassettes, followed by vacuum infiltration (15-20 minutes).

J. Sectioning and staining: For the most part, we use very routine, standard histological techniques in preparing slides for microscopic diagnosis. Sections are cut at 4-5 microns so that the full area of a sampled organ can be viewed (usually liver), and typically one or two sections are examined. If interesting lesions are present, serial sections are sometimes called for, but most of our diagnostic data comes from examination of a single, large section of liver. This is really a matter of pragmatics, because of the large number of specimens collected and examined annually (up to 10,000). Sections are routinely stained with Gill's hematoxylin and eosin-phloxine (similar to the AFIP method), with good results in terms of tissue definition and contrast. Special stains which are commonly used in our lab include the Prussian blue reaction for hemosiderin (used very effectively to demonstrate resistance to iron uptake in hepatic foci of cellular alteration and neoplasms); periodic acid-Schiff (for glomerular basal lamina evaluations, hepatocellular glycogen content estimations, and mycotic infections); Masson's trichrome (connective tissue components); Feulgen methods for DNA; Brown and Brenn's Gram stain for bacteria; Gomorri's methods for methenamine silver (mycotic infections) and reticulum fibers; and May-Grunwald Giemsa (for general identification of protozoan infections and infestations).

K. Tissue examination, diagnosis and quality control:
Slides should be read by an experienced fish histopathologist, with formal training in human, veterinary and/or comparative pathology, and preferably one who is familiar with the species being examined. To minimize bias in interpretation of the histology slides, we recommend use of a "blind" examination in which the histopathologist is not aware of the station of capture of the fish being examined. We accomplish this by using a "pathology" number on the slide label generated from a random number table (e.g., P3073) matched with the actual specimen number (e.g. 95-2502), the latter of which is only revealed to the examiner once examination of all slides for that particular

project is completed. Whenever possible, a standardized, concise, and consistent terminology for lesion description should be followed (e.g. Myers et al., 1987 for description of toxicopathic liver lesions in English sole). There is no substitute for experience in being able to recognize the normal histologic variation for a given species, and the intrinsic factors of sex, age, reproductive and nutritional status in establishing that range of normality. In our experience, this range of normality can be quite broad in a given species sampled from the natural environment. Our lab usually has 3-4 slide examiners working on a particular project at one time. Each slide reader in our lab must complete a training period of 3-9 months under the teaching and supervision of the chief histopathologist before the diagnostic data from that slide reader begins to be incorporated into the data base for any project. Such a training period helps to insure the consistency and accuracy of our diagnostic data. All unusual lesions are reserved by each slide reader for consultation with and confirmation by the chief histopathologist, and we have slide conferences, as necessary, to review these cases. Only when the chief histopathologist is confident of the slide reader's ability, accuracy and attention to detail does the diagnostic data from that person's examinations go into our data base for analysis.

For documentation of histopathologic diagnoses, we utilize a coding system for our histopathology data developed by our laboratory which was modeled after the old Systematized Nomenclature of Pathology (SNOP) coding system. This system permits the recording and computer entry of any lesion, whether infectious or idiopathic, in any organ; provides a standardized nomenclature for pathologic diagnosis; and permits assessment of distribution and severity of any lesion type encountered, including host response to infectious agents. With this system, which has been adopted for the National Benthic Surveillance Program, histopathologic data from multiple researchers throughout the country can be made relatively consistent and analyzed with confidence.

L. Statistical analyses of biological, chemical, biomarker (CYP1A, DNA adducts, bile FACs), and histopathology data: We recommend use of a statistical package that is capable of performing multivariate analyses such as stepwise logistic regression to identify significant relationships between potential biological and chemical risk factors and lesion occurrence. This epidemiological method is commonly used on binomial (e.g., presence/absence of lesion) or proportional (e.g., lesion prevalence) data to examine the influence of multiple risk factors on the probability of disease occurrence as well as exposure-reponse relationships , and most importantly, allows for the simultaneous adjustment for biological risk factors (e.g., fish age, sex) included in the regression by iterative model fitting. Using this method, one can calculate the odds ratio as an estimate of relative risk for lesions in individual fish in relation to the variables of site, sex, age, as well as biomarker data and chemical data measured in individual fish, in a stepwise fashion. One can also estimate relative risk of lesion occurrence as compared to reference or control sites. The most common application of this method to our data has been to determine the significance of the relationships between lesion prevalence at sampling sites to discrete risk factors, such as levels of contaminants in sediments and fish tissues, while simultaneously adjusting for mean fish age and sex ratio. We typically perform separate analyses for each contaminant class or risk factor (e.g., HMW PAHs, LMW PAHs, PCBs, DDTs, etc.), with results expressed as the proportion of variation in lesion prevalence that can be attributed to significant risk factors. A major caveat of this approach that limits the conclusiveness and resolution of the findings, but for which we have not found a satisfactory solution, is that because many of the chemical classes measured in sediments and tissues are significantly intercorrelated and covariant, it is not mathematically possible to include all chemical risk factors into a single multivariate analysis, and it has generally not been possible to describe the proportion of variation in lesion prevalence that can be independently attributed to exposure to particular classes of chemicals. For these statistical analyses, our laboratory utilizes the PECAN module (parameter estimation through conditional probability analysis) of the EGRET statistics package (Statistics and Epidemiology Research Corporation, 909 NE 43rd St, Suite 310, Seattle, Washington, USA 98105; tel. 206-632-3014).

M. Quantitation of lesions & other changes by morphometric analysis/image analysis: This approach takes much of the subjectivity out of histopathological analysis, and is assuming increasing importance as a adjunct tool for histopathology, expecially in quantifying alterations in proportions of cells in an organ, cell proliferation indices such as Proliferating Cell Nuclear Antigen (PCNA), differential cellular localization of P450 enzymes and other enzymes, changes in nuclear and cytoplasmic diameters, etc. It is especially useful when changes are subtle, and cannot be clearly discerned by subjective evaluation. Image analysis is very labor intensive and not generally applicable to large biomonitoring studies involving examination of thousands of fish, but better suited to laboratory studies or field studies with low sample sizes. Recent biomonitoring studies in winter flounder partially utilizing image analysis to quantify severity of hydropic vacuolization in the liver by Michael J. Moore (Mar. Pollution Bulletin, 1996, vol. 32(6), 458-470) concluded that " It is unlikely that the substantial labour increase due to the

use of image analysis can be justified in surveys such as this." Public domain software to do image analysis is available (NIH Image); in our lab we use OPTIMAS, from Optimas Corporation, 190 W. Dayton, Suite 103, Edmonds, Washington 98020, tel: 206-775-8000, FAX 206-775-3640. This objective method of quantifying changes in tissues and cells is becoming more important in documenting morphological changes, and investigators should become more familiar in its use and application.

N. Immunohistochemistry: Examples include lectins, other cell surface markers; xenobiotic metabolizing enzymes such as CYP1A, GST; tumor-specific markers; oncogene expression products such as for ras, P53; viruses; cytoplasmic filaments (cytokeratins) for cell identification, tumor type; bromodeoxyuridine (BrdU) incorp., and PCNA localization and quantitation for cell proliferation studies. Any of these more specific methods that give insight into the mechanisms of pathogenesis of a disease can all be used on a selective basis to better characterize lesions detected and in smaller mechanistic studies, but as biomarkers *per se* in large scale biomonitoring programs involving examination of thousands of fish, have limited application or utility; are relatively expensive compared to standard histopathology. For these reasons, our lab has not utilized these methods routinely in large scale biomonitoring studies, but we are open to selective application of certain of these markers.

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Annex 5.2 a

Cytochemistry of the liver of teleost fish species in frozen material - cellular/subcellular biomarkers

The tests for lysosomal perturbations described here are based on techniques transferred to fish by Köhler (1991; 1992) on the basis of techniques developed in invertebrates by Moore (1976). The tests using metabolic changes of the pentose phosphate pathway (G6PDH, PGDH) as markers during the multistep process of hepatocellular carcinogenesis is based on data obtained from a baseline study by Köhler and Van Noorden (submitted to Aquatic Toxicology). The descriptions contain details which appeared to be relevant during the practical application in large scale monitoring programms, like storage and simultaneous processing of a large number of samples.

The use of frozen material offers the opportunity to perform simultaneously in serial sections tests for biotransformation enzymes (NADPH cytochrom c P450 reductase, benzaldehyde dehydrogenase) and lysosomal latency, for metabolic altered cells during carcinogenesis, cell proliferation (PCNA) and other immunocytochemical biomarkers (N-ras, Ki-ras, P53, MDR) and to link pathomorphologial changes to metabolic and mutation changes caused by contaminants.

Tissue preparation

Immediately after sacrifice, the liver is dissected and cut into pieces of approximately 5x5x5 mm of size with a razor blade. The pieces are placed on a aluminium chuck which fits into the cyrostat used later for cutting the serial cryosections for the tests required. In general, liver pieces of 5 individual fishes can be placed on one chuck for simultaneous processing. For detailed investigations of metabolic disorders of tumor bearing livers it is recommended to freeze the nodules seperately on chucks in order to avoid loss of tissue.

The chucks are cooled on crunched ice during the tissue dissection and are then placed upside down for 1 min in hexane (aromatic hydrocarbon-free; boiling range 67-70° C), supercooled in liquid nitrogen. The chucks (plus quenched tissues) are stored on dry ice and later at -70°C (according to our present experience tissues can be used until 5 years of storage without problems).

Sectioning

Serial liver sections of constant thickness of 10 µm are cut with a motorised cryostat (Microm, Bright) with a cabinet temperature between -15 and - 23 °C dependent on the lipid content of the livers with a knife angle of 5°. Each two series of sections of the five liver blocks frozen on the chucks are placed on glass slides which had been kept at room temperature, on which the sections spread effectively. It is recommended to position the sections down at the end of the slides to guarantee that the buffer and incubatuon medium cover all 5 tissue sections. Dependent on the labilisation intervalls 14 slides are prepared. The sections are kept in the cryostat or -20 °C fridge until use, but not longer than 24 hours. When setting up the lysosomal latency test in various labs for monitoring purposes it is useful to agree about the storage period of the sections after sectioning. According to our experience it is useful to cut serial sections of five chucks (=25 individuals) and perform the test the following day.

Lysosomal Destabilisation Test

The test is based on the artificial labilisation of the lysosomal membrane in acid buffer and represents a measure of the membrane function and stability. Decreased lysosomal membrae stability reflects the onset of non-specific degenerative liver lesion and is measured in surrounding liver tissue of foci, adenoma and carcinoma in dab and flounder (Köhler, 1991; Köhler et al., 1992). Lysosomal stability is already recommended for the biological effect monitoring.

The period until the lysosomal membrane is destabilised by the acid treatment so that the substrate(s) can penetrate through the membrane and react with the latent enzyme(s)(hydrolases) is the destabilisation time (intervall).

Serial cryostat sections are subjected to acid labilisation in 0.1M citrate buffer (pH 4.5) at 37 ° C (shaking water bath) in a Hellendahl vial starting with the longest destabilisation period of 45 min eg, followed by the intervalls 40 min, 35 min, 30 min 25 min, 20 min, 15 min, 10 min, 8 min, 6 min, 4 min, 2 min, and 0 min. Following the acid labilisation the buffer is removed very quickly and the substrate incubation medium added. The substrate medium is made up of 20 mg naphthol AS-BI-N-Acetyl-ß-D-glucosamide (Sigma) dissolved in 2.5 ml 2-methoxyethanol at 37°C in a water bath. Shortly before use, the solution is made up to 50 ml with 0.1 M citrate buffer (pH4.5) and 3.5 g of low viscosity polypeptide as section stabiliser which had been thoroughly dissolved in buffer before! The sections are inubated at 37°C for 15 min. Afterwards the sections have to be rinsed properly in 3% NaCl at 37 ° C for several minutes to remove the polypeptide which could react with Violet B and produce artefacts. Sections are then transferred to 50 ml phosphate buffer (pH 7.4) containing 1 mg/ml Fast Violet B (Sigma) at 4°C (fridge) for 10 min. After rinsing for 5 min in tap water , the sections are fixed for 15 min in calcium formal at 4°C, rinsed in destilled water and mounted in aquous Kaiser's gelatine. The test can be performed with various substrates for the different lysosomal hydrolases.

The destabilisation (labilisation) period is determined as the time intervall of acid treatment required to fully permeabilise the lysosomal membrane which is indicated as the maximum of the reaction product in the lysosomes assessed microscopically. Four determinations per liver and the mean value is calculated for each animal. The measurements can be also performed using computerised Image Analysis.

Lysosomal enlargement, often accompanying a decrease of lysosomal membrane stability is determined microscopically as area/ liver unit by image analysis. The occurrence of enlarged heterogeneous lysosomes in fish liver cells under usual physiological conditions (no starvation, no spawning) can be generally considered as pathological and is indicative for increased (auto)digestive processes or inhibition of enzyme activity resulting in accumulation of unsaturated neutral lipid, phospholipids and contaminants (toxic phospholipidosis), as well as injured cell organells.

Detection of enzyme altered foci (G6PDH / PGDH) during carcinogenesis

The test for enzyme altered foci is regarded as sensitive to identify early putatively preneoplastic lesions which appear prior to those detectable in H&E stained wax- or methacrylate- embedded material and can be used optional. Further, a quantification of altered foci, adenomas and carcinomas in tissue areas can be done rapidly.

For the histochemical detection of G6PDH and PGDH activity, the tetrazolium salt methods as described by Van Noorden and Frederiks (1992) were used at a modified incubation temperature of 22°C (instead of 37°C) which is an adaption to the maximal natural environmental temperature of flounder. Before incubation, cryostat sections were adjusted to a temperature of 22°C for at least 10 min and all media and equipment were kept at this temperature during the experiment. The incubation medium for the demonstration of PGDH (EC 1.1.1.44) activity according to Jonges and Van Noorden (49) contained 18 g polyvinyl alcohol (PVA, weight average M_r 70 000-100 000, Sigma) in 100 ml 0.1 M phosphate buffer (pH 8.0) and 5 mM gluconate-6-phosphate (Böhringer, Mannheim, Germany), 0.8 mM NADP (Böhringer), 0.45 mM methoxyphenazine methosulphate (Merck), 5 mM $MgCl_2$, 5 mM sodium azide and 5 mM tetranitro BT (Sigma). The media were freshly prepared just before incubation, exactly as described in detail by Van Noorden and Frederiks (1992). Tetranitro BT was added after being dissolved in a heated mixture of dimethylformamide and ethanol (final dilution of each solvent in the medium was 2 %). The recommended incubation time for PGDH is 5 min. The incubation medium for the demonstration of G6PDH (EC 1.1.1.49) activity was similar to that of PGDH but phosphogluconate was replaced by 5 mM glucose-6-phosphate (Merck) and the pH of the medium was 7.45. The recommended incubation period is 3 min. Control reactions were performed in the absence of substrate and in the presence of coenzyme to be able to substract the conversion of endogenous substrates from the test reactions. Altered enzyme foci can be easily detected by altered formation of the formazan reaction product as well as the growth pattern (trabecular eg) and invasive growth (irregular border of lesion, invasive satelleites of carcinoma).

Image analysis and processing. For more detailed research on enzyme reaction rates the use of image analysis is recommended. A 3-chip CCD colour video camera (Sony, ATV Horn, Aalen, Germany) connected to the Zeiss Axioskop light microscope and coupled via a frame grabber (maximal size 786x512) to an image

analysis system with the KS 300 software package (Kontron, Eching, Germany). Camera signal and set up were adjusted according to the recommendations of Chieco et al. 1994. Tissue sections were viewed in white light with a x 10 objective (NA 0.03), a stabilised power supply, an infrared blocking filter and a monochromatic filter of a wavelength of 585 nm (43).

For standardization and validation, a series of grey filters were used and their images were applied to convert grey values to absorbance values. Absorbance values were converted in absolute units of enzyme activity on the basis of the Lambert-Beer law: $A = \varepsilon\ c\ d$ in which A=absorbance, ε=19.000 (molar extinction coefficient of tetranitro BT formazan), c= concentration of formazan and 1 mole of formazan is produced by 1 mole of substrate (glucose-6-phosphate or phosphogluconate) and d=0.001 cm (section thickness). Cytophotometric end point absorbance measurements were taken after 3 min of incubation in serial sections reacted for G6PDH and after 5 min for PGDH activity using the image analysis system Zeiss/Kontron KS 300 with the above described camera set up. Grey value measurements and conversion to absorbance values were performed with a sequence of functions recorded and executed by a macro in the KS 300 software.

Detection of storage disorders

Unsaturated Neutral Lipids (Triglycerides)

Fix duplicate cryosections for 15 min at 4°C for 15 min in 4% calcium formal. Rinse in destilled water and place in 60% triethylphosphate in distilled water for 3 min. Stain sections in1% solution of Oil Red O in 60 % triethylphosphate at 20° C for 15 min. The sections are then washed in 60% triethylphosphate in distelled water and are then mounted in glycerine gelatine. Lipid content is determined by image analysis with a 530nm filter as area/liver tissue with a minimum of 5 readings/sections. Gradings of lipid content can be made on the basis of photographs supplied by reference labs if an image analysis is not available.

References cytochemistry:

Jonges, G.N. and Van Noorden, C.J.F. 1989 *In situ* kinetic parameters of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase in different areas of the rat liver acinus. Histochem. J., 21, 585-594.

Köhler,A.1991. Lysosomal perturbations in fish liver as indicators for toxic effects of environmental pollution. Comp. Biochem. Physiol., 100,1/2,123-127.

Köhler,A., Deisemann, H. Lauritzen, B., 1992. Histological and cytochemical indices of toxic injury inthe liver of dab *Limanda limanda.* Mar. Ecol. Prog. Ser, 91, 141-153.

Köhler, A.Van Noorden, C.J.F. (subm.) Initial velocities of G6PDH and PGDH *in situ* and the expression of proliferating cell nuclear antigen (PCNA) are sensitive diagnostic markers during environmental hepatocellular carcinogenesis in marine flatfish, Aquatic Toxicology

Moore, M.N., 1976. Cytochemical demonstration of latency of lysosomal hydrolases in digestive cells inthe comon mussel, *Mytilus edulis,* and changes induced by thermal stress. Cell and Tissue Res., 175, 279-287.

Van Noorden, C.J.F., W.M. Frederiks. 1992. Enzyme Histochemistry, Laboratory Manua of current Methods, 26, RMS Microscopy Handbooks, Oxford Science Pulishers. 116 pp.

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Annex 5.2 b

Cytochemistry of the liver of teleost fish species in paraffin embedded sections

Immunocytochemical protocol for Paraffin sections using monoclonal antibody PCNA

I. **Paraffin sections cut at 4-5 microns mounted on Biotech Probe On Plus charged slides and air dried overnight.**

II. **Deparaffinize and rinse in Automation Buffer (Biomedia M30) for 5 min.**

A. 100ml *Automation Buffer* to 900 ml Deionized or distilled water. Make fresh at each use.

OR

B. *Automation buffer equivalent* (10X)
72.0g sodium chloride + 4.3g potassium phosphate monobasic + 1 liter dd H_2O, dissolve salts, then add 14.8g sodium phosphate, dibasic + 5 ml Tween 20
Adjust pH to 7.7 with NaOH. Store at room temprature
working solution: 100ml 10X + 900ml dd H_2O.

OR

C. *.01M PBS, 0.9% NaCl or Sigma PBS tablets* pH 7.6
- 1.42 g Sodium Phosphate, monobasic (Na_2HPO_4)+
  1.61 g Sodium Phosphate, Dibasic +($NaH_2PO_4 \cdot H_2O$)
  9 g Sodium Chloride (NaCl) + 1000ML ddH_2O

III. **Quench endogenous peroxidase with 3% H_2O_2 for 5 min.**

A. 20 ml. 30 % H_2O_2 in 180 ml De ionized water. (1/10 dilution)
This is a 1:10 dilution where $\frac{1.0 \text{ ml } H_2O_2}{9.0 \text{ ml dd } H_2O} = \frac{20 \text{ ml } H_2O_2}{180 \text{ ml dd } H_2O}$

B. Shake excess solution from slide

Note: If endogenous peroxidase activity needs further inhibiting or erythrocyte and macrophage is causing a non-specific reaction add a 0.1% sodium azide solution to the 3% H_2O_2 solution (Allen Warbritton, Nat'l Center for Toxicological Research, and DAKO Corp, Handbook of Immunochemical Staining methods, pg22, 1989). If endogenous peroxidase activity is still a problem try a methanolic H_2O_2 treatment
(1 part 3% H_2O_2 plus 4 parts methanol) or It may be advantageous to switch staining methods from streptavidin or ABC to Peroxidase-antiperoxidase (PAP- Enzyme anti-enzyme).

IV. **Gently water wash slides in distilled water for 5 min., then wash in automation buffer for 5 min.**

V. **Antigen Retrieval Solution**

A. For each plastic coplin jar: 20 mls antigen retrieval solution (Biogenex HK090-5K) to 60 mls dd H_2O (1: 3)
B. Microwave at full power until solution boils for 2 min.
C. Rest for 1 min. and replace evaporated fluid with dd H_2O
D. Continue heating till solution boils for 2 additional min.
E. Rest for 15 min.

Note: Antigen Retrieval Solution is used to unmask immunoreactive sites in tissues that are overfixed in formalin fixatives. This step may not be necessary if the tissues were fixed for a period less than 48 hrs. However, certain fish species and invertebrates staining quality may benefit from use of this solution regardless of them being over fixed.
Biogenex also has a new antigen retrievial solution called Citra that does not require boiling and is less toxic. antigen retrieval solution equivalant (Allen Warbritton): 1% anhydrous zinc sulfate in PBS (10X - Tween 20). This solution eliminates the lead disposal problem.
Biogenex has a new product called Citra, based on a sodium citrate buffer, which uses a gentle heating regimen.

PCNA Protocol Pg. 2

Antigen Retreival con't

From L. Ortego et al.

A. Use two containers and remove metal handels from the slide racks. Pour 250 mls of antigen retrieval solution into ech container and cover. Place the containers into another shallow container with 1/2 inch water. Using a 700 watt microwave, boil for two minutes. Rest one minute and check the level of solution and restore levels with (may ned to check levels after one minute. Boil for 2 minutes , rest for one minute, boil once more for 2 minutes then rest for 15 minutes.

NOTE: If you have less than twenty five slides, still use two containers, but place water in the 250ml vessel that does not contain slides.

B. *1M citrate buffer*
Prepare fresh.
63.75 g citric acid monohydrate in ddH_2O. After dissolution, add 58.75 g trisodium citrate dihydrate. Dilute up to 500ml.

VI. **Rinse in two changes of dd H_2O and then automation buffer for 5 min.**

VII. **Use pap pen to encircle sections on the slide.**

VIII. **Block nonspecific immunoglobulins binding for 20 min.**

A. 1% Bovine Serum Albumin (BSA)
- 0.1 gram BSA + 10 ml. dd H_2O

B. 1% non-fat Dry milk
- 0.1 gram milk + 10 ml. dd H_2O

C. Prior to use mix equal volums of BSA/milk then drop onto tissue sections.

D. After incubation, shake excess blocking solution from the slide but, DO NOT RINSE

VIV. **PCNA clone PC-10 and Appropriate controls**

A. 1:100 1° Mab in Automation Buffer 1 hour incubation
- 10µl Mab PCNA PC-10 + 990µl diluent [1]

1 ml(1000µl) of a 1: X required $= \frac{1000}{X}$ = µl stock reagent ; $1000 - \frac{1000}{X}$ = µl diluent

1 ml (1000µl) of a 1:100 $= \frac{1000}{100} = 10\mu l$ 1° Mab ; $1000 - \frac{1000}{100} = 990\mu l$ diluent

Diluent can be one of the following:
Biogenex Common Antibody Diluent (HK156-5K)
Automation buffer
.01M PBS, 0.9% NaCl or Sigma PBS tablets pH 7.6
- 1.42 g Sodium Phosphate, monobasic (Na_2HPO_4)+
  1.61 g Sodium Phosphate, Dibasic +($NaH_2PO_4 \cdot H_2O$)
  9 g Sodium Chloride (NaCl) + 1000ML ddH_2O

NOTE: I have been successful using 1:200 1° dilution
- Appropriate controls

A) **Isotype:** Equal dilution and and protien concentration as PCNA PC10
mouse IgG2a (Sigma M-9144)

Setp 1) Protein conc. 1° Mab g/l
Working dilution 1° Mab 1:X = protein conc. of diluted °1Mab g/l

Step 2) Protein conc. (isotype) IgG fraction g/l
Protien conc. of diluted 1°Mab g/l = dilution of IgG faction to give protien conc. equivalent to that of 1°Mab

B) **Positive:** 1°Mab with dilution equal to specimen treatment
Colon carcinoma
Tonsil
Esophagus
Gill

PCNA Protocol Pg 3

C) **Negative:**

A) 1° Mab with dilution equal to specimen treatment
Normal liver
Brain
Heart OR
B) Dako tissue culture supernatant of fetal calf serum (ordered from Gemini Bioproducts) at ther same dilution as 1° Mab.
Use an "intermediately" lesioned section of liver.

X. **Secondary biotinylated antibody for 30 min**
2° ab horse anti mouse
Dilute in PBS 1:20

- $\frac{1\mu l\ 2°\ ab}{20\mu l\ PBS} = \frac{X}{1000\mu l}$ = 50 µl 2°ab + 950 µl PBS

- Do not use any diluent that contains sodium azide as it will inhibit the the biotynaltion

XI. **Peroxidase-conjugated streptavidin for 30 min.**
Dilute in PBS 1:20

- $\frac{1\mu l\ Link}{20\mu l\ PBS} = \frac{X}{1000\mu l}$ = 50 µl 2°ab + 950 µl PBS

NOTE: Step X and XI use a kit from Biogenex: StrAviGen super sensitive (mouse) Cat# ZPOOO-UM

XII. **DAB chromagen for 1-6 min.**

10mg DAB / 20 ml tris + 6 µl 30% H_2O_2 / 10 ml DAB

- $\frac{10\ mg}{20ml} = \frac{X}{100} = X = 50$ mg DAB + 100 ml TRIS

TRIS Tablets (Sigma) 1 Tablet TRIS / 15 ml dd H_2O
$\frac{1}{15} = \frac{X}{100} = X = 7$ tablets / 100 ml dd H_2O

- $\frac{6\ \mu l\ 30\%\ H_2O_2}{10\ mls.\ DAB\ sol.} = \frac{X}{100\ mls\ DAB\ sol.} = X = 60$ µl 30% H_2O_2/100mls DAB

XIII. counter stain with harris hematoxylin about 5 sec. Check under scope for desired color.

XIV. Rinse with tap water and blue in PBS for 1 min.

XV. Dehydrate using 2x 95% ehtanol for 30 sec each, followed by 4X 100% ethanol 1 min each, then xylene or xylene substitute 3x 1 min. each. Coverslip.

P450 protocol
rev. 11/93

1993 P450 Protocol

I. **Section paraffin embedded tissues 4-5 microns, mount on coated slides,and air dry overnight.**

II. **Deparaffinize with 3-4changes of Shandon Xylene substitute at 5 min. each and follow with two changes 100% ETOH, 20 dips each and two changes 95% ETOH, 20 dips each.**

III. Remove alcohol
   A. Distilled water wash .
   B. Automation buffer (Biomedia) for 5 min.

IV. **Block endogenous peroxidase for 10 min.**
   A. 1% H_2O_2 in methanol or dd H_2O also works(preferrable if if this were a surface antigen).
      - 6.66 mls 30% H_2O_2+ 193.34 mls absolute methanol (dd H_2O)
   B. 3% H_2O_2 in methanol or dd H_2O.
      - 20mls 30% H_2O_2+ 180 mls methanol or dd H_2O

   Note: I have been using a methanolic 1% H_2O_2 for all runs since 1991

V. **Two 5 min. rinses with Automation buffer.**

VI. **Block with 5% Normal Goat serum for 10-20 min.**
   - 5 mls. PBS + 5 drops (250 µ*ls*) Normal Goat serum
   - Shake excessive NGS from slide prior to application of the primary Ab, but do not wash in buffer

VII. **1° Ab for 2 hrs at room temp or overnight at 4°C**
   - Rabbit-Anti Cod P450 1A1 IgG from Norway is Prediluted to 1:300
   - Working solution of 1:100

     C_1= 4.5 mls PBS* + 0.5 mls 1% BSA = 5 mls 0.1% BSA/PBS
     C_2 = 50 µ*ls* 1° Ab + 4950 µ*ls* 0.1% BSA/PBS (C_1)
     For volume or concentration change $C_1V_1 = C_2V_2$

   *PBS tablets from Sigma: 1 tablet in 200 mls dd H_2O

   - Following 1° incubation, rinse well, two changes at 5 min each in automation buffer or PBS.

VIII. **Controls**
   Substitution: Normal rabbit serum dilute 1:30000
   - C_1: 1:100 = 10µ*ls* NRS + 990 µ*ls* PBS or PBS/BSA Aliquot and store at -20°C
   - C_2 : 10µ*ls* C1 + 3000µ*ls* PBS/BSA

   Isotype:

P450 protocol
rev. 11/93

Negative: Tissue that would not normally stain for P450
Positive: Spleen

VIV. **2° Biotinylated goat Anti-rabbit 1:100 for 30-60 min**

- 3 drops (150 µl*s*)Normal GS + 2 drops (100 µl*s*) 2°Ab + 10 mls PBS
- Rinse well with automation buffer

X. **Avidin-Biotin Complex with HRP for 30-60 min. NOTE: Prepare 30 min before use.**

- 5 mls PBS + 2 drops (100 µl*s*) Reagent A + 2 drops reagent B MIX IMMEDIATLEY.
- Wash well in two changes of automation buffer, 5 min. each.

XI. **DAB chromagen 1-6 min**

- C_1 = 10 mg DAB / 20 ml. Tris
- C_2 = 6µl*s* 30% H_2O_2/ 10 mls DAB

Therefore,

$$\frac{10\text{ mg}}{20\text{ml}} = \frac{X}{100} = X = 50\text{ mg DAB} + 100\text{ ml TRIS}$$

TRIS Tablets (Sigma) 1 Tablet TRIS / 15 ml dd H_2O

$$\frac{1}{15} = \frac{X}{100} = X = 7\text{ tablets / 100 ml dd } H_2O$$

$$\frac{6\text{ µl 30\% } H_2O_2}{10\text{ mls. DAB sol.}} = \frac{X}{100\text{ mls DAB sol.}} = X = 60\text{ µl 30\% } H_2O_2\text{/100mls DAB}$$

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Annex 5.3 a

Biochemical marker techniques (R. Stagg)

These comprise measurements which are made on whole or homogenised tissues and will include:

- protein or enzyme measurements
- DNA damage
- the products of contaminant metabolites
- changes in levels of metabolites

Protein or enzyme measurements can be made by measuring the concentration or activity of the gene product or the activation of the gene and measurement of mRNA by the use of cDNA probes. In the case of measurements of protein level the most specific information is given by the use of antibody probes however other characteristics of the proteins may also be useful. Metallothionein for example can be measured by determination of metal binding (silver saturation assay) or by the determination of the cysteine content using spectrophotometry or pulse polarography or by antibody using RIA or ELISA. Induction is measured when contaminant exposure results in activation of the gene and enhanced expression (e.g. cytochrome P450, metallothionein, glutathione-S-transferase, UDP-glucuronyl transferase, vitellogenin) but reduced activity or expression is also observed when the mechanism of action is enzymic inhibition (e.g. acetylcholinesterase, ATPase, ALA-D).

Biochemical methods for the assessment of DNA damage can be measured by a varicty of techniques which vary in the specificity of the interaction measured. At one level the covalent binding of specific contaminants such as bulky PAH adducts can be measured using ^{32}P post labelling and damage to specific genes can be made by comparing the sequence of wild type and mutated alleles using amplified sequences of specific genes using PCR. Recent attention has focussed on genes involved in the control of the cell cycle such as ras and p53 which have been implicated in tumourogenesis as a consequence of their oncogenic and tumour suppressor activity respectively. On the other hand non-specific damage can be determined by studying the behaviour of DNA molecules using, for example, alkaline unwinding in the COMET assay.

Changes in the levels of metabolites of specific contaminants can indicate the exposure and reaction potential of environmental contaminants. The appearance of hydroxylated PAH derivatives in the bile of fish for instance is an established marker of exposure to these hydrocarbons.

Changes in the levels of blood or tissue metabolites have been used to indicate biological effects. Clinical approaches are also useful. The pigmented salmon syndrome illustrated the use of such an approach in diagnosing the causes of a hyperbilrubin-anaemia in fish exposed to effluents in the river Don. Here levels of plasma enzymes indicative of hepatic damage and the occurrence of metabolic indicators such as bilirubin, haemoglobin and alkaline phosphatase where diagnositic of the condition which was caused by the interactive effects of exposure to resin acids and hydrocarbons in effluents.

Advantages of protein biochemical techniques:

- Tend to be reasonably specific for contaminants and there is a good relationship between exposure and effect and a good understanding of the mchanism of action.

- Sensitivity. Enzyme responses are close to the initial molecular interaction of toxicant with the cell and are expected to yield sensitive response. For example dose response data has shown EROD activity to be more sensitive than adduct and pathological responses.

- Enzymic responses are rapid. Both induction and inhibition responses occur quickly, e.g. P450 induction takes place in 3 -4 days.

- Measurement of catalytic activity tend to be simple and cheap to run. (Some discussion on role of AHH and EROD latter simpler, safe and can be used at sea; the former possibly more representative of metabolism of aromatic hydrocarbons).

- QA is already under development. EROD has been the subject of two intercomparison exercises and in the MEDPOL programme MT measurments have been compared between different labs in the programme.

Disadvantages:

Contaminant interactions. Despite being specific other interactions with a wide range of contaminants do occur. These can be synergistic or inhibitory e.g. the interaction of resin acids (synergy) and TBT (inhibitory) with the measurement of EROD activity.
Biological factors shown to be important are:
Phenotypic and genetic variability
Sex
Sexual/life cycle stage
Niche
Environmental variables
Temperature
Season
Significance

Conclusions

Sampling & survey design
Integrated programmes
Risk analysis
Control sites

PIGMENTED SALMON

Haemolytic anaemia

Non-feeding migratory fish
Elevated plasma haemoglobin
Elevated plasma bilirubin
Increased nos imature RBC's
Induced P4501A
Liver damage
Plasma alkaline phosphatase
GOT

Environmental and biological interactions
Dose responsive.....Y
Time course generally short term depends on response measured mRNA hours, enzyme activity3-5 daysdepending on route
induction faster than reduction

Usually good understanding of cause and effect - mechanistic
Simple assays area available, basics can be done on ship,
Molecular techniques offer scope

Biochemical marker techniques: advantages, disadvantages limitations

Scope

Restrict to :

Enzyme/protein markers
Measured by: catalytic activity,
protein measurement (antibody, spectrophotometry, RT-PCR
mRNA measurements cDNA probe

Metabolites: Bile metabolites of PAH
Metallothioneins
Peroxidation

Genetic damage: Comet assay
^{32}P- postlabelling
Detection of mutations in specific genes

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Annex 5.3 b

An overview on the applicability of DNA adduct detection techniques for biomonitoring purposes

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The formation, structure and presumed biological significance of DNA adducts

The cytochrom P450 (CYP450) enzyme system, responsible for the detoxification of xenobiotics, catalyses the conversion of toxic compounds to more polar and hydrophilic forms that are more easily excreted by the organisms. The CYP450 catalyses the addition of oxygen to many polycyclic aromatic hydrocarbons (PAHs) forming an intermediate with a diol-epoxide structure. This metabolically activated PAH intermediate is chemically a highly reactive electrophil. Instead of being conjugated with stage II detoxification enzymes and excreted, these highly reactive electrophils may react with nucleophils in the cell, i.e. proteins, RNA or DNA, and bind covalently to these forming adducts.

In DNA the base guanine is considered to be most "prone" to adduct forming compounds. Different carcinogens are known to bind to different positions at guanine depending on their chemical properties. The binding of a carcinogen to DNA is not random. Stereochemical and electronic factors determine the binding properties. The genotoxic effect of PAHs depends on conformational changes in the molecules involved upon binding. The stability of DNA adducts formed is affected by factors like chemical instability, DNA repair mechanisms and cell turn over.

DNA adducts, if not repaired or removed prior to mitosis, can induce mutations which may irreversibly initiate altering of the cell to a preneoplastic lesion. DNA adducts have been shown to activate oncogenes and may also affect the expression of tumor suppressor and regulating genes. These early events are considered as critical steps in the induction of malignancy in chemical carcinogenesis. Once formed, the DNA adducts seem to be rather persistent and DNA adducts have therefore been suggested to be suitable as molecular dosimeters of carcinogen exposure.

DNA adducts as biomarkers in fish

In aquatic ecotoxicology, the use of DNA adducts for environmental biomonitoring purposes have by now been quite extensively tested on fish , both wild fish and after laboratory exposure. DNA adducts seem to be formed in almost all species studied. In the majority of studies where the extent of contamination of sediments (especially PAHs and PCBs) is known, it has been possible to establish a relationship between the extent of contamination and the levels of DNA adducts in the liver. TABLE 1.

The evidence for carcinogenic effects of PAHs via the formation of DNA adducts is still circumstantial. However, epizootiological studies on fish species, exhibiting high prevalences of preneoplastic and neoplastic liver lesions in areas polluted with antropogenic substances (PAHs) and exhibiting high hepatic DNA adduct levels as compared with fish from reference sites suggest that DNA adducts might be a valuable biomarker for environmental exposure to anthropogenic contaminants.

The detection and measurement of DNA adducts

Different methods for the detection of DNA adducts have been developed. The applicability and sensitivity varies depending on the method used. In short the different assays developed are based on 1) immunochemistry (RIA, ELISA), 2) fluorescence assays (scanning synchronous fluorescence spectroscopy, SFS) and 3) ^{32}P-postlabeling.

Immunochemistry assays require prior knowledge of the structure of the adducts studied, even though some crossreactivity of antibodies is possible. The limitation of fluorescence assays lies in the lack of quantification. For biomonitoring purposes the ^{32}P-postlabeling technique has recieved much attention since it is chemically unspecific and extremely sensitive. The drawback is that the assay is rather laborious. The ^{32}P-

postlabeling assay is still under development and the quantitative data obtained are often an underestimate of actual levels. Therefore unless an intercalibration has been performed, direct comparison of quantitative data reported by different laboratories should be avoided. Intercalibration studies have been done on the human side, but at the moment, to our knowledge, there are no reports on such intercalibrations from.laboratories working with fish.

For the analysis of DNA adducts using the ^{32}P-postlabeling assay, material should be frozen in liquid nitrogen immediately after killing the fish, the sample can be stored at -70°C. The extracted DNA is enzymatically digested to nucleotides and enriched for DNA adducts either by selective dephosphorylation of normal nucleotides with the enzyme nuclease P1 or by extraction of adducts with butanol. The nuclease P1-version is more common due to simplicity. The DNA adducts are postlabeled at 5' position with γ^{32}P-ATP using a kinase reaction. The hydrophobic character of DNA adducts allows for 2D-separation with thinlayer chromatography. The separated radiolabelled DNA adducts are visualised using screen enhanced autoradiography or the storage phosphor imaging technique. Quantification is performed by liquid scintillation counting or appropriate software for the image analysis. With all steps optimized, the sensitivity of the ^{32}P-postlabeling assay can reach a detection limit of 1 adduct / 10^8 or 10^9 nucleotides. Methods for identifying adducted molecules are presently developed.

References

Beach, A.C. & Gupta, R.C. 1992. Human biomonitoring and the ^{32}P-postlabeling assay. Carcinogenesis vol 13 (7): 1053-1074

Randerath, K., Reddy, M.V. & Gupta, R.C. 1981. ^{32}P-Labeling test for DNA damage. Proc.Natl.Acad. Sci. USA 78(10):6126-6129

Reddy, M.V. & Randerath, K.1986. Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. Carcinogenesis 7(9):1543-1551.

Reichert, W.L. & French, B. 1994. The ^{32}P -postlabeling protocols for assaying levels of hydrophobic DNA adducts in fish. U.S. Dep. Commer., NOAA Tech. memo. NMFS-NWFSC-14, 89 p.

Stein, J.E., Reichert, W.L. & Varanasi, U. 1994. Molecular epizootiology: assessment of exposure to genotoxic compounds in teleosts. Environ. Health Perspect.102(12):19-23

TABLE 1. Field studies on DNA adducts in fish. Highlighted studies have been able to show a relationship between the pollution grade and levels of DNA adducts in fish from a contaminated site as compared with a presumed clean area.

[1] The PAH pollution grade at sites not known.

[2] Another fish species used as control.

[3] By using DNA adducts as biomarker , the study couldn't discriminate between contaminated and reference areas.

[4] No results reported.

SPECIES	LOCAL : contam. gradient (g) reference	REFERENCES
Ictalurus nebulosus	**Buffalo River,NY** **Detroit River** Aquarium raised	Dunn *et al.* (1987) *Cancer Res.* 47:6543-6548
Leuciscus cephalus[3] *Barbus barbus*[3] *Abramis brama*[3] *Vimba vimba c.*[3] *Cyprinus carpio*[3] *Mugil auratus*[3]	Sava River,Zagreb Korana River, Karlovac Ravinji, Adriatic Sea	Kurelec *et al.*(1989) *Carcinogenesis* 10(7):1337-1339

Table continued

Parophrys vetulus *Pseudopleuronectes americanus*	**Puget Sound,WA** Useless Bay,WA **Boston harbor,MA**	Varanasi *et al.* (1989)*Cancer Res.*49: 1171-1177
Ictalurus nebulosus *Stizosteidon vitreum*[2)] *Catostomas commersoni*[2)] *Cyprinus carpio*[2)]	**Great lakes** **Buffalo River,NY** **Detroit River, M** in vivo	Maccubbin *et al.* (1990) *Sci. Tot. Environ.* 94:89-104
Tilapia mossambica	**Damsui River, China (g)**	Tsung-Yun Liu *et al.* (1991)*Bull. Environ. Contam. Toxicol.* 47:783-789
Parophrys vetulus *Lepidopsetta bilineata* *Platichtys stellatus*	**Puget Sound,WA**	Stein *et al.*(1992) *Environ. Toxicol. Chem.* 11:701-714
Opsanis tau	**Elizabeth River, Virginia** York River	Collier *et al.*(1993) *Environ. Sci.* 2:161-177
Chondrostoma nasus	**Rhône, Frankrike (g)**	Pfohl-Leszkowicz *et al.* (1993)in: Postlabelling Methods for detection of DNA adducts. Eds: Phillips, Castanegro & Bartsch. *IARC*
Anguilla anguilla	**4 sites, Amsterdam** Diemereedijk lake Gaaperplas	van der Oost *at al.* (1994) *Environ. Toxicol. Chem.*13(6):859-870
Cheilotrema saturnum *Genyonemus lineatus*[3)]	**San Diego Harbor, CA** Mission Bay Dana Point	Stein *et al.*(1994)Environ. health Perspec. 102(12):19-23
Rutilus rutilus[3)]	2 Amsterdam lakes, Holland	Vanderoost *et al.*(1994) *Chemosphere* 29(4):801-817
Catostomus commersoni	**St. Lawrence river** St. Francois river	Eladlouni *et al.* (1995) *Mol. cell. Biochem.*148(2):133-138
Perca fluviatilis	**Norrsundet, Baltic Sea** **Sundsvall** Häxvassen	Ericson *et al.*(1995) *Marine Env. Res.*39:303-307
Salvelinus fontinalis	Subarctic Labrador[1)] Newfoundland[1)]	Ray *et al.* (1995) *Chemosphere* 30(4):773-778
Oncorhynchus tshawytscha	**Puget Sound, WA (g)**	Stein *et al.* (1995) *Environ. Toxicol.Chem.* 14(6):1019-1029
Mullus barbatus *Serranus hepatus*[3)] *Serranus cabrilla*[3)]	**Northwestern** **Mediterranean (g)**	Burgeot *et al.* (1996) *Mar. Ecol. Prog. Ser.* 131:125-141
Gadus morhua	**Baltic Sea**[1)] Barents Sea	Ericson *et al.*(1996) Marine Env. Res. 42(1-4):119-123
Limanda limanda[4)]	Off the British coast	Lyons et al. *SECOTOX 96, 4th European conf.* 25-28 Aug, 1996. Metz, France (abstract)
Platichthys flesus	Northern Baltic Sea[1)]	Malmström & Bylund. *DNA adducts and mutations in human biomonitoring . Conf.* 9-13 June 1996,Stockholm, Sweden. (abstract)

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Annex 5.4

Integration of Chemical and Biological Effects Monitoring

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In 1979 Holdgate provided a sound framework for definition of monitoring programmes. He defined two broad categories of monitoring: Factor monitoring programmes designed to measure anything that may induce changes in living targets, and Target monitoring programmes designed to measure changes in distribution abundance or performance of living systems. Both types of monitoring have their advantages and drawbacks. Typically in factor monitoring programmes environmental quality objectives (EQOs) are set e.g. the protection of saltwater fish, and standards are set (EQS) in terms of concentrations of compounds not to be exceeded to achieve the objective. The limitation of applying this approach is that standards are set for individual compounds and assumptions are made i.e. about our ability to measure compounds at biologically active concentrations, whether or not we are measuring bioavailable fractions, the fate and effects of the compounds are unaffected by environmental factors and our sampling programmes are truly representative. The benefits are that the measurements provide a system for determination of spatial distribution and trends of contaminant concentrations and .a firm basis for management of contaminant inputs.

Target monitoring on the other hand is more relevant to the biological systems we are trying to protect, biological sentinels integrate the effects of all contaminants with time and do not rely on knowledge of the causal factor. However lack of understanding of causality is the difficulty when using such biological effects techniques for management of inputs.

The advent of diagnostic biomarker systems has in some ways bridged the gap between factor and target monitoring as some of these biological effects techniques are indicative of particular factors or small numbers of factors. Indeed in this context they are biologically relevant surrogates of factor measurements, e.g. DNA adduct determination is simply a measurement of certain genotoxic PAH compounds at a cellular level. Other biomarkers fall into the category of those indicating general effects e.g. lysosomal stability and more comfortably fit into the target monitoring category. Clearly the development of such techniques does not invalidate the factor/target monitoring scheme and it is still valid to consider how fish disease measurements compliment factor and target monitoring of environmental quality.

As a component of factor monitoring e.g. PAH or PCBs, our ability to link cause to effect is considerably strengthened by not only determining concentrations of the compounds in relevant environmental matrices e.g. PAH in sediment and shellfish tissues but also to include measurements of CYP1A, PAH metabolites in bile, DNA adducts and liver disease. This linked set of determinands has been set out recently as appropriate for PAH monitoring by OSPAR. In a similar way for PCBs concentrations in both sediments and shellfish tissues linked to CYP1A and liver disease would allow better development of risk factor models for disease induced by PCBs.

The far more difficult area is identifying causal factors when fish disease is used for target monitoring of environmental quality. To date very few appropriate targets for biological effects monitoring have general acceptance, bioassays such as the oyster embryo have been used in UK and European programmes along with benthic ecology and fish disease. It is only in the bioassay field where there have been recent developments to identify causal agents by testing complex environmental samples with the bioassay system and then, by as series of chemical separation schemes, resolve the complex mixture into simple fractions for re-testing. Further separations of the toxic fractions leads to isolation and identification of the toxic components. Some success has been achieved, but the procedure itself has a number of difficulties. At the very least the system has allowed the identification of contaminants that should be considered in factor monitoring programmes.

A similar approach has also led to the identification of oestrogens and xenoestrogens in environmental samples at biologically active concentrations and such components may provide anther risk factor in disease induction.

When using liver disease in a target monitoring approach the advice for accompanying analytical support must be - measure everything or nothing. Without some additional mechanism for determining causality it is difficult to envisage how the data may be used for risk assessment unless there is considerable data available for the genotoxicological properties of each compound determined.

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Annex 8.1

Standardisation of monitoring techniques

8.1 Field sampling
8.2 Tissue processing for histopathology/histochemistry

1. Sampling requirements:

- Selection of sample sites
- Fishing gear appropriate for providing live fish for examination
- Avoid spawning season for sampling
- Fish species (dab, flounder, winter flounder)
- Organ (liver)
- Sample weight, age, size, length, sex and, preferably, stage of maturation
- Examine for and record external diseases and anomalies

2. Suitable sample selection, preparation and preservation procedures:

Note 1:
Details of methods for sampling, macroscopic examination and recording fish diseases are detailed in the ICES Training guide for the identifiction of common diseases and parasites of fish in the North Atlantic (Bucke et al. 1996).

Note 2:
All information should be recorded individually per specimen.

- Collect and maintain live fish prior to individual sampling.
- Select 50 fish (or the number required to fulfil statistical requirements*) of female fish per station from the mid-length sizes, e.g. for dab, 20-24 cm, for flounder, 25-30 cm
- Sacrifice fish individually by severing spinal cord just posterior to brain.
- Carefully dissect out liver avoiding damage to gall bladder and record weight. Examine and record macroscopic lesions. Cut a 3 mm slice longitudinally from centre axis of the liver using a sharp blade. (e. g. No. 24). For enzyme histochemistry, take pieces of 5 mm^3 (see Figure 1) and place on a cold, coded chuck at refrigerated temperature. The tissue and chucks are then quenched (supercooled) in n-hexane to -70 °C and stored as described in Köhler *et al.* (1992) .
- Record macroscopic lesions in section and place into wide mouthed container with 20x fixative :sample. Leave for 24 hours at ambient temperature then transfer to 70% alcohol for storage.
- Remove otoliths and store in dry container.
- Process samples for light microscopy to paraffin wax blocks, section blocks at 5µm, stain with haemotoxylin and eosin.(H&E) (Bucke, 1989).
- Archive block and stained section.

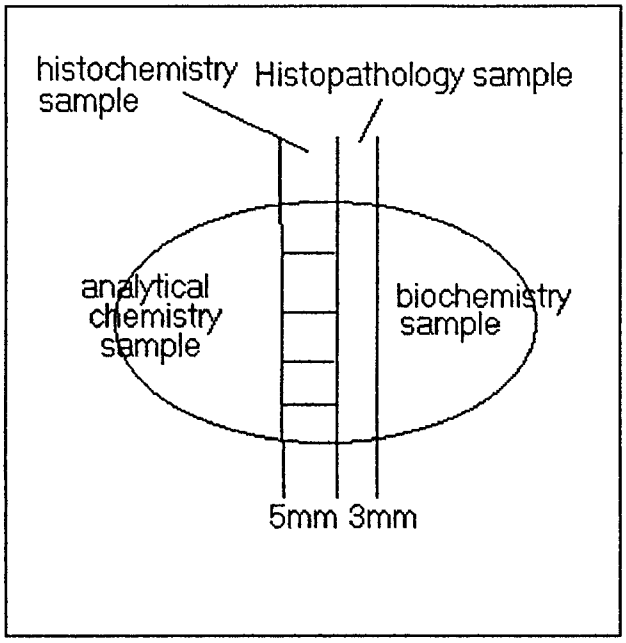
*** Example:**
for 95% confidence of detection of a 2 % disease prevalence in a population: 150 specimens,
for 95% confidence of detection of a 5 % disease prevalence in a population: 60 specimens
for 95% confidence of detection of a 10 % disease prevalence in a population: 30 specimens

3. Standard reagents required:

- Fixative: 10 % neutral buffered formalin* (or other fixatives that will allow immuno-histochemical analysis).
- 70 % alcohol (industrial methylated spirit (IMS))
- Reagents for haemotoxylin and eosin staining* (Bancroft & Cook, 1994).

* Methods appended.

Figure 1: Sampling procedure for multiple analysis of liver.



*** Formula for 10% Neutral buffered formalin;**

Formaldehyde solution - 37% w/v	500 ml
Sodium di-hydrogen orthophosphate	22.75 g
Sodium chloride	22.5 g
Di-sodium hydrogen orthophosphate	32.5 g
Distilled water	2.0 l

*** Example procedure for Haematoxylin and Eosin Stain (H&E)**

Solutions required:

- Clearing agent
- Graded alcohols
- Acid/alcohol (1% hydrochloric acid in 70% alcohol)
- 1% aqueous Eosin Y
- Haematoxylin (Gill 3 formula) (Surgipath - Huntingdon, England).

Method:

Part 1: Taking slides to water

- Place slides in 'Clearene' (Surgipath UK) to remove wax for a **minimum** of 2 minutes.
- Repeat step 1 in fresh Clearene.
- Place in 100% alcohol to remove Clearene for a **minimum** of 2 minutes.
- Repeat step 3 in fresh 100% alcohol.
- Wash slides in running tap water for 2-5 minutes, slides should be **clear**, not cloudy.

Part 2: Staining

- Place in haematoxylin for 3 minutes.
- Blue in running tap water for at least 10 minutes (It is not possible to "over blue").
- Differentiate in acid/alcohol for a **maximum** of 10 seconds.
- Rinse in running tap water until blue.
- Microscope check for clear cytoplasm and blue nuclei.
- Place in aqueous eosin for 3 minutes.
- If necessary wash for up to 1 minute in running tap water to differentiate eosin (take care not to over differentiate).

Part 3: Dehydration, clearing and mounting;

- Rinse well in 70% alcohol for 30 seconds.
- Place in 100% alcohol for 1 -2 minutes.
- Repeat, using fresh alcohol.
- Place 50/50 alcohol /Clearene for 1 - 2 minutes.
- Place into Clearene 2 minutes.
- Repeat, using fresh Clearene.
- Mount in D. P. X. and leave to dry.

Results:

Nuclei:	blue
Muscle fibre:	red
Red blood cells:	bright red (depending on fixative)
Collagen:	pink

References:

Bancroft, J.D., & Cook, H.C. (1994) Manual of Histological Techniques and their Diagnostic Applications. Churchill Livingstone, UK. 457 PP.

Bruno D W and Poppe T T. (1996) A Colour Atlas of Salmonid Diseases. Academic Press, London 194pp.

Bucke, D. (1989) Histology. In "Methods for the microbiological examination of fish and shellfish. Eds. B. & D.A. Austin. Ellis Horwood Ltd., Chichester, UK.. cH. 5: 69-97.

Bucke, D. Vethaak, D., Lang, T. &Mellergaard, S. (1996) Common diseases and parasites of fish in the North Atlantic: Training guide for identification ICES No. 19, 27 pp.

Köhler, A.., Deisemann, H.&Lauritzen, B. (1992) Histological and cytochemical indices of toxic injury in the liver of dab *Limanda limanda*. Mar. Ecol. Prog. Ser. 91: 141-153.

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Annex 8.3

Diagnostic criteria for liver histopatholgy

The following table is based on histopathological diagnosis of lesions in paraffin - embedded, H&E-stained sections of the liver of teleost fish species.The numbers in brackets indicate the relative importance (1-3) of the lesions to indicate contaminant exposure.

General (non-specific) (2)
necrotic/degenerative changes
- coagulative necrosis (pyknosis, karyorrhexis) - hepatocytes or biliary epithelial cells
- hydropic degeneration (hepatocellular, biliary epithelial cells)
- increased single cell necrosis, apoptosis
- hyaline inclusion bodies-hyalinization

Unique degenerative lesions (1)
- hepatocellular and nuclear polymorphism (1)
- megalocytic hepatosis, hepatic megalocytosis (1)
- spongiosis hepatis (3)
- hydropic vacuolisation of biary epithelial cells and/or hepatocytes **(1, especially in flounder)**

Storage conditions (3)
- lipidosis (steatosis, fatty change, fatty degeneration)
- hemosiderosis
- variable glycogen content
- phosholipidosis
- fibrillar inclusions-paracrystalline arrays, hepatocellular macrotubules

Inflammatory changes (3)
- macrophage aggregates, melanomacrophage centers
- lymphocytic / monocytic infiltration
- fibrosis, fibroplasia, cirrhosis
- granuloma

Non-neoplastic proliferative lesions (2)
- hepatocellular regeneration diffuse throughout the liver parenchyma
- bile preductular epithelial cell proliferation (oval cells?)
- bile duct hyperplasia
- cholangiofibrosis / adenofibrosis

Vascular abnormalities (3)
e.g.
- peliosis hepatis

Foci of cellular alterations (FCA) (tinctorial / staining properties) (1)

- clear cell focus (glycogen storage)
- vacuolated focus (lipid storage)
- eosinophilic fous
- basophilic focus

*includes amphophilic focus
(mixed cell foci are placed into categories above based on the dominant staining type)

Morphological criteria for foci of cellular alterations (FCA)

- dicrete focal lesion
- lack of compression, continuity of tubules with the surrounding parenchyma
- normal tubular architecture and tubular thickness
- < 10 cells in diameter, no limits on upper size
- relatively normal cytomorphology, rare or absent mitotic figures
- relative absence of MMCs, pancreatic tissue, bile ducts

Benign neoplasms (1)

- hepatocellular adenoma

staining properties

- basophilic
- eosinophilic
- clear cell

- cholangioma
- pancreatic acinar cell adenoma
- hemangioma

Morphological criteria for benign hepatocellular adenoma

- hepatocellular tubular thickening (> 3 cell layers) with mild architectural disorganisation
- clear, distinct separation of tubules composing the tumour from those in surrounding tissue
- relative absence of melanomacrophage centres (MMCs), pancreatic tissue and bile ducts

Malignant neoplasms (1)

- hepatocellular carcinoma
- cholangiocarcinoma
- mixed hepatobiliary carcinoma
- pancreatic acinar cell carcinoma, pancreatic adenocarcinoma
- hemangiosarcoma
- hemangiopericytic sarcoma

Morphological criteria for malignant tumours

- metastasis
- invasion, irregular borders, obvious "satellite" foci
- loss of normal architectural organisation of tubules
- cytologic atypia, such as nuclear and/or cellular pleomorphism within tubules,loss of cellular polarity within the tubules, anaplasia
- increased mitotic index
- relative absence of macrophage aggregates, pancreas, hepatic bile ducts (if not a biliary tumor)

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Annex 8.4

Quality assurance for monitoring of liver pathology in fish

The purpose of quality assurance is to ensure that monitoring data can be used with confidence, to identify data sets of poor quality, to increase convergence of data, and to enhance international cooperation.
The intercalibration programme should address both the processing of samples and the diagnosis of histopathological and histochemical changes. The programme should be coordinated by a reference laboratory (the MAFF Fish Diseases Laboratory, Weymouth, UK is recommended) that will identify suitable reference material and distribute it to the participating laboratories, compile the results, and organize intercalibration workshops if necessary. The following are the essential elements of such a programme.

1. Good laboratory practice (GLP)

The following elements are needed:

- standard operating procedures (SOP)
- an adequate training programme
- sample and data traceability
- sample archiving
- a quality control programme

For liver histopathology and histochemistry, standard operating procedures for the following processes are required:

- sampling of fish
- dissection and macroscopic examination
- preparation of liver tissue for each of the analyses required
- fixation and preservation
- processing (embedding, sectioning, staining, histochemical labelling)
- quantification of histopathological/histochemical changes
- archiving and reporting of data

2. Reference materials

- a colour atlas with common histopathological liver changes
- representative slides exhibiting a range of typical histopathological liver changes
- fixed or frozen material for processing by each laboratory

Every laboratory involved should produce slides showing a wide range of liver pathology and distribute them to each of the other laboratories for quality assessment. In order to facilitate comparison, it is important that these are sequential sections.

3. Regulation of analytical quality control

Pathological diagnosis is by necessity somewhat subjective and may vary with time. To control for this variability, there should be regular but random exchange of blind processed samples both between laboratories and, where there are multiple pathologists, between individuals within the laboratory.

4. Training requirements

Within laboratories, there should be an adequate training programme to ensure that individuals reach the required standard defined by the quality assurance programme.

Training should be achieved through the use of training material such as the reference material detailed above, videos and workshops. Workshops are an important tool for the establishing of consensus among scientists concerning the diagnostic criteria to be used for liver pathology and the training of scientists to use these criteria. This will ensure that data from different laboratories are consistent.

5. Performance limits

In histopathology, performance criteria will not be absolute rather they are based on consensus and agreements between pathologists of the nature of the pathology observed. Therefore, the function of quality assurance is to enhance the convergence of diagnoses. This will require that, in the beginning of a monitoring programme, frequent intercalibration activities are needed.

6. Action to be taken if there is poor agreement

If there is consistent disagreement about a particular pathological change, this lesion should not be used for the assessment of biological effects of contaminants until the problem has been resolved. Workshops should be arranged to deal with such uncertain cases in order to better define the diagnostic criteria to be used.

If there are consistent differences between individual laboratories compared with the majority, data derived from these laboratories should not be used in the international assessment.