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Report of the ICES/BSRP Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM)

5–12 December 2005

International Council for the Exploration of the Sea
Conseil International pour l'Exploration de la Mer

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Executive summary

The ICES/BSRP Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM) was held 5–12 December 2005 on board the German RV 'Walther Herwig III'. It was organised by the ICES Study Group on Baltic Ecosystem Health Issues in Support of BSRP (SGEH) and the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDM) and was co-chaired by T. Lang (Germany) and G. Rodjuk (Russia). The Workshop was attended by scientists from Estonia, Finland, Germany, Latvia, Lithuania, Poland, Russia, and Sweden and it started and ended in Gdynia, Poland.

The report provides the following elements: an overview of present activities in Baltic Sea countries related to fish diseases, background information (the status of fish disease monitoring in the marine environment on a national and international scale, methods for fish disease data handling and analysis, approaches for integrated chemical and biological effects monitoring), a description of the practical work carried out during the workshop and its results, a proposal for the construction of a Fish Health Index as an assessment tool, and methodological guidelines for fish disease monitoring in the Baltic Sea.

Based on the results and the discussions, a number of recommendation were made, highlighting the importance of fish disease studies in the context of integrated ecosystem health monitoring and assessment, the need for improved quality assurance and for further activities (e.g. a workshop on diseases in coastal fish in the Baltic Sea and a workshop on ecosystem health of the Gulf of Finland).

It is hoped that the workshop and related future activities will build the basis for the incorporation of coordinated and standardised fish disease studies into national marine monitoring and assessment programmes of the Baltic Sea countries and eventually into the HELCOM monitoring programme.

1 Opening of the meeting

The ICES/BSRP Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM) was held 5–12 December 2005 onboard the German Research Vessel 'Walther Herwig III' and started and ended in Gdynia, Poland. The workshop was co-chaired by T. Lang (Germany) and G. Rodjuk (Russia) and was attended by scientists from Estonia, Finland, Germany, Latvia, Lithuania, Poland, Russia, Sweden and the UK (see Annex 1).

After arrival of all participants, RV 'Walther Herwig III' left the port of Gdynia in the afternoon of 5 December heading for the first sampling area selected. T. Lang welcomed the participants onboard on behalf of ICES and the German Ministry of Food, Agriculture and Consumer Protection operating the RV and introduced the experts acting as trainers (G.D. Stentiford (UK), W. Wosniok (Germany) and K. Lehtonen (Finland)). The Co-chair thanked the Polish Sea Fisheries Institute in Gdynia, and in particular M. Podolska, for their support in organising the stay of the RV in Gdynia.

The workshop was organised according to the timetable shown in Table 1 and consisted of practical work and training with flounder (*Platichthys flesus*), cod (*Gadus morhua*) and herring (*Clupea harengus*) as target fish species as well as of theoretical work addressing aspects such as:

- **Practical work:** Methods for fish sampling, disease diagnosis (externally visible diseases/ parasites and liver histopathology), intercalibration exercises, sampling for histopathology., fixation and preservation techniques, age determination, data entry software, hydrographic measurements, sampling for biomarker measurements.
- **Theory:** Overview of national and international programmes and databases (e.g. ICES Data Centre), sampling design, data recording, analysis and assessment, development of health indicators, confounding factors with impact on diseases (host-specific, site-specific), quality assurance (e.g. BEQUALM) etc.

Practical work was carried out in five Baltic Sea areas encompassing sampling sites in German, Polish and Lithuanian waters (see Figure 1 and Table 2).

Table 1: Timetable for the ICES/BSRP Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM)

4 Dec. 2005	RV Walther Herwig III arrives in port of Gdynia
5 Dec. 2005	Participants arrive in Gdynia, start of workshop on board RV Walther Herwig III
6–11 Dec. 2006	Field work and training at selected sampling sites (see Figure 1)
11 Dec. 2005	RV Walther Herwig III returns to Gdynia, reception on board with participants and invited guests
12 Dec. 2005	End of workshop, RV Walther Herwig III leaves Gdynia

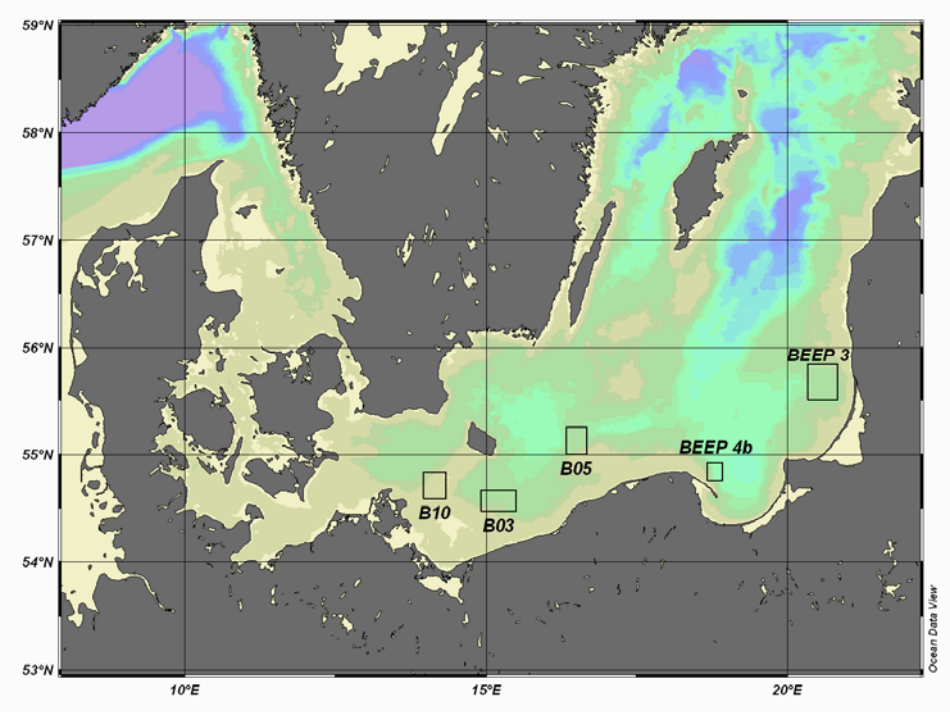

Figure 1: Location of sampling areas of the ICES/BSRP Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM)

Table 2: Geographical position of sampling areas of the ICES/BSRP Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM)

SAMPLING AREA	LATITUDE	LONGITUDE
B10	54° 35'N - 54° 50'N	13° 58'E - 14° 20' E
B03	54° 28'N - 54° 40'N	14° 55'E - 15° 30'E
B05	55° 00'N - 55° 15'N	16° 20'E - 16° 40'E
BEEP 3	55° 30'N - 55° 50'N	20° 20'E - 20° 50'E
BEEP 4b	54° 45'N - 54° 55'N	18° 40'E - 18° 55'E

2 Terms of reference, adoption of the agenda

The participants took note of the Terms of Reference of the workshop (Annex 2). A draft Agenda was circulated and adopted without change (Annex 3).

3 General Introduction and rational for the workshop

From a large variety of studies there is general consensus today that fish diseases are an appropriate indicator of ecosystem health and that the prevalence of diseases/parasites responds to natural and anthropogenic environmental change, including exposure to contaminants. Furthermore, many fish diseases/parasites are of ecological and economical relevance since they may affect growth, reproduction and survival in affected fish populations and may even cause human health problems. Therefore, a number of ICES Member Countries carry out fish disease surveys as part of their national marine monitoring and assessment programmes and results from these surveys are being used for internationally coordinated assessments, e.g as part of the OSPAR JAMP/CEMP and the HELCOM Periodic Assessments.

In the Baltic Sea, only Poland, Germany and Russia are presently conducting regular fish disease monitoring programmes. However, from data assessments carried out by the ICES

Working Group on Pathology and Diseases (WGPDMO), there has been indication of methodological problems, particularly regarding the comparability of disease prevalence data, and a clear need for more intercalibration has, thus, repeatedly been emphasised.

Besides these countries, there is also interest in other Baltic Sea countries to implement fish disease monitoring as part of the national coastal or offshore monitoring, but there has been an apparent lack of either capacities or experience. Within the Baltic Sea Regional Project (BSRP), this has been realised and funding was provided for capacity building related to fish disease monitoring in the BSRP beneficiary countries. The AtlantNIRO, Kaliningrad, Russia, was appointed as BSRP Lead Laboratory for Fish Diseases, Parasites and Histopathology in order to coordinate relevant activities and the ICES Study Group on Baltic Ecosystem Health in support of the BSRP (SGEH) is reviewing progress made.

Since ICES has a long-term experience in developing and intercalibrating methodologies for fish disease surveys and organised a number of practical practical methodological workshops before (e.g. 1994 in the Baltic Sea, co-sponsored by the Baltic Marine Biologists, BMB), it was recommended in the ICES SGEH and the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) to organise an ICES/BSRP sea-going training workshop in December 2005, aiming at a standardisation and intercalibration of methodologies, addressing aspects from fish sampling, disease diagnosis, data reporting to statistical data assessment.

Based on an invitation by the German Ministry of Food, Agriculture and Consumer Safety, it was decided to organise the workshop on board RV Walther Herwig III as part of Cruise No. 281 with T. Lang (Germany) and G. Rodjuk (Russia) as co-chairs. An ICES Council Resolution was adopted at the 2005 ICES Annual Science Conference/Statutory Meeting:

ICES Council Resolution 2005/2/BCC02

An **ICES/BSRP Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea** [WKFDM] (Co-Chairs: Thomas Lang, Germany, and G. Rodjuk, Russia) will meet 7–10 days in December 2005 onboard RV Walther Herwig III to:

- provide training and intercalibration related to methodologies applied in fish disease monitoring in the Baltic Sea,
- further develop and assess health indicators and indices appropriate for monitoring and assessment purposes,
- establish a closer collaboration between institutes involved in fish disease monitoring in the Baltic Sea,
- build the basis for incorporation of fish disease surveys into the revised HELCOM monitoring programme.

The idea for the workshop was to gather scientists from all 9 Baltic Sea countries involved in fish disease work and to invite some specialists in the field of fish disease monitoring whose responsibility was to act as trainers for various aspects related to fish disease monitoring.

Gdynia, Poland, was chosen as starting and ending point for the workshop because of its geographical position that made it easily accessible for all participants.

4 Background information – setting the scene

4.1 Status of wild fish disease monitoring and the role of ICES in intercalibration and standardisation of methodologies

T. Lang presented an overview of the present status of fish disease monitoring on a national and international level and of ICES's role in the development of guidelines and standardised operating procedures.

4.1.1 Monitoring of diseases in wild marine fish stocks: the present status

Diseases of wild marine fish have been studied on a regular basis by ICES Member Countries for more than two decades. At present, annual or biannual fish disease surveys in the North Sea are carried out by Germany (BFA Fisheries), The Netherlands (RIKZ) and the UK (Cefas, FRS Marine Laboratory). In the Baltic Sea, Germany (BFA Fisheries), Poland (Sea Fisheries Institute) and Russia (AtlantNIRO) are carrying out regular fish disease monitoring. However, more data is available from monitoring programmes that were terminated in the 1990s or early 2000s (e.g. carried out by Belgium, Denmark, Estonia, Finland and Sweden). More information on current national programmes in the Baltic Sea can be found in section chapter 5 of the present report..

Many of these national programmes have increasingly evolved into integrated monitoring programmes, including studies on chemical contamination and on biological effects of contaminants, as part of national monitoring programmes aiming at an assessment of the health of the marine environment, in particular in relation to the impact of human activities (Lang, 2002).

Long-term programmes have largely focused on externally visible diseases and, only partly, parasites. Since the end of the 1980s, studies on liver anomalies (mainly neoplastic liver lesions (tumours and their pre-stages)) have increasingly been added. Studies are being conducted in a variety of fish species, including dab (*Limanda limanda*) (main target species for the North Sea and adjacent areas such as the English, Channel, Celtic Sea, Irish Sea and western Baltic Sea), flounder (*Platichthys flesus*) (in coastal/estuarine North Sea areas and in the entire Baltic Sea) and cod (*Gadus morhua*) (at present, mainly in the Baltic Sea) and methodologies are easily adaptable for other species such as plaice (*Pleuronectes platessa*) and other flatfish species, whiting (*Merlangius merlangus*) and haddock (*Melanogrammus aeglefinus*). Methodologies and diagnostic criteria involved in the monitoring of contaminant-specific liver nodules and liver histopathology have largely been developed based on studies with flatfish species, in Europe mainly dab and flounder, but can also be adapted to other flatfish species (e.g. plaice) or long rough dab (*Hippoglossoides platessoides*) and possibly also to bottom-dwelling roundfish species, such as viviparous blenny (= eelpout) (*Zoarces viviparus*).

On an international level, fish disease data have been used for environmental assessments in the framework of the North Sea Task Force and its Quality Status Report (North Sea Task Force, 1993), the OSPAR Quality Status Report 2000 (OSPAR Commission, 2000) and in the 3rd and 4th HELCOM assessments (HELCOM, 1996, 2002). Studies on externally visible diseases, liver nodules and liver histopathology are on the list of techniques for general and contaminant-specific biological effects monitoring as part of the OSPAR Co-ordinated Monitoring Programme (OSPAR, 2004).

4.1.2 Quality assurance of fish disease monitoring: the role of ICES

Since the early 1980s, ICES has played a leading role in the initiation and coordination of fish disease surveys and has contributed considerably to the development of standardised methodologies. Through the work of the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), its offspring, the Sub-Group/Study Group on Statistical Analysis of Fish Disease Data in Marine Stocks (SGFDDS) (1992–1994) and the ICES Secretariat, quality assurance procedures have been implemented at all stages, from sampling of fish to submission of data to ICES and to data assessment.

Three practical ICES sea-going workshops on board research vessels were organised by WGPDMO in 1984 (southern North Sea), 1988 (Kattegat) and 1994 (Baltic Sea, co-sponsored by the Baltic Marine Biologists, BMB) in order to intercalibrate and standardise methodologies for fish disease surveys (Dethlefsen *et al.*, 1986; ICES, 1989; Lang and Mellergaard, 1999) and to prepare guidelines. Whilst first guidelines were focused on externally visible diseases and parasites, WGPDMO developed guidelines for macroscopic and microscopic inspection of flatfish livers for the occurrence of neoplastic lesions at a later stage. Further intercalibration and standardision of methodologies used for studies on liver pathology of flatfish were a major issue of the 1996 ICES Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants (ICES, 1997). This formed the basis from which the BEQUALM programme developed for the application of liver pathology in biological effects monitoring (Feist *et al*., 2004).

A fish disease data bank has been established within the ICES Marine Data Centre, consisting of disease prevalence data of key fish species and accompanying information, submitted by ICES Member Countries. Submission of fish disease data to the ICES Marine Data Centre has been formalised by the introduction of the ICES Environmental Reporting Format designed specifically for the purpose. This is used for fish disease, contaminant and biological effects data. The programme includes internal screening procedures for the validation of the data submitted providing further quality assurance.

With the new ICES Environmental Reporting Format (version 3.2), the fish disease database will be extended to include data from other species and maritime areas as well as data on studies into other types of diseases, e.g. liver histopathology. To date, the data comprise information from studies on the occurrence of externally visible diseases and macroscopic liver lesions in the common dab (*Limanda limanda*) and the European flounder (*Platichthys flesus*) from the North Sea and adjacent areas, including the Baltic Sea, Irish Sea, and the English Channel. In addition, reference data are available from pristine areas, such as waters around Iceland. In total, data on length, sex, and health status of more than 500.000 individual specimens, some from as early as 1981, have been submitted to ICES, as well as information on sampling characteristics (Wosniok *et al.*, 1999).

Current ICES WGPDMO activities have focussed on the development and application of statistical techniques for an assessment of disease data with regard to the presence of spatial and temporal trends in the North Sea and western Baltic Sea (Wosniok *et al.* 1999; Wosniok *et al*. in press). An output of WGPDMO's activities is the ICES web-based report on wild fish diseases, consisting of trend maps and associated information. In a subsequent, more holistic approach, pilot analyses have been carried out combining the disease data with oceanographic, nutrient, contaminant and fishery data extracted from the ICES data banks in order to improve the knowledge about the complex cause-effect relationships between environmental factors and fish diseases (Lang and Wosniok, 2000; Wosniok *et al.*, 2000). These analyses constituted one of the first attempts to combine and analyses ICES data from various sources and can, therefore, be considered as a step towards a more comprehensive integrated assessment.

Quality assurance is in place for externally visible diseases, liver nodules and liver histopathology via the ongoing BEQUALM programme (Biological Effects Quality Assurance in Monitoring programmes) (http://www.bequalm.org/about.htm.) Regular intercalibration and ring-test exercises are conducted and a histopathology workshop next workshop is planned for March 2006. The basis for QA procedures are provided in two key publications in the ICES TIMES series (Bucke *et al.*, 1996, Feist *et al*., 2004)).

As indicated above, ICES has developed requirements for the international reporting of fish diseases over many years in order to minimise variation between laboratories regarding the accuracy and reproducibility of data generated. These have been reviewed by BEQUALM and produced in CD-ROM format. Each grossly visible disease (lymphocystis, acute and healing skin ulcerations, epidermal hyperplasia/papilloma and liver nodules) has a minimum requirement for reporting and severity is assessed according to criteria allocated to three stages (lymphocystis, ulcerations and epidermal hyperplasia/papilloma only). Macroscopic liver nodules are only recorded if the minimum diameter exceeds 2 mm. Each case of a liver nodule has to be verified histologically to exclude the possibility that nodules are the response to parasites, cysts, necrotic or inflammatory foci.

With regard to the application of liver histopathology as a tool in biological effects monitoring, the activities undertaken in ICES and within BEQUALM have been successful in the establishment of the methodology and diagnostic criteria. The diagnostic key developed provides clear criteria to discriminate between the lesion types, thus minimising the possibility of mis-diagnosis. Ring tests and other intercalibration exercises are regularly undertaken in order to minimise inter-observer variation and to establish acceptable limits of variation. These are carried out as an ongoing process in order to ensure continuous quality assurance of data obtained.

These quality assurance procedures implemented are a crucial prerequisite for the establishment of assessment criteria and reference or threshold values applied by all institutions involved in fish disease monitoring in order to take decisions on further actions. However, the definition of reference and threshold values related to disease prevalence or incidence has only recently been started and an implementation will require considerable further work. Reference and threshold values are likely to be determined from comparisons of the prevalence or incidence of disease conditions between reference site(s) and on the basis of quantitative change over time (trends) in a given area.

4.1.3 How to interpret fish disease data: environmental variables that influence fish diseases and liver pathology

The multifactorial aetiology of diseases, in this context in particular of externally visible diseases, is generally accepted. Therefore, externally visible disease have correctly been placed into the general biological effect component of the OSPAR CEMP. Most wild fish diseases monitored in past decades are caused by pathogens (viruses, bacteria). However, other endogenous or exogenous factors may be required before the disease develops. One of these factors can be environmental pollution, which may either affect the immune system of the fish in a way that increases its susceptibility to disease, or may alter the number and virulence of pathogens. In addition, contaminants may also cause specific and/or non-specific changes at various levels of biological organisation (molecule, sub-cellular units, cells, tissues, organs) leading to disease without involving pathogens.

The occurrence of significant changes in the prevalence of externally visible fish diseases can be considered a non-specific and more general indicator of chronic rather than acute (environmental) stress, and it has been speculated that they might, therefore, be an integrative indicator of the complex changes typically occurring under field conditions rather than a specific marker of effects of single factors. Because of the multifactorial causes of diseases,

the identification of single factors responsible for observed changes in disease prevalence is difficult, and scientific proof of a link between contaminants and fish diseases is hard to achieve. Nevertheless, there is a consensus that fish disease surveys should continue to be part of national and international environmental monitoring programmes since they can provide valuable information on changes in ecosystem health and may act as an "alarm bell" potentially initiating further more specific studies on cause and effect relationships.

In the statistical analysis of ICES data on externally visible diseases (lymphocystis, epidermal hyperplasia/papilloma, acute/healing skin ulceration) of dab from different North Sea regions, it could be demonstrated that there were significant spatial differences, both in terms of absolute levels and the temporal changes in disease prevalence in the North Sea. While data from the 1990s revealed stable or decreasing disease prevalences in the majority of sampling sites, some areas in the North Sea showed increasing trends for some of the diseases, indicating a change in environmental conditions adversely affecting the health status of dab (Wosniok *et al*., 1999). The results from the subsequent multivariate analysis on the relationship between the prevalence of the diseases with potentially explanatory environmental and host-specific factors (also extracted form the ICES fishery, oceanography and environmental databases) clearly highlighted the multifactorial aetiology of the diseases under study. A number of natural and anthropogenic factors (stock composition, water temperature, salinity, nutrients, contaminants in water, sediments and biota) were found to be significantly related to the temporal changes in disease prevalence. However, depending on area, time range and data availability, different sets of factors were identified. This reflects the multifactorial aetiology of the diseases covered, but was also attributed to some high correlations among the explaining quantities (Lang and Wosniok, 2000; Wosniok *et al*., 2000).

The presence of liver nodules and of histopathological liver lesions is a more direct indicator of contaminant effect and has been used for many years in environmental monitoring programmes around the world. Liver nodules (representing macroscopically visible liver tumours) and neoplastic histopathological liver lesions are likely to be associated to exposure to carcinogenic contaminants, including PAHs, and are therefore considered appropriate indicators for general and for PAH-specific biological effects monitoring. Therefore, monitoring of liver nodules in the CEMP should not only be part of the CEMP general biological effects monitoring but also of the CEMP PAH-specific biological effects monitoring. The study of liver histopathology (incorrectly termed liver neoplasia/hyperplasia in the JAMP Guidelines for General biological effects monitoring, see below under 6. Final remarks) comprises the detection of more lesion categories (non-specific, neoplastic and non-neoplastic toxicopathic lesions), reflecting responses to a wider range of contaminants (including PAHs) but also to other environmental stressors and is, therefore, considered an appropriate indicator for both General and PAH-specific biological effects monitoring.

The liver is the main organ involved in the detoxification of xenobiotics and several categories of hepatocellular pathology are now regarded as reliable biomarkers of toxic injury and representative of biological endpoints of contaminant exposure (Myers *et al*, 1987, 1992, 1998; Stein *et al*., 1990; Vethaak and Wester, 1996; Stentiford *et al*., 2003; Feist *et al*., 2004). The majority of lesions observed in field collected animals have also been induced experimentally in a variety of fish species exposed to carcinogenic compounds, PAHs in particular, providing strong supporting evidence that wild fish exhibiting these lesions have been exposed to such environmental contaminants.

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4.2 Liver histopathology as a tool in fish disease monitoring

4.2.1 Introduction

G.D. Stentiford gave a presentation explaining the rationale, the general strategies applied and the types of liver lesions recorded for monitoring purposes. He focused on the structure and function of the normal liver, on mechanisms involved in the chemical induction of liver cancer (in particular in relation to PAH exposure), on the pathogenesis of liver cancer, and on the use of data generated in relevant monitoring programmes (see Annex 4)

It was pointed out that, in addition to externally visible diseases, the presence of liver tumours (neoplasms) is recorded routinely in dab (*Limanda limanda*) and flounder (*Platichthys flesus*) populations sampled as part of, e.g., the Dutch, German and UK monitoring programmes. In the flatfish liver, the presence of neoplasia has been classified as a more direct indicator of contaminant exposure and likely represents a biological endpoint of historic exposure to chemicals that initiate and promote the carcinogenic pathway. As a result, the presence of grossly visible liver tumours has been used for many years in environmental monitoring programmes around the world. At some offshore sites in the North Sea, liver tumour prevalence in wild flatfish has exceeded 10% in recent years while prevalence in estuarine species can be significantly higher.

4.2.2 Guidelines for studies on liver histopathology

Liver tumours in dab and flounder are recorded according to guidelines developed by ICES (Bucke *et al.*, 1996, Feist *et al.*, 2004) and within the Biological Effects Quality Assurance in Monitoring (BEQUALM) programme (http://www.bequalm.org/about.htm). This involves the quantification of macroscopic liver nodules > 2 mm in diameter in a minimum of 50 fish per sampling site of the size group ≥ 25 cm (for dab) and ≥ 30 cm (for flounder) total length, respectively. If no sufficient numbers of fish are available, smaller fish (20–24 cm in dab; 24–29 cm in flounder) may be taken in order to fill up the sample Tissue samples of all nodules identified have to be taken and fixed for subsequent histological confirmation of the neoplastic nature of the lesion. Guidelines for histological techniques to be applied are provided in Feist *et al.* (2004).

In addition to the assessment of grossly visible tumours, histopathological assessment of randomly taken liver samples from flatfish populations collected under such programmes allows for the diagnosis of microscopic lesions not visible during whole fish assessments. According to BEQUALM guidelines, samples should be taken from the central part of 50 specimens of the size group ≥ 20 cm total length (both for dab and flounder). The lesions recorded using this approach include those thought to precede the development of benign and

malignant lesions (such as foci of cellular alteration, FCA), non-neoplastic toxicopathic lesions (such as nuclear and cellular polymorphism) and lesions associated with cell death, inflammation and regeneration. Currently, 31 categories of liver lesion are classified under the BEQUALM programme (see Table 3). The diagnosis of these lesion types in the dab and flounder liver follows guidelines recently set out by Feist *et al*. (2004). Similar guidelines exist for diagnosis of liver lesions in the medaka (*Oryzias latipes*) (Borman *et al.,* 1997) and in English sole (*Parophrys vetulus*) (Myers *et al.,* 1987).

4.2.3 Conclusions

As conclusions, the following item were highlighted:

- Liver neoplasia is significantly more common in wild flatfish inhabiting European waters than in human populations.
- Diagnostic criteria for flatfish liver histopathology is largely based upon description of lesions in mammals.
- Significant progress toward quality assurance in lesion diagnosis (in dab and flounder) has been achieved under the BEQUALM programme. However, more partners are required.
- Institutes from Baltic Sea countries conducting fish disease studies (both on externally visible diseases and on liver histopathology) should take part in the BEQUALM programme.
- More effort is required to directly compare the lesions observed in the fish liver with those seen in human patients.
- Molecular based approaches (e.g. via genomics, proteomics and metabolomics) are enhancing our understanding of tumour pathogenesis in flatfish in relation to environmental conditions**.**

4.2.4 References

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Table 3: Categories of liver lesions and lesion types recommended for monitoring in dab (*Limanda limanda*) and flounder (*Platichthys flesus*) (according to BEQUALM)(Feist *et al.,* 2004, modified)

LESION CATEGORY	LESION	BEQUALM CODE
No abnormalities detected (NAD)	-	1
Early non-neoplastic toxicopathic lesions	Phospholipidosis	2
	Fibrillar inclusions	3
	Hepatocellular and nuclear polymorphism	4
	Hydropic degeneration	5
	Spongiosis hepatis	6
Foci of cellular alteration	Clear cell foci	7
	Vacuolated foci	8
	Eosinophilic foci	9
	Basophilic foci	10
	Mixed foci	11
Benign neoplasms	Hepatocellular adenoma	12
	Cholangioma	13
	Hemangioma	14
	Pancreatic acinar cell adenoma	15
Malignant neoplasms	Hepatocellular carcinoma	16
	Cholangiocarcinoma	17
	Pancreatic acinar cell carcinoma	18
	Mixed hepatobiliary carcinoma	19
	Hemangiosarcoma	20
	Hemangiopericytic sarcoma	21
Non-specific inflammatory lesion	Coagulative necrosis	22
	Apoptosis	23
	Lipoidosis	24
	Hemosiderosis	25
	Variable glycogen content	26
	Melanomacrophage centres	27
	Lymphocytic/monocytic infiltration	28
	Granuloma	29
	Fibrosis	30
	Regeneration	31

4.3 Handling and analysis of fish disease data

W. Wosniok provided a presentation on the handling and analysis of data on fish diseases. He focused on the aims of such analyses, on methods for the analysis of fish disease data, on statistical requirements and on data recording and processing. Methods described were related to:

- the calculation of disease prevalence and its precision,
- sample size to determine a prevalence with specified precision,
- testing an observed prevalence against a reference value,
- sample size for comparing a prevalence to a reference value,
- comparing two empirical prevalences,
- sample size to detect differences between two empirical prevalences,
- assessing time series of disease prevalence,
- investigating the relation between disease prevalence and suspected explaining factors.

He further reported on experience with the analysis of ICES data with regard to fish diseases and environmental factors.

A summary of the presentation is given in Annex 5.

4.4 Integrated monitoring of contaminant concentrations and their biological effects in the Baltic Sea environment

4.4.1 Introduction

K. Lehtonen provided a presentation on the present status of biological effects monitoring in the Baltic Sea, highlighting the need for implementing integrated monitoring and assessment programmes on contaminants and their biological effects, an important part of which is the monitoring of fish diseases. Furthermore, an outlook on future activities was given. The presentation is summarised below.

4.4.2 Background

Concentrations of a vast majority of hazardous substances remain undetected in routine chemical monitoring programmes. Although the levels of PCBs and DDTs in the Baltic Sea marine environment have reduced during the past two decades it may still be assumed that pollution by a wide spectrum of hazardous substances is higher today than ever before. Effective monitoring of the numerous "new" chemicals in the marine environment is an extremely demanding and expensive task. Even if substantially extended from its current status the fact that chemical monitoring does not give any information on toxic effects to organisms has to be acknowledged, regardless on the concentrations measured. Pollution usually occurs as a mixture of a variety of compounds present at different levels with complex interactions with physicochemical and biotic factors. Thus, in order to improve the reliability of assessments regarding the pollution status of the Baltic marine environment, biological effects of contaminants should be included into monitoring programmes.

Biological effects of contaminants can be detected and monitored at different biological levels:

- biomarkers: biological responses to environmental chemicals at the individual level or below (i.e. molecular, cellular or "whole-organism" levels),
- effects at tissue level: histopathological alterations,
- effects at organism level: pathology, diseases, reduced performance (e.g. fitness, reproduction),
- effects at population level: changes in population parameters (e.g. abundance, sex ratio),
- effects at community level: changes in community structure,
- effects at ecosystem level: disturbance in the functioning of the whole (local) ecosystem.

Biomarker responses indicate that the organism has been exposed to effective levels of pollutants that are bioavailable in a given environment. Therefore, a battery of "screening biomarkers" can be used to detect the presence of pollutants unnoticed by routine chemical monitoring. The difference between early-warning indicators (i.e. biomarkers) and "ecologically-relevant" effects (population and community effects) should be understood in a way that the former act both as detectors of pollution and potential higher level effects while the latter represent states and processes that have taken a longer time period to develop.

In regard to biomarkers, responses to exposure to contaminants and their effects in organisms can be recorded using parameters indicating e.g. genotoxicicity, biotransformation and transport of xenobiotics, oxidative stress, general stress, cellular protection, immune system

responses, neurotoxicity, energy metabolism and bioenenergetics, and endocrine disruption. When a toolbox of biological effects methods is constructed for monitoring, important things to consider whether they are are cost-effective, rapid, robust, and informative, how they cover all the different biological levels listed above, and whether they indicate responses to specific groups of compounds or general stress.

4.4.3 Developing an integrated chemical-biological pollution monitoring programme for the Baltic Sea

In the Baltic Sea area, the monitoring of pollution has traditionally focused on the measurement of chemical concentrations in water, sediment and biota. To obtain reliable assessments of the hazards of chemical pollution in the marine environment the developments in other sea areas (e.g. North Atlantic, the Mediterranean) concerning the implementation of biological effects methods into monitoring programmes in these areas should be followed (Lehtonen and Schiedek, 2006). An integrated monitoring programme in the Baltic Sea could be based on these already existing or developing practices. However, due to the very specific biotic and abiotic conditions prevailing in the Baltic Sea a direct transfer of methodologies or selection of target species into such a programme is not realistic and more research is obviously needed. However, the recent experiences gained in the extensive pan-European EU project BEEP (Biological Effects of Environmental Pollution in Marine Coastal Ecosystems) (Lehtonen *et al*., 2006) serve as an excellent basis for designing a monitoring programme for the Baltic Sea.

Main objectives of an integrated chemical and biological monitoring programme for pollution proposed are

- assessing of temporal and spatial development in environmental concentrations of hazardous substances of major concern to the Baltic Sea,
- detecting of early-warning stress signals of exposure to various anthropogenic contaminants and their higher-level biological effects on the Baltic Sea biota (e.g. effects on reproduction of organisms),
- identifying the possible relationships between changes in the levels of selected contaminants in environmental matrices and variability in the measured indicators of biological effects,
- assessing the pollution status of different regions in the Baltic Sea.

When constructing the monitoring programme the following main items need special attention:

- selection of target species,
- overall sampling strategy,
- contaminants to be monitored (regularly monitored and screening of a wider spectrum of chemicals),
- set of methods to measure general and contaminant-specific biological effects at different levels of biological organisation,
- supporting parameters,
- development of assessment tools and criteria,
- QA/QC procedures,
- data storage and handling.

4.4.4 The BONUS programme and integrated monitoring of pollution in the Baltic Sea

BONUS is a project involving national research funding agencies in the Baltic Sea region with a main aim to enhance coordination of marine research activities in the Baltic Sea. Recently,

BONUS has been selected as a near-future EU research programme with additional funding from the EU. It should be considered of major importance that a project encompassing biological effects of pollution with a practical deliverable in the form of a design for a programme for integrated monitoring of pollution will be included in the BONUS programme.

Ideally, the new project should include all the following topics:

- biomonitoring studies, transplantation experiments, laboratory and mesocosm exposure studies, novel methods.data treatment (multivariate analyses and integrated approaches),
- evaluation and determination of chemical contamination in the Baltic Sea,
- evaluation and operational use of biological effects methods in the Baltic Sea,
- QA/QC,
- sampling design and statistical analysis, guidelines and formulation of the integrated monitoring programme,indicators of ecosystem health,socio-economic effects of pollution in the Baltic Sea.

4.4.5 References

Lehtonen, K.K., Schiedek, D. 2006. Monitoring biological effects of pollution in the Baltic Sea: neglected – but still wanted? Marine Pollution Bulletin (in press).

Lehtonen, K.K., Schiedek, D., Köhler, A., Lang, T., Vuorinen, P.J., Förlin, L., Baršienė, J., Pempkowiak, J., Gercken, J. 2006. The BEEP project in the Baltic Sea: overview of results and outlines for a regional biological effects monitoring strategy. Marine Pollution Bulletin (in press).

4.5 Conclusions

In the discussion of the presentations, there was consensus that studies on fish diseases (externally visible diseases and parasites, liver neoplasms, liver histopathology) are an important component of ecosystem health monitoring in the Baltic Sea. Although guidelines for fish disease surveys have been developed (largely by ICES) to a great extent, there still seems to be a need for improvement, particularly in studies conducted in the Baltic Sea (see further below in section 5 of the present report).

The WKFDM participants emphasised that integrated monitoring of contaminants (and other anthropogenic stressors) and their biological effects (including fish diseases) is the way to go in future because the results of such a programme would allow a much more comprehensive and holistic assessment of ecosystem health than traditional programmes that are often not harmonised or coordinated.

It was suggested that Baltic Sea countries and HELCOM may consider to modify the sampling scheme in their monitoring programmes. Traditionally, all countries involved are responsible for measuring, e.g., contaminants and biological effects in a certain geographical region allocated to the country. However, the parameters measured are more or less the same in all countries. The WKFDM participants felt that this strategy may lead to an inefficient use of resources and that ways for improvement should be explored. One possibility noted was to organise joint sampling campaigns and to appoint expert laboratories in Baltic Sea countries that are responsible for conducting measurements of certain parameters for all countries. The WKFDMO agreed that this idea should be put forward to Baltic Sea countries and to HELCOM as a recommendation.

In this context, a suggestion was put forward to organise an international research-vessel-based workshop or demonstration project in the Gulf of Finland, the aims of which would be to provide baseline data on ecosystem health of that region and to assess the feasibility of coordinated sample collection an analysis. The participants endorsed this proposal and it was

agreed that Baltic Sea countries, HELCOM and the ICES Study Group on Baltic Ecosystem Health Issues in Support of BSRP (SGEH) should consider to organise the workshop in the years 2007 or 2008.

4.6 Recommendations

The WKFDM recommends that:

i) Baltic Sea countries harmonise the components of their national marine monitoring and assessment programmes in order to implement an integrated programme on contaminants (and other anthropogenic stressors) and their biological effects,

ii) Baltic Sea countries and HELCOM investigate the potential for an internationally coordinated integrated monitoring programme in the Baltic Sea, encompassing joint sampling campaigns and the involvement of appointed expert laboratories in the Baltic countries responsible for the conduct of specific analytical measurements,

iii) ICES/BSRP, HELCOM and Baltic Sea countries organise an international demonstration project in 2007 or 2008 on the ecosystem health of the Gulf of Finland, providing baseline data and assessing the feasibility of coordinated sample collection and analysis.

5 National reports on fish disease monitoring

Each country represented at the workshop provided a report on national activities on research and monitoring activities related to diseases wild fish stocks. The reports are summarised in the following sections.

5.1 Report from Estonia

There have been studies on marine pollution and fish diseases in the Baltic Sea but there is still lack of systematic monitoring and assessment of the situation in Estonian waters.

In 1998, the **Estonian Marine Institute** closed its department of fish diseases. Until that time, studies on diseases (including neoplasms) and parasites of flounder and other species had been undertaken. In the 1980s, neoplastic lesions of Baltic fish neoplasms were also investigated in the **Institute of Experimental and Clinical Medicine**. However, these studies were terminated in 2000.

The **Department of Animal Health** of the Food and Veterinary Agency is monitoring only fish farms for Infectious Salmon Anaemia (ISA), Viral Haemorrhagic Septicaemia (VHS), Infectious Pancreas Necrosis (IPN) and Spring Viraemia of Carp (SVC).

Parasitological studies have been conducted in coastal fish species by the **Marine Institute of Tartu University**.

5.2 Report from Finland

K. Lehtonen presented a report on current activities in Finland. **The National Veterinary and Food Research Institute of Finland (EELA)** presently does not carry out research or monitoring of fish diseases directly related to environmental factors. However, assessments are being carried out focusing on infectious diseases of natural fish populations relevant for mariculture. Annual surveys on maternal salmonids *Salmo salar* and *Salmo trutta* m. *trutta* and whitefish *Coregonus lavaretus* (L.) cover Infectious Haemopoietic Necrosis (IHN), Viral Haemorrhagic Septicaemia (VHS), Infectious Pancreas Necrosis (IPN) and Bacterial Kidney Disease (BKD). These samples are taken regarding to EU legislated monitoring programme, and have been regularly carried out since 1995. The samples are taken from wild broodfish.

In addition, spawning lampreys have been studied during transfer from seawater to river water. Natural fish samples sent by private citizens from various parts of the Baltic Sea have also been studied for infectious fish diseases. A research project on VHS has been going on in EELA for a couple of years, and the effects of algal toxins have also been studied in co-operation with the **Finnish Game and Fisheries Research Institute**.

5.3 Report from Germany

T. Lang presented a report on current activities in Germany. The German fish disease monitoring in the Baltic Sea and in the North Sea is carried out by the **Federal Research Centre for Fisheries, Institute of Fishery Ecology** on an annual basis. First systematic studies started end of the 1970s. Until 1998, only winter surveys (Nov/Dec) took place, but since 1999, winter and summer (Aug/Sept) surveys are conducted.

Areas in the southern Baltic Sea, from Kiel Bight to Gulf of Gdansk, are sampled (see Figure 2), and additional sampling areas have been added for research purposes (e.g., as part of the EU-funded BEEP project, 2001–2004).

The main target species in the Baltic Sea are flounder (*Platichthys flesus*) and dab (*Limanda limanda*) (externally visible disease/parasites and liver histopathology), cod (*Gadus morhua*) (externally visible diseases/parasites) and herring (*Clupea harengus*) (externally and internally visible diseases/parasites). If resources permit, additional species are examined, e.g. whiting (*Merlangius merlangus*), plaice (*Pleuronectes platessa*) and 4-bearded rockling (*Rhinonemus cimbrius*).

Methodologies applied are according to ICES standard operating procedures (Bucke *et al.*, 1996; Feist *et al.*, 2004). Data on diseases in dab and flounder generated in the programme are submitted annually to the ICES Environmental Data Centre.

The diseases monitored are (target species in parentheses):

- Lymphocystis (flounder, dab, herring),
- acute/healing skin ulcerations (flounder, dab, cod),
- acute/healing fin rot/erosion (flounder, dab, cod),
- epidermal hyperplasia/papilloma (flounder, dab, cod),
- skeletal deformities (flounder, dab, cod),
- Hyperpigmentation (flounder, dab, cod),
- Pseudobranchial swelling (x-cell disease) (cod),
- *Cryptocotyle lingua/concavum* (flounder, dab, cod),
- *Lepeophtheirus pectoralis* (flounder, dab),
- *Lernaeocera branchialis* (cod),
- *Anisakis simplex* larvae (herring),
- *Ichthyophonus hoferi* (herring),
- macroscopically visible liver nodules (flounder, dab),
- liver histopathology (flounder, dab),
- gross liver parasites (flounder, dab).

Additional measurements/samples are done/taken on:

- Sex, length (cm below), age, weight, organosomatic indices,
- Contaminants (inorganic, organic, radioactivity; largely limited to HELCOM monitoring, commitments),
- Biomarkers (so far limited, e.g. EROD, PAH metabolites),
- Hydrography (water temperature, oxygen, salinity).

Literature cited:

Bucke, D., Vethaak, A.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, 19. 27 pp.

Feist, S.W., Lang, T., Stentiford, G.D., Köhler, A. 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.) for monitoring. ICES Techniques in Marine Environmental Sciences, 38. 42 pp.

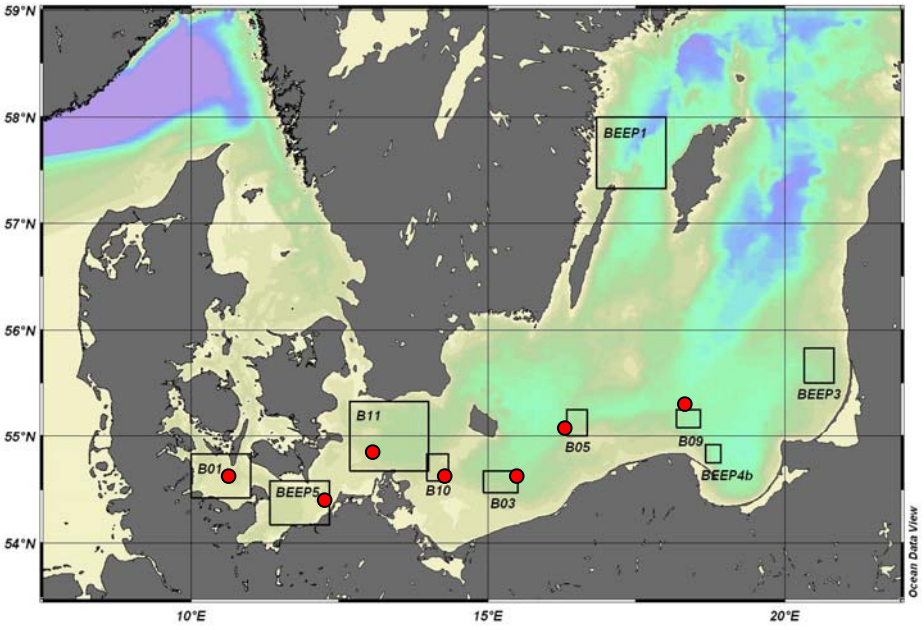

Figure 2: Sampling areas of the German long-term fish disease monitoring programme in the Baltic Sea (areas with red dots are routine stations visited on a regular basis, the rest are sites visited only temporarily, e.g. as part of the EU-funded BEEP project, 2001–2004)

5.4 Report from Latvia

M. Kirjušina presented a report on current activities in Latvia and on national organisations involved in fisheries and marine environmental research.

The **Latvian Fish Resources Agency (LATFRA)** is responsible for fisheries research and provides the elaboration of scientific advice concerning the sustainable exploitation and enhancement of living marine and freshwater resources. However, previous fish disease activities in the Baltic Sea have been terminated.

The scientific areas of responsibility of the **Institute of Aquatic Ecology (IAE)** are:

- Environmental monitoring of the Baltic Sea and the Gulf of Riga,
- Investigation of ecosystems of the Gulf of Riga estuaries and recreation areas under the conditions of anthropogenic load,
- Balance of heavy metals in the Gulf of Riga,
- Interaction of marine sediment micro- and macrobenthos,
- Turnover of organic carbon and nutrients in the Gulf of Riga,

- Aerobic-anaerobic biotransformation of contaminants in the sediments of brackish water basins,
- The influence of toxic algae on different levels of the marine trophic chain.

The **State Veterinary Medicine Diagnostic Centre (SVMDC)** performs functions of the National Reference Laboratory in the animal infection diseases examination. The animal infection diseases state control plan for 2005 encompasses obligatory state control activities for aquatic animals related to Viral Haemorrhagic Septicaemia (VHS) (B401) (salmonidae, pike, grayling), Infectious Haemopoietic Necrosis (IHN) (B405) and Infectious Salmon Anemia (ISA) (salmonidae). Once a year, five samples are taken from each hatchery for clinical and laboratory testing. In addition, five samples per year are taken from a number fish species from hatcheries, ponds and the Gulf of Riga for examination of parasitological diseases.

The **Marine and Inland Waters Administration's (MIWA)** responsibilities are:

- controls compliance with environment protection regulations in Latvian marine waters and fishing in Latvian marine and inland waters, international waters and waters of the EU member states and third countries,
- issues licenses and logbooks for fishing in Latvian marine and inland waters,
- ensures the functioning of fishing vessel monitoring system and fish landing control system in Latvian ports,
- approves contingency plans for ports, wharfs and terminals, and port waste management plans,
- collects, compiles and provides information on marine environment quality and use of natural resources in frames of its competency,
- carries out other duties stipulated in bylaws of MIWA.

5.5 Report from Lithuania

E. Bacevicius presented a report on activities and relevant organisarions in Lithuania.

The **Laboratory for Toxicology** (Vilnius University, Institute of Ecology, Department of Aquatic Ecosystems) investigates ecotoxic effects of environmental pollution and ecological changes in freshwater and marine ecosystems, including the assessment of genotoxic, cytotoxic and mutagenic effects of chemical compounds in aquatic organisms. Target species are molluscs (*Macoma baltica*, *Mytilus edulis*) and flounder (*Platichthys flesus*). The Laboratory has been invloved in a number of projects:

- The EU-funded project Biological Effects of Environmental Pollution in Marine Coastal Ecosystems (BEEP),
- The Baltic Sea Regional Project (BSRP)
- Development of environmental genotoxicity assays in fish and mussels for the application in monitoring of oil contamination (North, Barents and Mediterranean Seas) .

Some relevant references:

Baršienė J. 2002. Genotoxic impacts in Klaipėda Marine Port and Būtingė Oil terminal areas. Marine Environmental Research, 54: 475–479.

Baršienė, J. Lang, T., Broeg, K., Lehtonen, K.K., Vuorinen, P.J., Pempkowiak,, J., Šyvokienė, J., Dedonytė, V., Rybakovas, A. 2005. Biological effects of Environmental pollution in Fish and Mussels Inhabiting Klaipėda- Būtingė area (Baltic Sea). Jūra ir aplinka/ Sea and Environment, 1(12): 51–55.

Baršienė, J., Lazutka, J., Šyvokienė, J., Dedonytė, V., Rybakovas, A., Bagdonas, E., Bjornstad, A., Andersen, O. 2004. Analysis of micronuclei in blue mussels and fish from the Baltic and North Seas. Environmental Toxicology, 19(4): 365–371.

The **Lithuanian Centre for Sea Research, Subdivision for Ecotoxicology** (Department of Ministry of Nature Protection) conducts studies on:

- Monitoring of contaminant effects in marine ecosystems,
- Analysis of environmental genotoxic effects in freshwater and marine organisms,
- Assessment of environmental genotoxicity using passive and active (deployment) ecological monitoring.

The following projects are being carried out:

- Evaluation of the environmental state of the sea area in Lithuanian territorial waters and the economic zone adjacent to the Russian oil platform D-6. (Lithuanian Center for Sea Research, Finnish Institute of Marine Research and Vilnius University, Institute of Ecology),
- Assessment of genotoxic, cytotoxic and mutagenic effects of chemical compounds in aquatic organisms. Genotoxicity (Micronulei frecquency) in marine and freshwater ecosystems. Target species: molluscs *Macoma baltica* and *Mytilus edulis*), and flounder (*Platichthys flssus*).

The **Fishery Research Laboratory** of the Lithuanian State Center for Pisciculture and Fishery Research is involved in the monitoring of metazoan parasites of marine comercial and non-comercial fishes and the monitoring of external grossly visible diseases of cod, flounder, turbot, eelpout, sprat and herring, focusing on diseases such as skin ulcers, lymphocystis, papillomatosis, skeletal deformities, syndroms of fish development disturbances (cod dwarfism, nanism etc.).

5.6 Report from Poland

K. Trella presented the national report for Poland.

Regular studies on the health status of Baltic fish in the Polish EEZ (ICES Subdivisions 24, 25, 26) have been conducted by the **Sea Fisheries Institute** in Gdynia since 1981. These focus on the examination of externally visible symptoms of diseases in herring, sprat (*Sprattus sprattus*), cod and flounder and on the occurrence of *Anisakis simplex* larvae in herring.

Data are collected from research and commercial vessel catches and the sampling is carried out on a monthly basis. Observations on diseases are conducted during biological analyses and length measurements. The number of fish examined range from 70 000 to 100 000 per year. Parameters recorded in addition to the occurrence of diseases are total body length, weight, sex, gonad developmental stage (Maier's scale) and age.

Diseases recorded in cod, flounder and sprat are:

- Ulceration (5 developmental stages)
- Lymphocystis
- Skeletal deformites (dwarfism, vertebral column anomalies, pugheadednes)
- Developmental anomalies
- Reverse or incomplete eye migration in flatfish
- Opercular deformities
- Melanism, albinism, epidermal hyperemia and fin rot

Symptoms of disease are classified based upon standards established for each of fish species examined. Data recorded are:

- specification of disease (eg. lymphocystis)
- progression (phase of disease)
- location on fish body (for cod and flounder 15 locations in total)

In Baltic herring, regular studies are being carried out on spatial and temporal patterns in the infestation with larval nematodes (*Anisakis simplex*). The results show that the prevalence increases with fish length, is high in the 1st and 2nd quarter, is higher in coastal areas than in open sea, and has decreased over time since the end of the 1990s. The intensity of infestation (number of nematode larvae per infested herring) increases with fish length, increases eastward and decreased over time since the beginning of the 2000s.

In addition to the disease studies, biomarkers have been measured in flounder (acetylcholinestarase inhibition, glutatathion-S-transferase) and herring (acetylcholinestarase inhibition)

5.7 Report from Russia

N. Chukalova presented a report on current activities in Russia related to fish disease studies in the Baltic Sea.

In Russia, only the **Atlantic Scientific Research Institute of Marine Fisheries and Oceanography (AtlantNIRO)** in Kaliningrad conducts annual monitoring in the Baltic Sea as well as in the Curonian and the Vistula Lagoons since 1996. These investigations include two parts:

- examination of environmental factors and
- biological research of fishes.

Environmental monitoring is carried out by chemical and radiobiological laboratories and include studies of water temperature, pH, salinity, oxygen and ammonia concentrations, chemical and radioactivity contaminants. These laboratories also conduct the examination of fishes. Studies of fishes and water contaminants have been carried out since 2001. The main results of these investigations have been published in different scientific journals and in annual AtlantNIRO reports.

Biological research includes studies on stock structure, biology and distribution of fishing objects and investigations of parasites and fish diseases. Data about length, weight, age, somatic indices of gonad, stage of gonad maturation and population structures of fishes are collected by scientists of the Laboratory of the Baltic Sea and its Lagoons.

Scientists of the parasitological laboratory carry out monitoring of fish diseases and parasites. A total of about 5800 specimens of herring, sprat, flounder, cod and other fish species from the southern Baltic Sea and 4400 specimens of bream (*Abramis brama*), eel (*Anguilla anguilla*), perch (*Perca fluviatilis*), pikeperch (*Sander lucioperca*), roach (*Rutilus rutilus*) and other fish species from the Baltic Sea lagoons were investigated with different methods in the years 2004 and 2005. At present, fish disease monitoring involves investigations of parasitic infestation, external diseases, hematological indices and microflora composition.

Studies of fish diseases are based on the detection and description of visible evident changes on fish skin and in internal organs. Diseases symptoms in internal organs are investigated based on visual examination according to standardized morphopathological methods (Reshetnikov *et al*., 1999). Degrees of pathological processes in internal organ and on skin are estimated by using a normalized index of pathological changes in fish (Vasiljev, 2002).

Parasitological research includes the detection and identification of all parasitic species (protozoan and metazoans) in fish muscles and in internal organs (Bychovskaya- Pavlovskaya, 1985) and an estimation of indices of parasitic infestation (Margolis *et al*., 1982).

Microbiological investigations include studies on quantity and species composition of microflora and an identification of pathogenic factors among detected microorganisms.

Problems encountered with the conduct of regular fish diseases monitoring are a lack of modern equipments and trained scientists.

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Reshetnikov J.S., Popova O.A., Kashulin N.A., Lukin A.A., Amundsen P.- A., Staldwick F. 1999. Ozennka rybnoy chasty vodnogo soobzchestva s pomozchju morphopathologicheskogo methoda (Estimate of health of fishes part of the water community using morph pathological method)// Uspechi sovremennoy biologii (Progress of modern biology), 199 (2): 165–177 (in Russian).

Vasiljev A.S. 2002. Morphopathologicheskiy analis lezcha i sinza Rybinskogo vodochranilizsha (Morphopathological analysis of bream (*Abramis brama*) and blue bream (*Abramis ballerus*) from Rybinsk reservoire).// Voprosy rybolovstva (Problems of fisheries), Vol. 3, No. 4(12), p. 605–613 (in Russian).

5.8 Report from Sweden

A report on Swedish monitoring programmes on fish diseases in the Baltic region was presented by A. Alfjorden.

In the **Swedish Board of Fisheries** there are two institutes running continuous/ongoing programmes for external disease monitoring in this region. At the **Institute of Marin Research** there are two Baltic Sea stock assessment expeditions each year and during these expeditions cod and flounder are screened for externally visible lesions (P-O. Larsson, pers. communication). Almost all the cod and parts of the flounders (> 15 specimens) are examined for skin lesion (> 1mm) (skin ulcerations), lymphocystis and skeletal deformities. No investigations are done on inner lesions.

In the more coastal Swedish waters, the **Institute of Costal Research** carries out fish disease monitoring for externally visible lesions on the majority of all the fish species caught (mostly gillnet fishing). Lesions recorded are skin lesions (> 1 mm), skeletal deformities, tumours, fin rot/fin erosion, lymphocystis and other outer lesions. No investigations are done on lesion of internal organs. The fishes monitored are mostly from shallow areas caught during the warm season, mainly in August.

These investigation are part of several monitoring programmes: integrated fish monitoring (since 1983), regional fish monitoring (since 1991), recipient programmes (since 1962). Some of the sampling sites are integrated in the HELCOM COBRA (Coordination Organ for Baltic Reference Areas) programme.

5.9 Report from the United Kingdom

G. D. Stentiford presented a report on activities in the UK as an example of integrated fish disease monitoring programmes carried out in non-Baltic Sea countries.

Fish disease monitoring in the UK is undertaken by the **Centre for Environment, Fisheries and Aquaculture Science (Cefas)** as part of the integrated biological effects monitoring cruise of the UK National Marine Monitoring Programme (NMMP).

The cruise occurs in June and July each year and covers a range of sites in the North and Irish Seas (see map). The NMMP is an ongoing annual programme. It seeks to develop time trend data for a number of sites around the UK, with the core programme being augmented by special surveys. The programme manual (the 'Green Book') is available in a downloadable format from the Scottish Environmental Protection Agency (SEPA) website at: www.sepa.org.uk/marine. The work of the UK NMMP, which covers contaminant monitoring in sediments, water and benthos; benthic ecology; biological effects; aggregate extraction activities and sea disposal is organised into publicly available reports known as Aquatic Environment Monitoring Reports (AEMRs). These are available from the Cefas website: www.cefas.co.uk.

The fish disease component of the UK NMMP has been carried out for over 20 years and centres on the use of dab (*Limanda limanda*) as a sentinel species collected from offshore sites. Cod (*Gadus morhua*) are also assessed where captured. At estuarine sites, flounder (*Platichthys flesus*) is utilised in this role. Where sufficient numbers of other species are caught, a disease assessment is also undertaken on these species. Such species include plaice (*Pleuronectes platessa*), Dover sole (*Solea solea*), haddock (*Melanogrammus aeglefinus*), herring (*Clupea harengus*), whiting (*Merlangius merlangus*) and four-bearded rockling (*Rhinonemus cimbrius*). Sampling protocols follow those established by ICES for external disease assessments (Bucke *et al.,* 1999).

From all dab examined for external disease that harboured liver nodules greater than 2 mm in diameter, a section of the liver containing the nodule is collected for histological confirmation of the lesion type. Collection of this material and diagnosis of lesion type follows protocols set out by Feist *et al.* (2004) and following the Quality Assurance procedures set out under the Biological Effects Quality Assurance in Monitoring Programme (BEQUALM). In addition, standard sections of liver, gonad, kidney and spleen are sampled from 50 dab (greater than 20 cm total length) at each site, with the first 20 of these also being sampled for a range of biomarkers (including EROD, PAH metabolites in bile and DNA adducts). The otoliths are collected from each fish for age confirmation. Liver pathologies are assessed under the broad categories of (see Feist *et al.,* 2004):

- non-specific and inflammatory lesions,
- non-neoplastic toxicopathic lesions,
- pre-neoplastic lesions,
- benign neoplastic lesions and
- malignant neoplastic lesions.

Diseases measured externally in dab (on-board assessment at point of capture) include: lymphocystis (grades 1–3), epidermal papilloma (grades 1–3), skin ulceration (grades 1–3), skin hyperpigmentation (grades 1–3) and presence of gross liver nodules (diameter of lesion recorded). Histopathological analysis of the liver contributes a further 31 categories of data to individual fish. Overall, up to 50 individual data points may be collected for each specimen.

This data is applicable to multivariate analysis of disease in appropriate software packages. All data for external disease is formatted in accordance to the requirements of ICES and is submitted on an annual basis to the ICES databank.

References

Bucke, D., Vethaak, A.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, 19. 27 pp.

Feist, S.W., Lang, T., Stentiford, G.D., Köhler, A. 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder

(*Platichthys flesus* L.) for monitoring. ICES Techniques in Marine Environmental Sciences, 38. 42 pp.

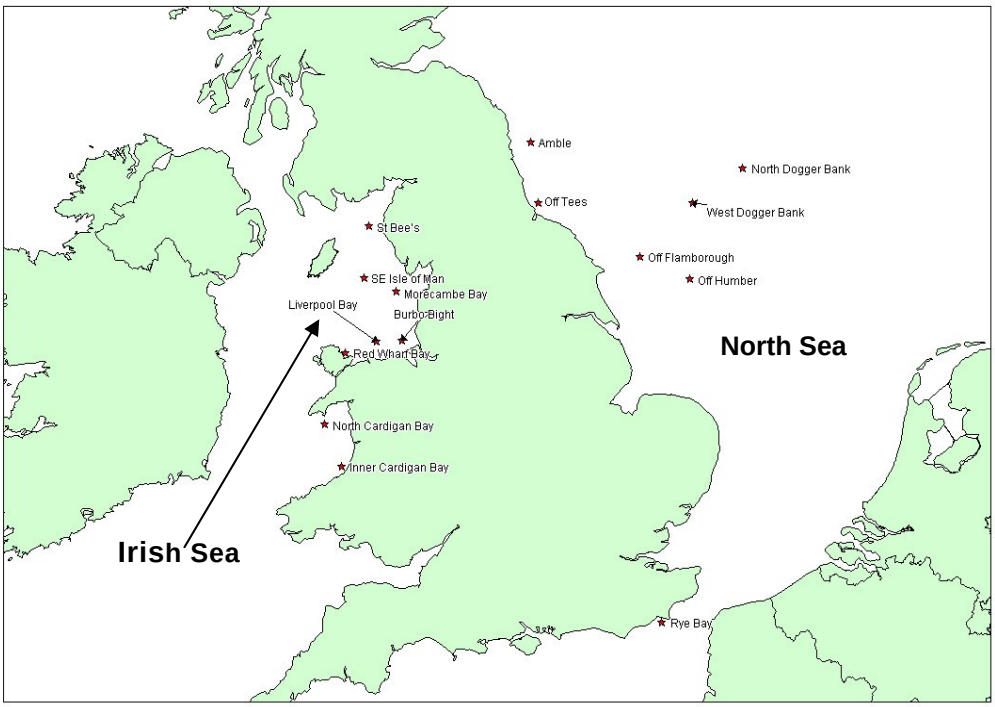


Figure 3: A typical range of sites from which fish disease data is collected during the annual Biological Effects Monitoring cruise of the UK NMMP.

5.10 Conclusions

The workshop participants emphasised the importance of studies on diseases and parasites in wild fish as part of ecosystem health monitoring and assessment programmes in the Baltic Sea and that, therefore, all Baltic Sea countries should use fish disease as a 'top-level indicator' of health in national monitoring and assessment programmes.

Based on the information presented in the national reports it was acknowledged that all Baltic Sea countries are carrying out studies on diseases of wild marine fish. However, it was noted that the extent to which this is being done is variable. The most extensive programmes are conducted by Germany and Poland and, to a certain degree, by Russia. Only these programmes can be considered as monitoring programmes because they are conducted on a regular basis.

However, it was noted with concern that the methodologies used in these programmes are partly different and may lead to incomparable data products. Therefore, it was highlighted that there is still an urgent need for improvements regarding standardisation. The workshop participants emphasised that the workshop constitutes an important step into this direction. Further improvements on a continuous basis will be achieved through participation in the Biological Effects Quality Assurance in Monitoring Programme (BEQUALM).

5.11 Recommendations

The WKFDM recommends that:

i) Baltic Sea countries use fish diseases as 'top-level indicators' of health in national integrated chemical and biological effects monitoring programmes

ii) Baltic Sea institutes involved in fish disease monitoring in the Baltic Sea are encouraged to participate in the Biological Effects Quality Assurance in Monitoring Programme (BEQUALM).

6 Practical work during the workshop

As part of the training programme, externally visible diseases and parasites of cod (*Gadus morhua*) and flounder (*Platichthys flesus*) were recorded. In addition, flounder livers were examined for the presence of liver nodules and parasites and herring were inspected for the presence of larval stages of parasitic nematodes (*Anisakis simplex*) and macroscopic cysts in the heart caused by *Ichthyophonus hoferi*. Plates A6.I–A6.III in Annex 6 provide images of these diseases/parasites.

6.1 Methodology

6.1.1 Sampling

Fishing was performed in five sampling areas (see Figure 1) by means of bottom trawling with a 140 ft standard bottom trawl. The towing time was 1 h, the speed 3–4 nm. Target fish species (cod, flounder, herring) were sorted from the catches and were immediately examined. The flounder were kept alive in running seawater of ambient water temperature.

Table 4: Grossly visible diseases recorded in Baltic cod (*Gadus morhua*), flounder (*Platichthys flesus*) and herring (*Clupea harengus*) and their causes (for illustrations, see Plates A6.I–A6.III in Annex 6)

COD (*GADUS MORHUA*)		FLOUNDER *(PLATICHTHYS FLESUS*)		HERRING (*CLUPEA HARENGUS*)	
DISEASE/PARASITE	AETIOLOGY	DISEASE/PARASITE	AETIOLOGY	DISEASE/PARASITE	AETIOLOGY
Acute/healing skin ulcers	Bacterial	Lymphocystis	Viral	Lymphocystis	Viral
Skeletal deformities	Multifactorial (natural and anthropogenic stressors)	Acute/healing skin ulcers	Bacterial	Skeletal deformities	Multifactorial (natural and anthropogenic stressors)
Pseudobranchial swelling (X-cell disease)	Parasitic (Amoeba-like)	Acute/healing fin rot/erosion	Bacterial	*Anisakis simplex*	Larval nematodes
Cryptocotyle lingua	Metacercariae of a parasitic digeneans	Skeletal deformities	Multifactorial (natural and anthropogenic stressors)	*Ichthyophonus hoferi*	Fungus-like parasite
Lernaeocera branchialis	Parasitic copepods	*Cryptocotyle* spp.	Metacercariae of two parasitic digenean species (C. lingua, C. concavum)		
		Liver nodules > 2 mm (macroscopical liver neoplasms)	Carcinogenic contaminants likely		
		Liver parasites	Nematodes, Acanthocephalans		

6.1.2 Examination for diseases

The inspection for externally visible diseases and parasites largely followed ICES guidelines (Bucke *et al.,* 1996) (see Annex 6) and focused on the body surface including the spread-out fins and the gill and mouth chambers. Prior to inspection for diseases, the fish were cleaned in water, sexed, weighed and length-measured (total length to the cm below). In some specimens, otoliths were removed for demonstration purposes.

For the inspection of flounder livers for nodules and parasites (again following ICES guidelines (Feist *et al*., 2004) (see Annex 6), the fish were anaesthetised by a blow on the head and killed by severing of the spinal cord.

The workshop participants created three groups focussing either on flounder, cod or herring and were advised regarding the standard operating procedures for disease examination and diagnosis. After a sufficient amount of training and intercalibration, the target species for the groups were changed.

Target diseases looked for in flounder, cod and herring are listed in Table 4

6.1.3 Biomarker sampling

At two sampling sites in the area BEEP3 (see Figure 1) flounder samples were also taken for the analysis of selected biomarkers (activities of ethoxyresorufin *O*-deethylase, catalase, glutathion-S-transferase, metabolites of polycyclic aromatic hydrocarbons, micronuclei test, acetylcholinesterase inhibition, metallothioneins, liver histopathology) and contaminants in tissues (heavy metals and phenols). The samples will be processed in a Finnish, Lithuanian and German collaboration.

6.1.4 Bacteriological sampling

Samples from cod with various stages of skin ulceration were taken for bacteriological examinations. The samples will be processed at the Swedish National Veterinary Institute, Uppsala.

6.2 Results of practical work

Results of the examination of flounder and cod for externally visible diseases and parasites are shown in Figures 4 and 5.

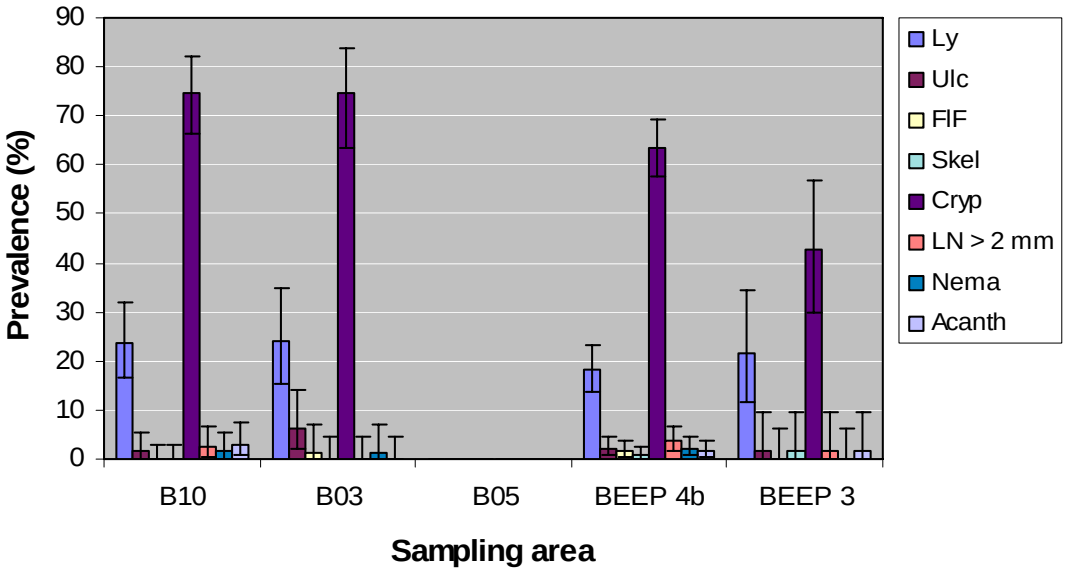


Figure 4: Prevalences (with 95% confidence intervals) of externally visible diseases in Baltic flounder (*Platichthys flesus*) recorded during the ICES Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM) (Ly: lymphocystis; Ulc: acute/healing skin ulcerations; FlF: acute/healing fin rot/erosion; Skel: skeletal deformities; Cryp: *Cryptocotyle* spp.; LN > 2 mm: liver nodules > 2 mm in diameter (not yet histologically confirmed); Nema: liver nematodes; Acanth: liver acanthocephalans)

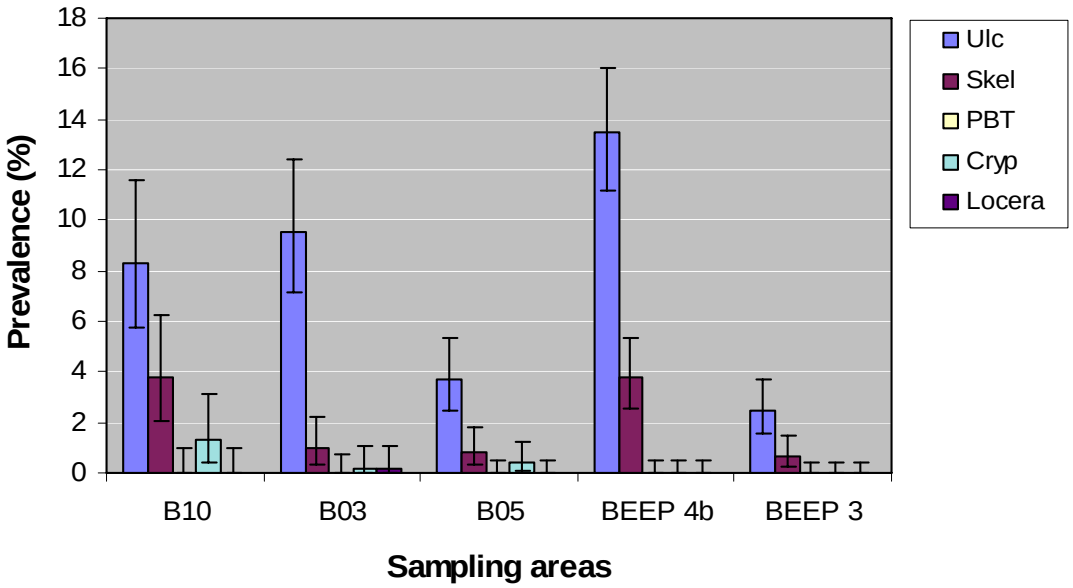


Figure 5: Prevalences (with 95% confidence intervals) of externally visible diseases in Baltic cod (*Gadus morhua*) recorded during the ICES Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM) (Ulc: acute/healing skin ulcerations; Skel: skeletal deformities; PBR: pseudobranchial swelling; Cryp: *Cryptocotyle lingua*; Locera: *Lernaeocera branchialis*)

In flounder, the presence of cysts of *Cryptocotyle* spp. was by far the most prevalent condition, followed by lymphocystis. The prevalence of the other disease conditions was low. Statistical differences between sampling areas (as to be seen from the confidence intervals) were only evident for *Cryptocotyle* spp. Information for area B05 is lacking because of a lack of flounder in that area.

In cod, acute and healing stages of skin ulcerations and skeletal deformities were most prevalent. Psudobranchial swelling was absent and the prevalence of *Cryptocotyle lingua* and

Lernaeocera branchialis was low. The data confirm earlier findings indicating that *Cryptocotyle lingua* is more common in the western compared to the eastern Baltic. There were some statistically significant differences between sampling areas for lymphocystis and for skin ulcerations.

The results of the examination of herring in areas BEEP 3 and BEEP 4b are shown in Table 5 Skeletal deformities and *Ichthyophonus hoferi* were absent and the prevalences of *Anisakis simplex* larvae and lymphocystis were low.

Table 5: Results of the examination of herring (*Clupea harengus*) from two sampling sites in the Baltic Sea for diseases and parasites during the ICES Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM)

AREA	BEEP 3	BEEP 4B
N examined	153	254
Size range	13–36 cm	13-28 cm
Prevalence of lymphocystis	0.00 %	0.39 %
Prevalence of skeletal deformities	0.00 %	0.00 %
Prevalence of *Anisakis simplex* larvae	0.00 %	1.57 %
Prevalence of *Ichthyophonus hoferi*	0.00 %	0.00 %

6.3 On board workshop on liver histopathology

A liver histopathology workshop was organised on board RV Walther Herwig III, providing the participants the possibility to familiarise with histopathological liver lesions commonly found in flatfish species and in particular in flounder and dab. Specimens demonstrated were examples from the UK National Marine Monitoring Programme in coastal and offshore areas of the North Sea and adjacent regions.

The participants were also made aware of possibilities for data recording an treatment, e.g. by using specific Excel spreadsheets.

6.4 Conclusions

The workshop participants emphasised the importance of intercalibration and standardisation of methodologies applied in fish disease monitoring. Since the workshop focused on offshore regions, it was suggested that another workshop should be organised in 2006 or 2007 on diseases in coastal fish. This would be of particular interest in relation to the HELCOM coastal fish monitoring in which externally visible lesions are recorded already now. However, guidelines are still lacking. The workshop could be carried out in the framework of the BSRP and could be organised by ICES (SGEH and WGPDMO). As venues, the AtlantNIRO in Kaliningrad, Russia, or the Estonian Marine Institute, Tallinn, were suggested.

6.5 Recommendations

The WKFDM recommends that:

i) ICES/BSRP organise a land-based workshop on methodologies for coastal fish disease monitoring. The workshop could be held in 2006 or 2007 at the AtlantNIRO, Kaliningrad, Russia, or at the Estonian Marine Institute, Tallinn.

6.6 References

Bucke, D., Vethaak, A.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, 19. 27 pp.

Feist, S.W., Lang, T., Stentiford, G.D., Köhler, A. 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder

(*Platichthys flesus* L.) for monitoring. ICES Techniques in Marine Environmental Sciences, 38. 42 pp.

7 Proposals for assessment tools: development of a fish health index

W. Wosniok and T. Lang presented information on the development of a fish health index.

7.1 Introduction

Most fish species monitored for diseases may be affected by a considerable number of diseases (externally visible diseases/parasites and histopathological liver changes) at a varying degree of severity/intensity and assessments on spatial and temporal disease prevalence patterns made so far were largely based on the analysis of data for individual diseases. However, the health status of a fish is composed of the sum of its diseases and their severity. Therefore, the workshop participants discussed possibilities to develop an index that summarise disease prevalence and intensity data for a set of diseases ideally in one simple figure and, thus, represents quantitative information on the health status of an individual fish. Such an index is considered to have the potential to be used as a tool for the development of Ecological Quality Objectives (EcoQOs) and consequently for quantitative ecosystem health assessments.

It was emphasised that sufficient empirical data exist from long-term monitoring programmes carried out, e.g., in the North Sea (mainly for dab, *Limanda limanda*) and the Baltic Sea (mainly for flounder, *Platichthys flesus*, and cod, *Gadus morhua*) that can be used to create and validate a health index. Particularly the North Sea data have been submitted to the ICES Data Centre and can be utilised.

It was emphasised that, for the development of such a health index, it is not only crucial to take into account data on the presence or absence of a disease, but also on the severity of the disease if present. Furthermore, it was considered important to take into account confounding factors known to affect the disease prevalence or intensity, in order to get an adjusted health/disease index which is independent from such factors and reflects only the impact of the target factor to be assessed, e.g. contaminant exposure. Confounding factors known to affect the disease prevalence or severity are, e.g., host-specific demographic factors (sex, age, size) or site-specific chemical or physical factors (salinity, oxygen, temperature).

7.2 Example for the construction of a fish health index

Table 6 shows an example of how such a health index can be constructed for flounder from the Baltic Sea, by using three test flounders of different length (18, 25 and 30 cm). The index is based on 4 components:

- a set of diseases/parasites
- information on the severity of the diseases
- a disease-specific weight
- an adjustment factor for size effects

Disease included are externally visible diseases/parasites (lymphocystis, skin ulcerations, *Cryptocotyle* spp.) and on pre-neoplastic and neoplastic histopathological liver lesions (foci of cellular alteration, benign tumours, malignant tumours). For each of these diseases, three grades (1–3) are assigned, reflecting the severity of the condition. The disease-specific weight (1–4) reflect the suspected impact of the disease on the host. As an example of the adjustment for confounding factors, a size adjustment factor has been incorporated in the model which is based on the natural relationship between the size of the fish and the disease prevalence. Since

this relationship may differ depending on the type of disease, disease-specific size adjustment factors have to be generated based on empirical data. In the present model, only two types or relationship were assumed, one reflecting the situation for lymphocystis and the other one for neoplastic liver lesions. For lymphocystis, empirical data indicate an increase in prevalence with increasing length of the fish to a length between 20 cm and 25 cm and, thereafter, a decline possibly due to acquired immunity or selective mortality. For neoplastic liver lesions, data available suggest an increase in prevalence with increasing length and age, respectively. For the other diseases (ulcerations and *Cryptocotyle* spp.), no relationship between the length of the fish and the prevalence was assumed in the model. The resulting curves for the length-specific adjustment factors (see Figures in Table 7) consequently have the opposite form. It has to be emphasised that these estimates are of a purely hypothetical nature and require validation before entering a final model applied on real data.

7.3 Calculation of the fish health index

Based on the model components, scores are calculated for the individual combination of disease, severity, weight and adjustment factor, which are added to raw scores for each of the three fish. The Health Index is created by calculating the percent proportion of the raw scores compared to the maximum score possible (see Table 6). The higher the index is the better is the health status of the individual fish.

7.4 Conclusions

In the discussion of the model there was an agreement that the approach is promising and should further be developed. A number of items were raised:

- Disease data in the ICES Databank so far do not include information on disease grades (indicating the severity) and ways should be explored how this can be changed.
- A critical point in the calculation of the Health Index is the use of disease-specific weights because these have a major impact on the Health Index. Although there was consensus that the definition of the weights will ultimately have to be based on expert judgement (more or less a 'wet finger approach'), as much scientific data on effects of diseases on the host as possible should be compiled as a basis for the definition of disease-specific weights.
- Another way to apply disease-specific weights would be to use them as a reflection of the responsiveness of a given disease to environmental stressors (either as a general or as a specific stress marker, depending on the purpose of the assessment) rather than as a reflection of the effects of the disease on the host.
- More adjustment factors (e.g. for age and gender) should be incorporated.
- There is a need to validate the model by using empirical data.
- If the approach will find a wider application, guidelines including information on minimum requirements for information needed to calculate a Health Index should be developed (types of disease, disease grades, adjustment factors, techniques to fill data gaps).

The participants were informed that the development of assessment tools for externally visible diseases/parasites and for liver histopathology is also on the agenda of the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) for its 2006 meeting. It was agreed that progress made by WKFDM in this context will be reported to WGPDMO.

7.5 Recommendations

The WKFDMO recommends that:

i) the ICES Working Group on Pathology and Diseases (WGPDMO) takes note of the proposals made by the WKFDM regarding the Fish Health Index and

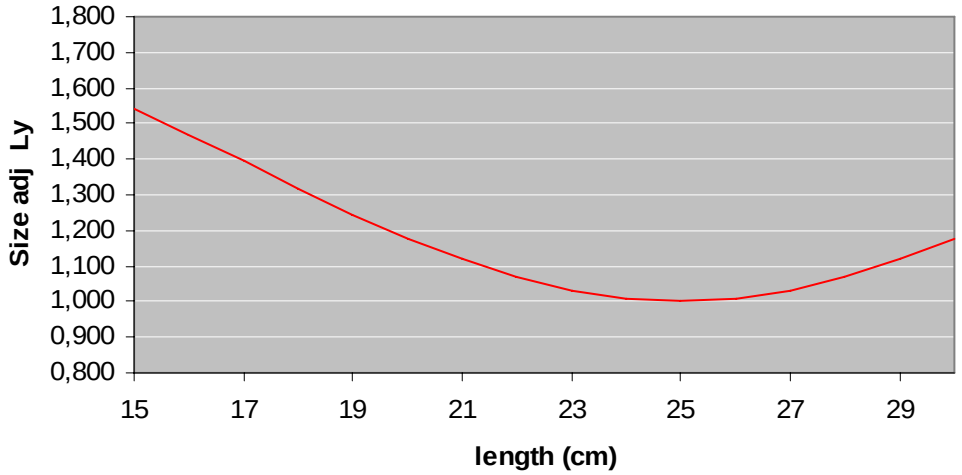
conducts work on its further development based on the conclusions made by WKFDM.

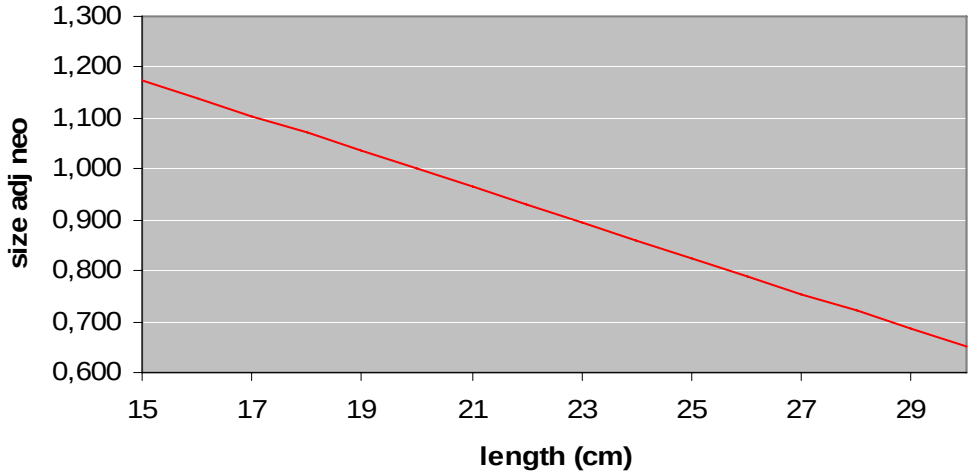
Table 6: Model for the calculation of a Health Index for flounder (*Platichthys flesus*), using hypothetical data on selected externally visible diseases/parasites and liver histopathologies

			Fish 1 (18 cm)			Fish 2 (25 cm)			Fish 3 (30 cm)		
Disease	**Grade**	**Disease-specific weight**	**Presence of disease**	**Size adjustment factor**	**Score**	**Presence of disease**	**Size adjustment factor**	**Score**	**Presence of disease**	**Size adjustment factor**	**Score**
Lymphocystis	1	2	1	1,32	2,64	0	1,00	0,00	0	1,18	0,00
	2	2	0	1,32	0,00	0	1,00	0,00	0	1,18	0,00
	3	2	0	1,32	0,00	0	1,00	0,00	1	1,18	7,06
Acute/healing skin Ulcers	1	2	0	1,00	0,00	1	1,00	2,00	0	1,00	0,00
	2	2	1	1,00	4,00	0	1,00	0,00	0	1,00	0,00
	3	2	0	1,00	0,00	0	1,00	0,00	1	1,00	6,00
Cryptocotyle spp.	1	1	0	1,00	0,00	1	1,00	1,00	0	1,00	0,00
	2	1	0	1,00	0,00	0	1,00	0,00	0	1,00	0,00
	3	1	1	1,00	3,00	1	1,00	3,00	1	1,00	3,00
Foci of cellular alteration	1	2	1	1,07	2,49	1	0,83	1,65	0	0,65	0,00
	2	2	0	1,07	0,00	0	0,83	0,00	0	0,65	0,00
	3	2	0	1,07	0,00	0	0,83	0,00	0	0,65	0,00
Benign tumours	1	3	0	1,07	0,00	0	0,83	0,00	0	0,65	0,00
	2	3	0	1,07	0,00	0	0,83	0,00	0	0,65	0,00
	3	3	0	1,07	0,00	0	0,83	0,00	1	0,65	5,85
Malignant tumours	1	4	0	1,07	0,00	0	0,83	0,00	0	0,65	0,00
	2	4	0	1,07	0,00	1	0,83	6,60	0	0,65	0,00
	3	4	0	1,07	0,00	0	0,83	0,00	0	0,65	0,00
			Raw score		***11,78***			***14,25***			***21,91***
			Health Index		***72,22***			***69,75***			***62,09***

Table 7: Calculation of size adjustment factors to compensate for effects of length on the presence of diseases

	Size adjustment factor lymphocystis	Size adjustment factor liver tumours and FCA
Param a	25,000	0,500
Param b	8,000	0,035
Size (cm)		
15	1,542	1,175
16	1,469	1,140
17	1,393	1,105
18	1,318	1,070
19	1,245	1,035
20	1,177	1,000
21	1,118	0,965
22	1,068	0,930
23	1,031	0,895
24	1,008	0,860
25	1,000	0,825
26	1,008	0,790
27	1,031	0,755
28	1,068	0,720
29	1,118	0,685
30	1,177	0,650
max	1,542	1,175





8 Guidelines for fish disease monitoring in the Baltic Sea

8.1 Introduction

As outlined , e.g., in section 4 of the present report, guidelines exist for the monitoring of

- externally visible fish diseases and parasites (Dethlefsen *et al*. 1986; ICES, 1989; Bucke *et al*., 1996)
- macroscopic liver tumours (liver nodules) (Bucke *et al*., 1996; Feist *et al.*, 2004)
- liver histopathology (Feist *et al*., 2004)

These have largely been developed and published through ICES activities and as part of the fish disease component of the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM) programme that started as an EU-funded project and has evolved into a self-funding QA programme in the meantime. Guidelines (based on the ones above) also exist in the Technical Annexes to the OSPAR Guidelines for general biological effects monitoring (OSPAR, 1997) and those for PAH-specific biological effects monitoring (OSPAR, 2003) since fish disease monitoring has been incorporated in the OSPAR Coordinated Environmental Monitoring Programme to be carried out in waters of the North-East Atlantic (OSPAR, 2004)[1].

8.2 Conclusions

The workshop participants discussed the existing guidelines for fish disease monitoring and agreed that completely new guidelines for the Baltic Sea are not needed because methodologies detailed in the existing ones are sufficiently comprehensive to be used for Baltic Sea studies, at least those focusing on dab (only in the western Baltic Sea), flounder and cod. However, it was felt beneficial to summarise the main items relevant for the Baltic Sea situation in the present report and to add some information on studies in other fish species to the present report as an annex (Annex 6).

8.3 Recommendations

The WKFDM recommends that:

i) Baltic Sea countries conducting fish disease studies should apply the guidelines developed by ICES and through the BEQUALM programme with the amendments proposed by WKFDM summarised in the present report as Annex 6.

8.4 References

Bucke, D., Vethaak, A. 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, 19. 27 pp.

Dethlefsen, V., Egidius, E., McVicar, A. H. 1986. Methodology of fish disease surveys. Report of an ICES Sea-going Workshop held on RV 'Anton Dohrn' 3–12 January 1984. ICES Cooperative Research Report, 140. 33 pp.

[1] At its 2005 meeting, the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) identified a need for some amendments in the OSPAR Guidelines for fish disease monitoring. Recommendation were published as ICES Advice (ICES 2005. ICES Advice http://www.ices.dk/committe/acfm/comwork/report/2005/may/Technical%20Annex%20to%20the%20review%20of%20WKIMON.pdf

Feist, S.W., Lang, T., Stentiford, G.D., Köhler, A. 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.) for monitoring. ICES Techniques in Marine Environmental Sciences, 38. 42 pp.

ICES. 1989. Methodology of fish disease surveys. Report of an ICES Sea-going Workshop held on RV U/F 'Argos' 16–23 April 1988. ICES Cooperative Research Report, 166. ICES, Copenhagen.

ICES. 1997. Report of the Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants. ICES CM 1997/F:2.

OSPAR. 1997. JAMP Guidelines for general biological effects monitoring. OSPAR Commission Ref. No. 1997-7.

OSPAR. 2003. JAMP Guidelines for contaminant-specific biological effects monitoring, OSPAR Commision Ref. No. 2003-10.

OSPAR. 2004. OSPAR Coordinated Environmental Monitoring Programme (CEMP). OSPAR Commission Ref. No. 2004-16.

9 General conclusions, analysis of progress with tasks

The participants considered the workshop to be successful since the Terms of Reference were fulfilled.

There was an agreement that the results of the workshop should be communicated to a wider forum, including responsible national authorities and international organisations coordinating marine environmental monitoring programmes.

A number of follow-up activities were suggested, building on the experience made during the sea-going workshop.

A number of recommendations were made provided in Annex 7.

Annex 1: List of participants

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Kari Lehtonen	Finnish Institute of Marine Research POB 2 FI-00561 Helsinki Finland	+358961394566/+35893232970	lehtonen@fimr.fi
Kaja Lotman	The Matsalu National Park Administration, Penijoe 90305 Lihula Läänemaa Estonia	+3725247899/-	kaja@matsalu.ee

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Grant D. Stentiford	CEFAS Weymouth Laboratory Weymouth Dorset DT4 8UB UK	+441305206722/+441305206601	grant.stentiford@cefas.co.uk
Kordian Trella	Sea Fisheries Institute ul. Kollataja 1 81-332 Gdynia Poland	+480587356266/+480586202831	trella@mir.gdynia.pl
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Annex 2: WKFDM Terms of Reference 2005

2005/2/BCC02 An **ICES/BSRP Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea** [WKFDM] (Co-Chairs: Thomas Lang*, Germany, and G. Rodjuk*, Russia) will meet from 5–12 December 2005 onboard RV Walther Herwig III to:

a) provide training and intercalibration related to methodologies applied in fish disease monitoring in the Baltic Sea;

b) further develop and assess health indicators and indices appropriate for monitoring and assessment purposes;

c) establish a closer collaboration between institutes involved in fish disease monitoring in the Baltic Sea;

d) build the basis for incorporation of fish disease surveys into the revised HELCOM monitoring programme.

WKFDM will report by 31 January 2006 for the attention of the Baltic Committee.

Supporting Information

PRIORITY:	ACME welcomed the plan for the workshop and emphasised that it will constitute a major step forward in the establishment of a coordinated fish disease monitoring programme in the Baltic Sea.
SCIENTIFIC JUSTIFICATION AND RELATION TO ACTION PLAN:	Action Plan No.: Since there is an apparent need to further intercalibrate methodologies to be used for fish disease monitoring in the Baltic Sea, the ICES Study Group on Ecosystem Health in Support of BSRP (SGEH) and the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) suggested to hold a practical sea-going workshop under the auspices of ICES/BSRP with specialists in this field and with trainees from the eastern countries. The workshop will be held onboard the German RV Walther Herwig III with Gdynia as port of embarkation. The major target fish species will be flounder (*Platichthys flesus*), herring (*Clupea harengus*), sprat (*Sprattus sprattus*) and cod (*Gadus morhua*). These species will be sampled on a transect with selected sites representing different environmental conditions. If appropriate, samples can be taken for subsequent lab-based measurements, e.g. on biomarker responses (e.g. as part of the planned BSRP BIODEMO Project on Biological Effects of Contaminants).
RESOURCE REQUIREMENTS:	-
PARTICIPANTS:	Twelve scientists will participate, including training experts (on methodologies for fish disease surveys in the Baltic Sea, epidemiology of infectious and non-infectious diseases and parasites, liver histopathology, data assessments, quality assurance) and trainees from Baltic Sea countries, with priority given to eastern BSRP countries.
SECRETARIAT FACILITIES:	None
FINANCIAL:	Funding (travel and per diem) will be required for scientists from the eastern recipient countries, for a trainer representating the BEQUALM lead laboratory on fish diseases and liver histopathology at CEFAS, Weymouth, UK, whose participation is essential in order to guarantee compliance with the BEQUALM quality assurance activities, and for a western expert from the Univ. Bremen, Germany, on survey design and statistical requirements. Ship time, accommodation and food on board, the use of equipment as well as time allocation by western experts constitute a significant in-kind contribution by western countries to the BSRP.
LINKAGES TO ADVISORY COMMITTEES:	ACME
LINKAGES TO OTHER COMMITTEES OR GROUPS:	MHC, MCC, BC
LINKAGES TO OTHER ORGANISATIONS:	HELCOM, BSRP
SECRETARIAT MARGINAL COST SHARE:	ICES 100%

Annex 3: Agenda and timetable

DATE	TIME	ACTIVITY
05.12.	11:30/12:00	*Lunch*
	15:00	RV W.H. III leaves Gdynia Port heading for the first sampling area
	15:30	Official start of the workshop, welcome (*T. Lang*)
		Introduction of participants
		Presentation of the workshop programme (*T. Lang*)
		Distribution of tasks (e.g. selection of rapporteurs)
		Security instructions, tour around the ship
	17:30/18:00	*Dinner*
	19:00	Previous efforts to standardise methodologies (e.g. ICES) (*T. Lang*)
	20:00	Icebreaker Party
06.12.	07:30	*Breakfast* Sampling area: B03
	08:00	First catch on deck
		Create teams
		Familiarise with the working procedures
		Lab work
	11:30/12:00	*Lunch*
	12:30	Continue practical work
	17:30/18:00	*Dinner*
	19:00	Presentation of national reports (*all*)
07.12.	7:30	*Breakfast* Sampling area: B10
	8:00	Practical work
	11:30/12:00	*Lunch*
	12:30	Practical work
	17:30/18:00	*Dinner*
	19:00	Introduction histopathology (*G. Stentiford*)
08.12.	7:30	*Breakfast* Sampling area: B05
	8:00	Histopathology, practical work
	11:30/12:00	*Lunch*
	12:30	Histopathology, practical work
	17:30/18:00	*Dinner*
	19:00	Data treatment (*W. Wosniok*)
09.12.	7:30	*Breakfast* Sampling area: BEEP 3
	8:30	Practical work
	11:30/12:00	*Lunch*
	12:30	Practical work
	17:30/18:00	*Dinner*
	19:00	Integrated monitoring (*K. Lehtonen*)
		Discussion on Health Indicators/Indices
10.12.	7:30	*Breakfast* Sampling area: BEEP 4b
	8:30	Practical work
	11:30/12:00	*Lunch*
	12:30	Practical work
		Cleaning of working area
	17:30/18:00	*Dinner*
	19:00	Drafting of report sections
11.12.	7:30	*Breakfast*
	8:00	Wrap-up session
		Recommendations
		Status of Report (*T. Lang*)
		Miscellaneous
	11:30/12:00	*Lunch*
		Berthing in Gdynia Port
	18:00	Reception/farewell party on board with invited guests
12.12.	7:30	*Breakfast*
	9:00	End of the workshop, participants leave
		RV W.H. III leaves Gdynia Port

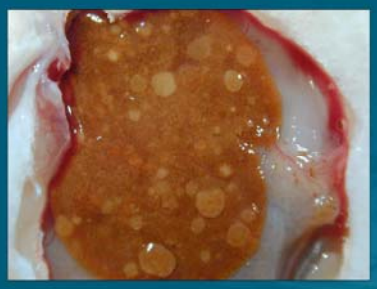
Annex 4: The status of monitoring liver histopathology in wild marine fish

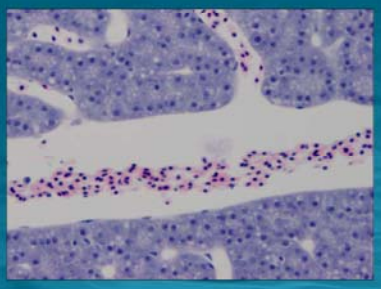
by
G. D. Stentiford

Liver histopathology

ICES/BSRP Sea-going workshop on fish disease monitoring in the Baltic Sea

5-12 December 2005





Grant D. Stentiford and Steve W. Feist

Cefas Weymouth Laboratory, Barrack Road, Weymouth, Dorset DT4 8UB

Cefas

The liver

- Special function as a guardian between the digestive tract and the rest of the body
- Blood channels running in to the liver import a large variety of endobiotics and xenobiotics (e.g. nutrients and toxic substances derived from the gut)
- Major function in uptake, storage and metabolic conversion of nutrients (e.g. carbohydrates, amino acids, lipids). Subsequent release to blood and bile.
- Involved in biotransformation: conversion of hydrophobic substances to water soluble derivatives for export in urine or bile.
- Liver also integrated into immune defence system: e.g. phagocytosis
- Functional unit is the hepatocyte : these enclose a complex network of blood vessels and bile canals.
- Due to high metabolic activity of hepatocytes, they have a large array of organelles (ER, Golgi, mitochondria, lysosomes, peroxisomes)

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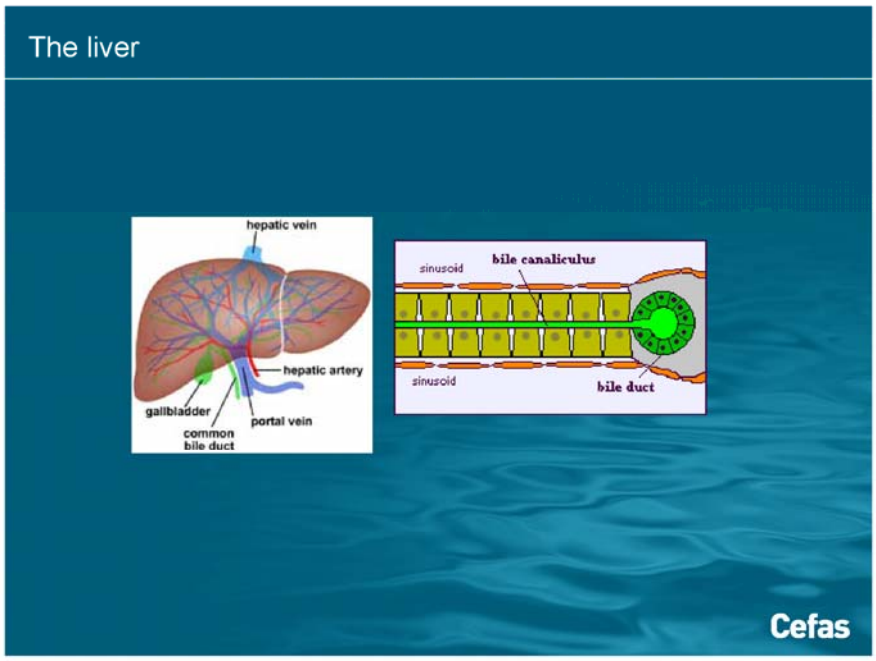
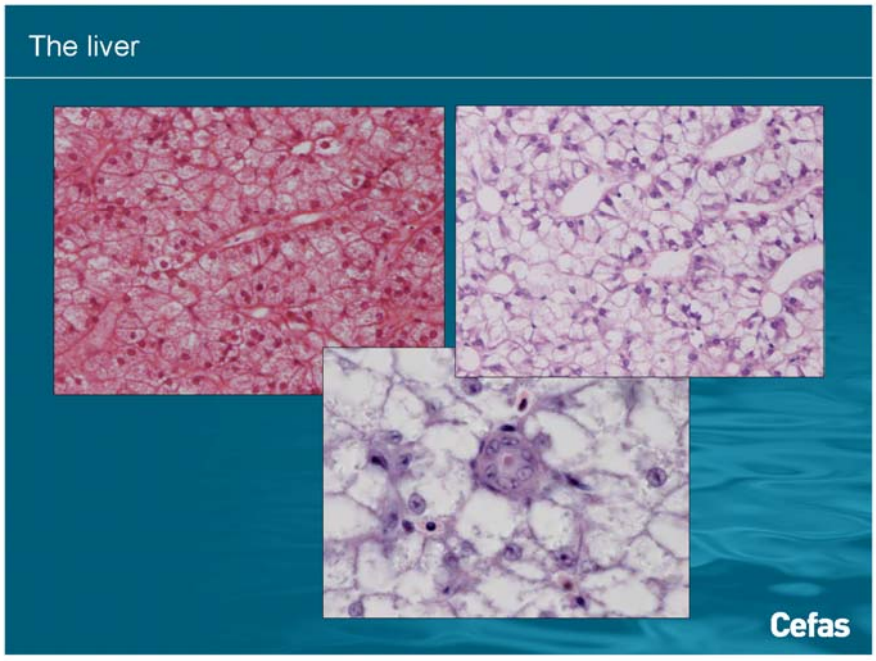

The liver
hepatic vein
hepatic artery
gallbladder
portal vein
common bile duct
sinusoid
bile canaliculus
sinusoid
bile duct
Cefas


The liver
Cefas

Chemical induction of liver cancer

- 7th most frequent cancer in humans (10/100,000)
- Large body of evidence linking environmental chemicals to cancer in human and animals (particularly via feeding studies)
- Several naturally occurring compounds can also induce tumours (e.g. aflatoxin β1 from *Aspergillus* moulds).
- Parent compounds may act as 'complete carcinogens' – BUT often it is their metabolites (produced within the hepatocyte) that are carcinogenic ('metabolic activation')
- Metabolic activation can create metabolites that have electron-deficient sites that can bond to cellular macromolecules such as DNA
- Therefore, 'procarcinogens' are transformed to 'ultimate' carcinogens that interact with the cell macromolecules to induce neoplasia.
- 'Adducts' are formed between ultimate carcinogens and macromolecules (e.g. with the nucleic acid guanine in DNA). Some are very stable.
- Mutation of genes during DNA repair following adduct formation are likely involved in neoplastic process (e.g. genes responsible for controlling cell cycling).

Cefas

Chemical induction of liver cancer

- Complete carcinogen: any agent, in single or repeated application, resulting in the development of malignant hepatic neoplasia (initiate, promote and progress liver cells).
- Initiating agent: capable of initiating cells but not causing further development of neoplastic potential.
- Promoting agent: results is amplification of initiated cells in a clonal and reversible manner. Incapable of initiation or progression.
- Progressing agent: capable of directly inducing measurable chromosomal abnormalities (including karyotypic alterations), but incapable of initiation or promotion.

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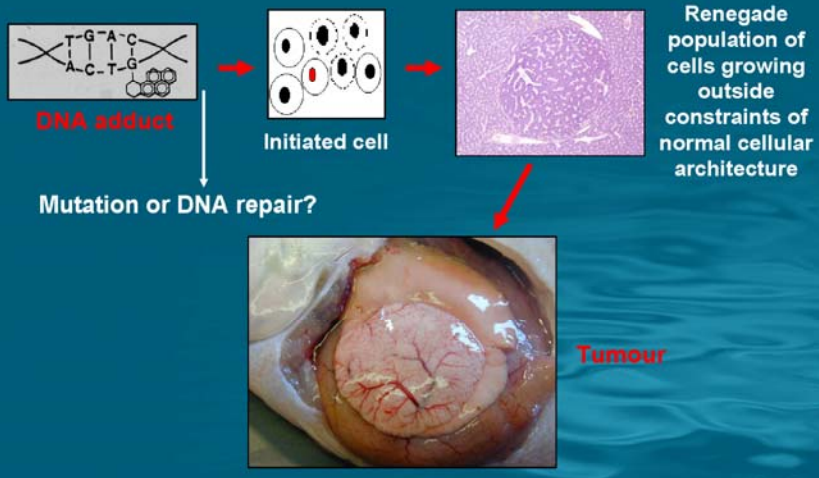
Biological effects of PAH exposure - carcinogenesis



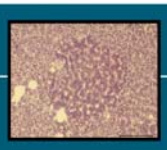
Cefas

Pathogenesis of liver neoplasia

- Stepwise process – at least three major stages (Initiation, Promotion and Progression).
- Different parent chemicals and their metabolites may play different roles in this process – dose and order of exposure is likely to influence carcinogenesis.
- 'Down the microscope' – this is likely reflected in the appearance of 'foci of cell alteration' (preneoplastic lesion) and hepatocellular adenoma and carcinoma (neoplastic lesions).
- FCA (or enzyme altered foci) have potential to develop to malignancy (carcinoma) – but quantitative studies in rats show that most do not. FCA can also occur in the liver of 'control' animals.
- Cell division (e.g. during liver regeneration) is likely to be critical in multistage carcinogenesis (can occur following toxic insult, damage or infection).
- Reversibility of lesions induced by initiators and subsequently promoted is commonly observed in rodent cancer studies.
- Also, once cells are initiated, re-growth (and progression) of FCA to neoplasia appears irreversibly maintained in the tissue.

Cefas

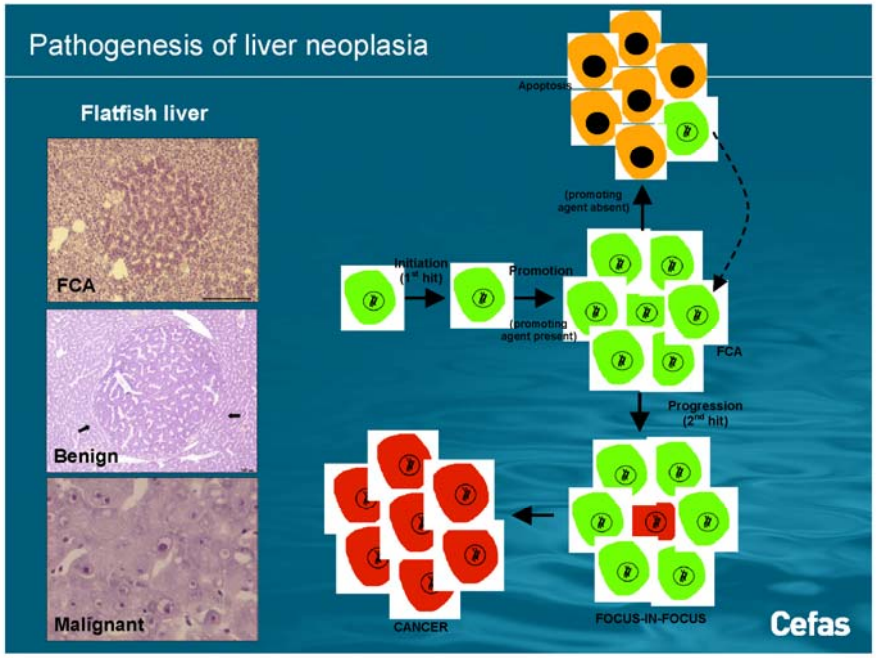
Pathogenesis of liver neoplasia



- FCA apparently depend on the continued presence of promoting agents for their development to neoplasia (removal of promoter causing apoptosis of lesion).
- Age, hormonal and dietary influences also affect the promotion stage of carcinogenesis.
- Initiation forms irreversibly altered hepatocytes with their clonal progeny being reversibly expanded by promoting agents.
- The Progression stage of carcinogenesis is irreversible and leads to formation of benign or malignant neoplasms.
- Chemical agents that act only in the interface between promotion and progression are not well characterised.
- Gross morphological, molecular and growth changes are seen in lesions undergoing progression.

Cefas

Pathogenesis of liver neoplasia



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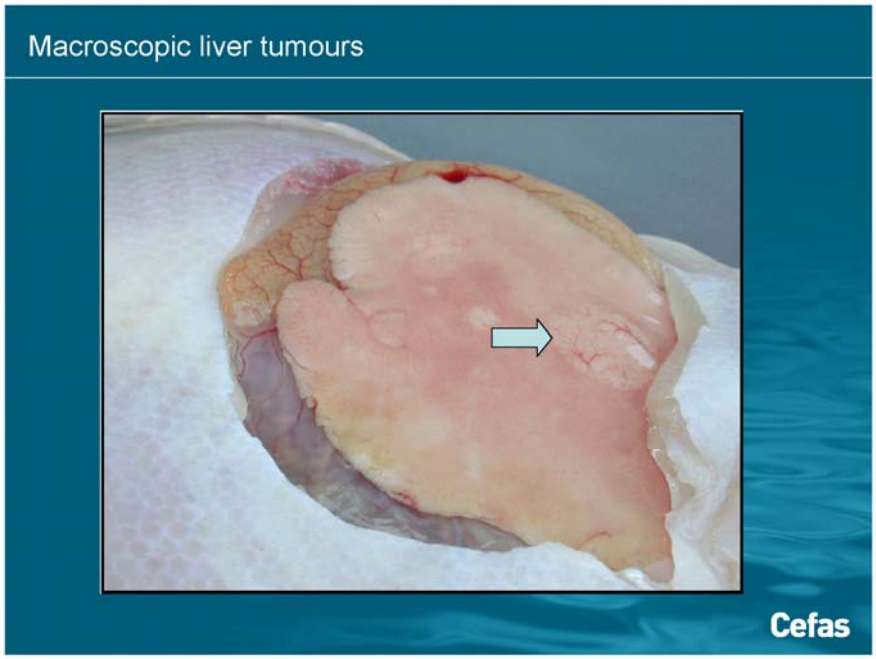
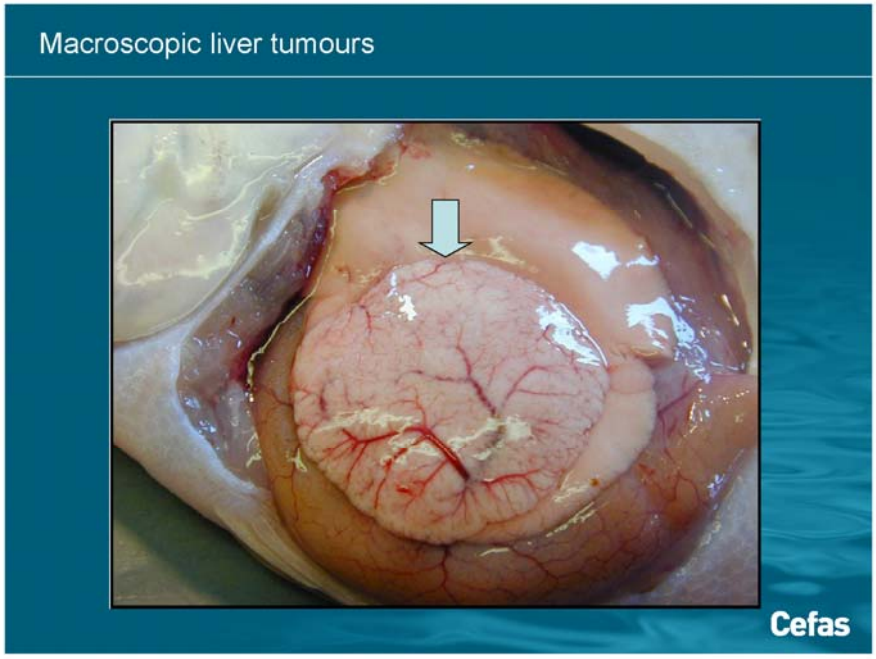
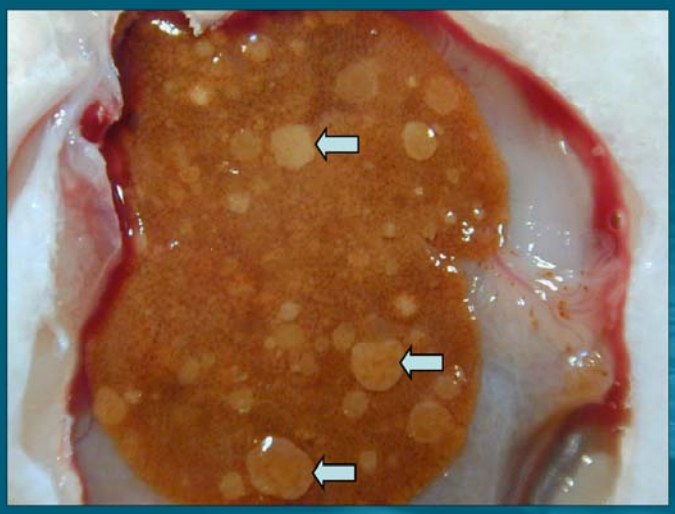

Macroscopic liver tumours
Cefas


Macroscopic liver tumours
Cefas

Macroscopic liver tumours



Cefas

BEQUALM

- Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM).
- Provides standard protocols for monitoring.
- Several work packages covering a range of biological effects techniques.
- Cefas Weymouth laboratory (UK) leads the work package on fish diseases.
- Ring tests and inter-calibration exercises aim to assess the performance of participating laboratories and serve to identify areas that require attention.
- Coherence to QA standards likely to be required for submission of data to ICES
- Next workshop in March 2006 (Weymouth, UK)

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Liver lesion classification

1. No Abnormalities Detected (NAD)

EARLY NON-NEOPLASTIC TOXICOPATHIC LESIONS
2 Phospholipidosis
3 Fibrillar inclusions
4 Hepatocellular and nuclear polymorphism
5 Hydropic degeneration
6 spongiosis hepatis

FOCI OF CELLULAR ALTERATION
7 Clear cell
8 Vacuolated
9 Eosinophilic
10 Basophilic
11 Mixed

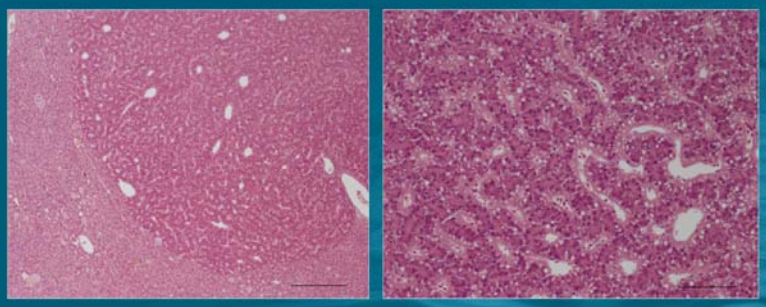
BENIGN NEOPLASMS
12 Hepatocellular adenoma
13 Cholangioma
14 Hemangioma
15 Pancreatic acinar cell adenoma

MALIGNANT NEOPLASMS
16 Hepatocellular carcinoma
17 Cholangiocarcinoma
18 Pancreatic acinar cell carcinoma
19 Mixed hepatobiliary carcinoma
20 Hemangiosarcoma
21 Hemangiopericytic sarcoma

NON SPECIFIC INFLAMMATORY LESION
22 Coagulative necrosis
23 Apoptosis
24 Lipoidosis
25 Hemosiderosis
26 Variable glycogen content
27 Melanomacrophage centres
28 Lymphocytic/monocytic infiltration
29 Granuloma
30 Fibrosis
31 Regeneration

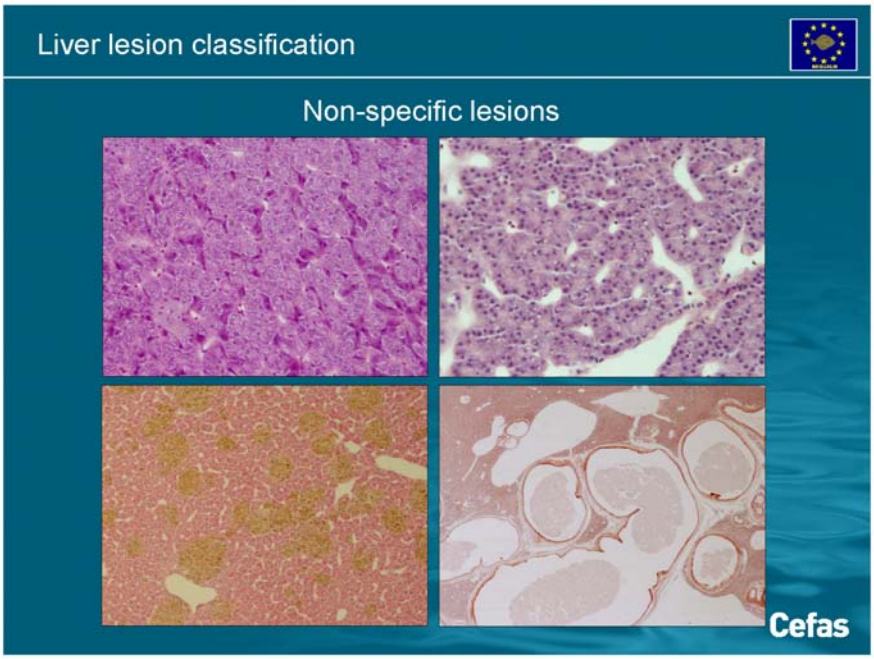
Cefas

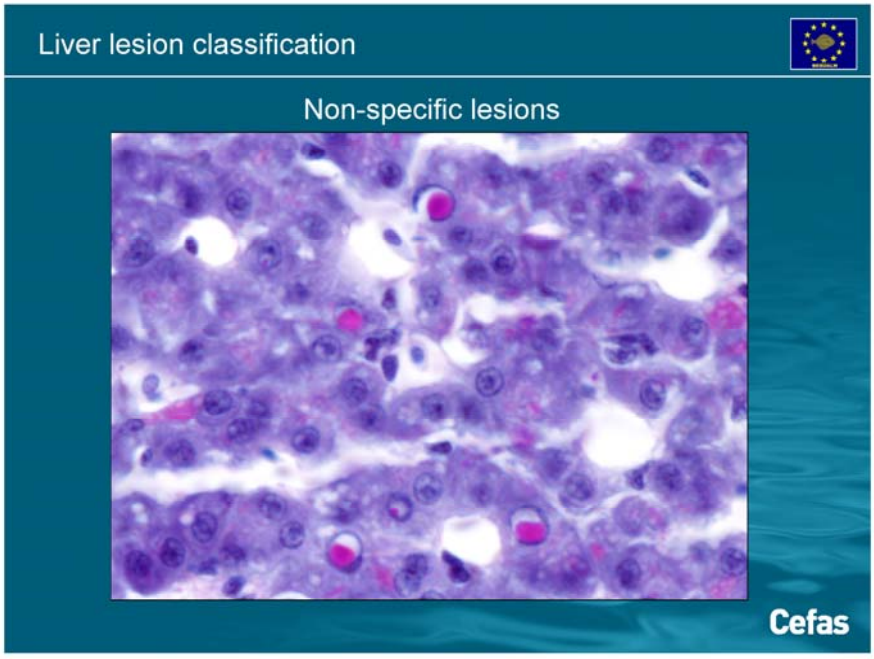
Liver histopathology – quality assurance

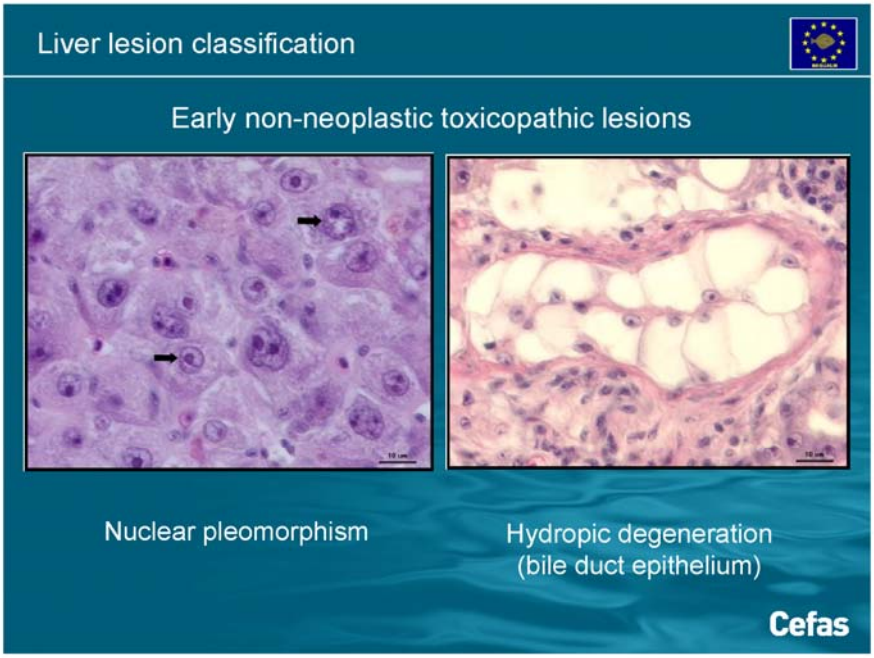


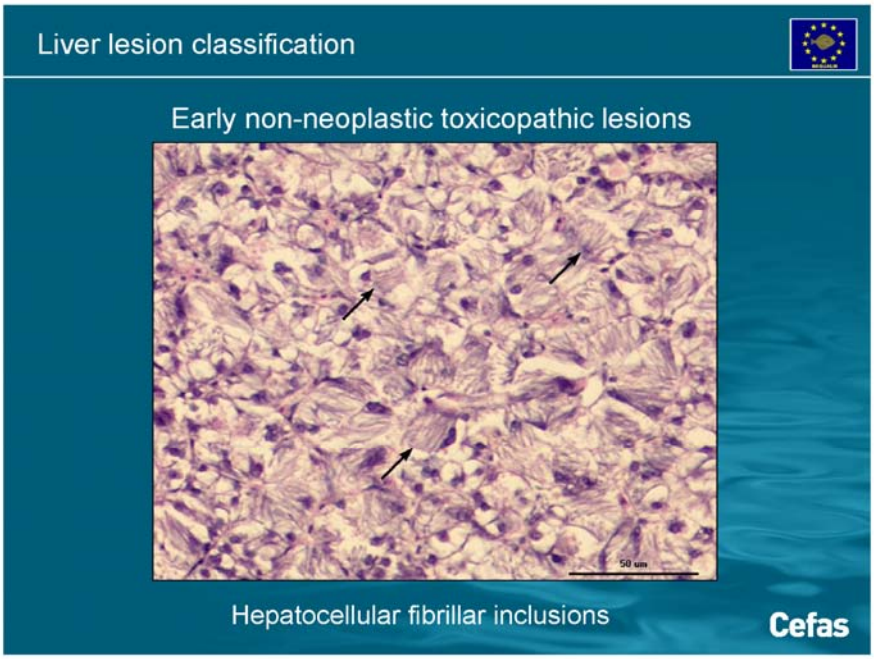
Feist, S.W., Lang, T., Stentiford, G.D., Koehler, A. (2004). Use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L..) for monitoring. ICES Techniques in Marine Environmental Sciences 38. 42pp.

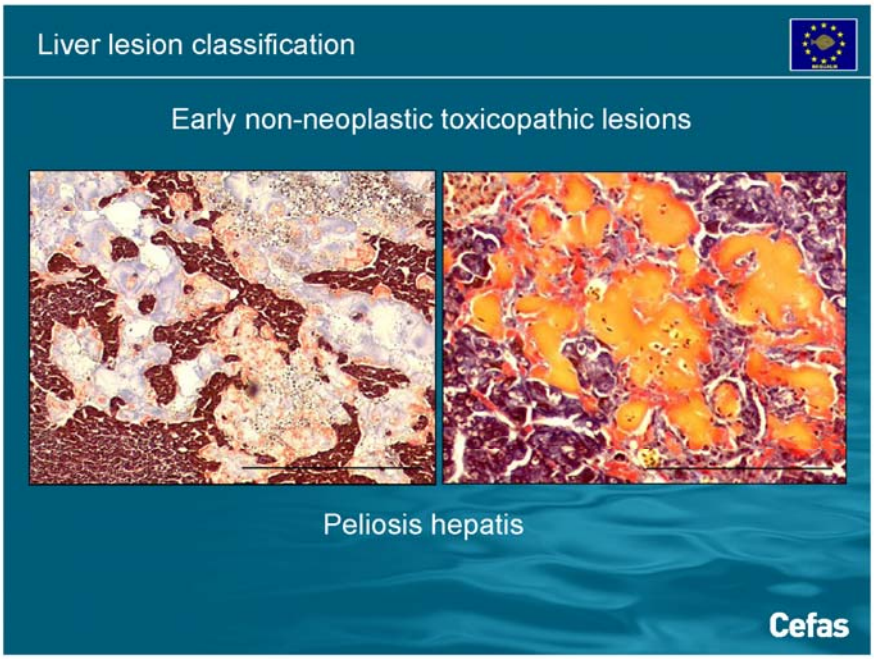
Cefas

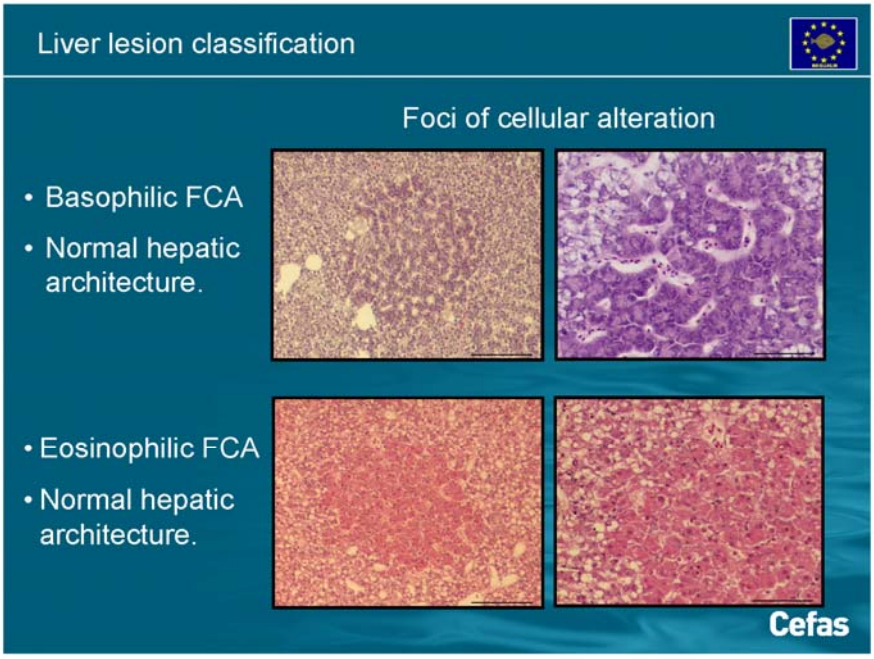

Liver lesion classification
Non-specific lesions
Cefas

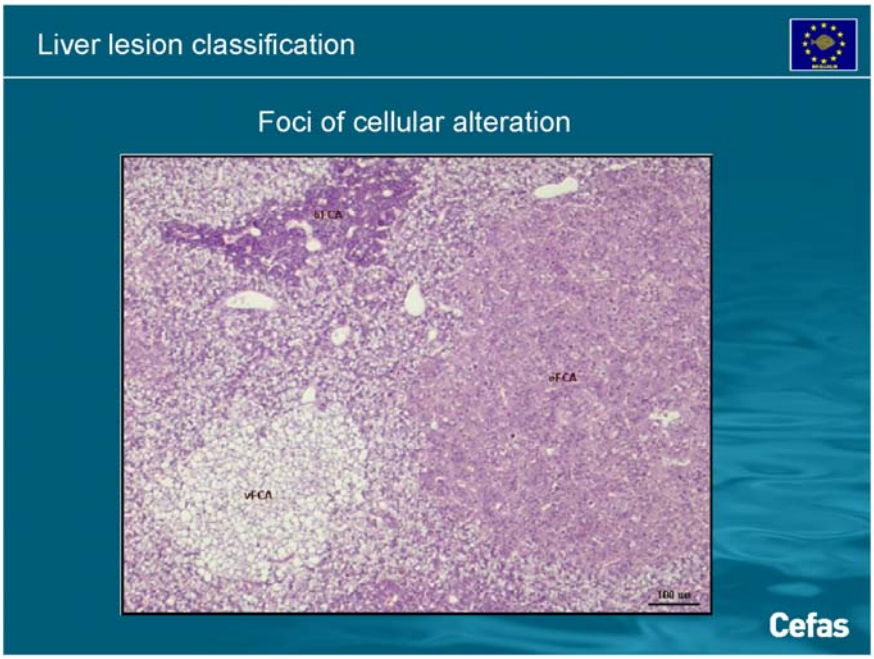

Liver lesion classification
Non-specific lesions
Cefas

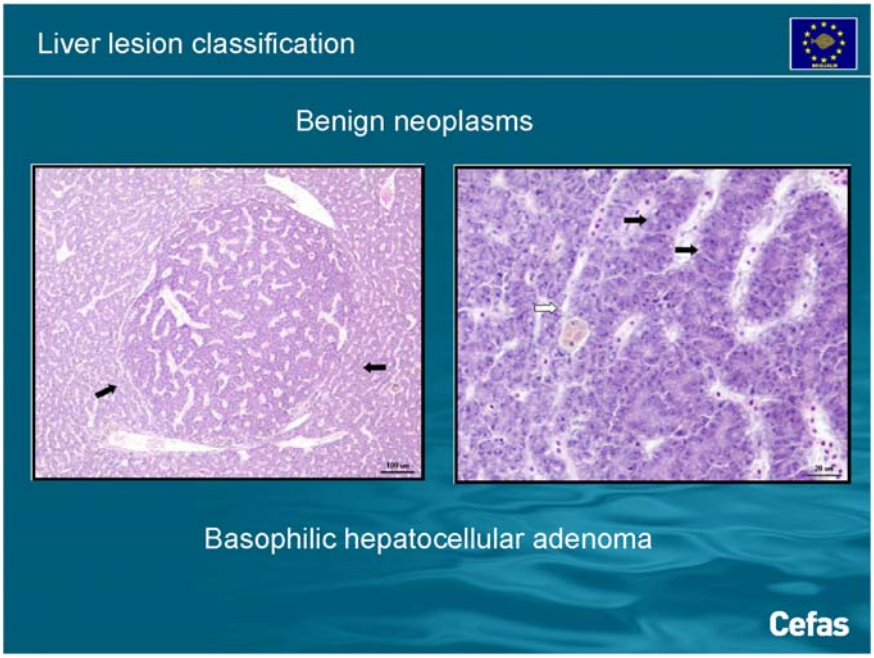

Liver lesion classification
Early non-neoplastic toxicopathic lesions
Nuclear pleomorphism
Hydropic degeneration
(bile duct epithelium)
Cefas

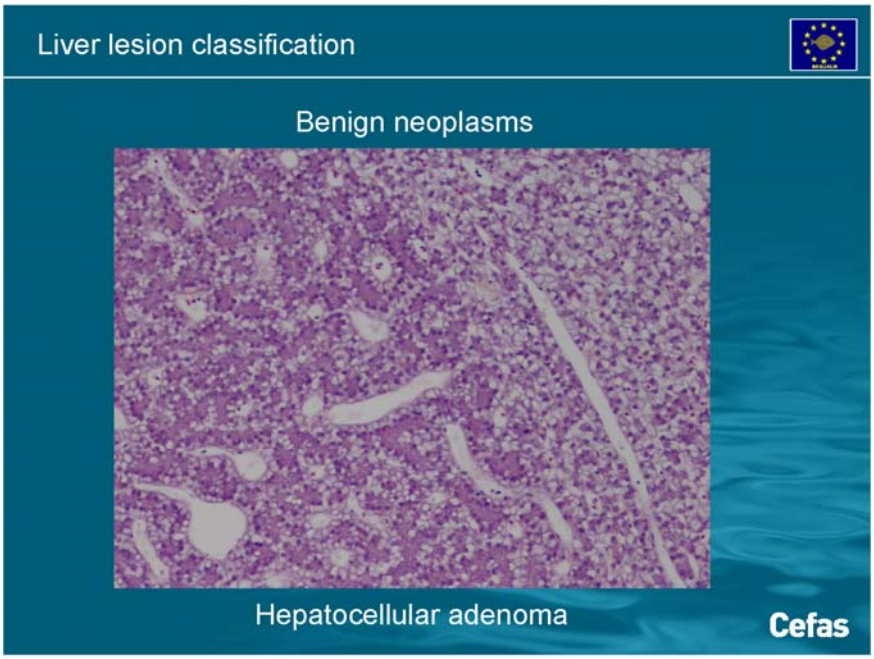

Liver lesion classification
Early non-neoplastic toxicopathic lesions
Hepatocellular fibrillar inclusions
Cefas

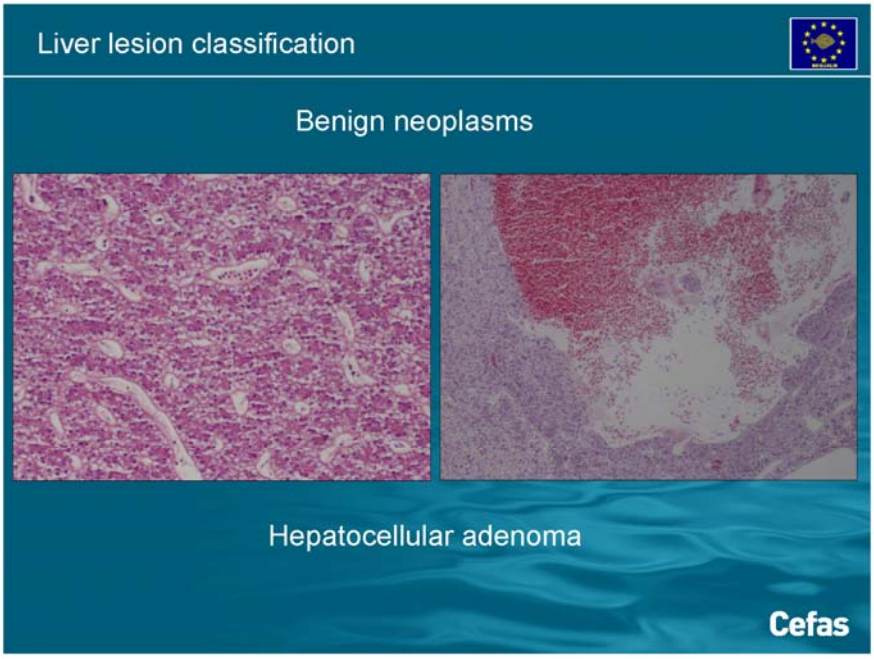

Liver lesion classification
Early non-neoplastic toxicopathic lesions
Peliosis hepatis
Cefas

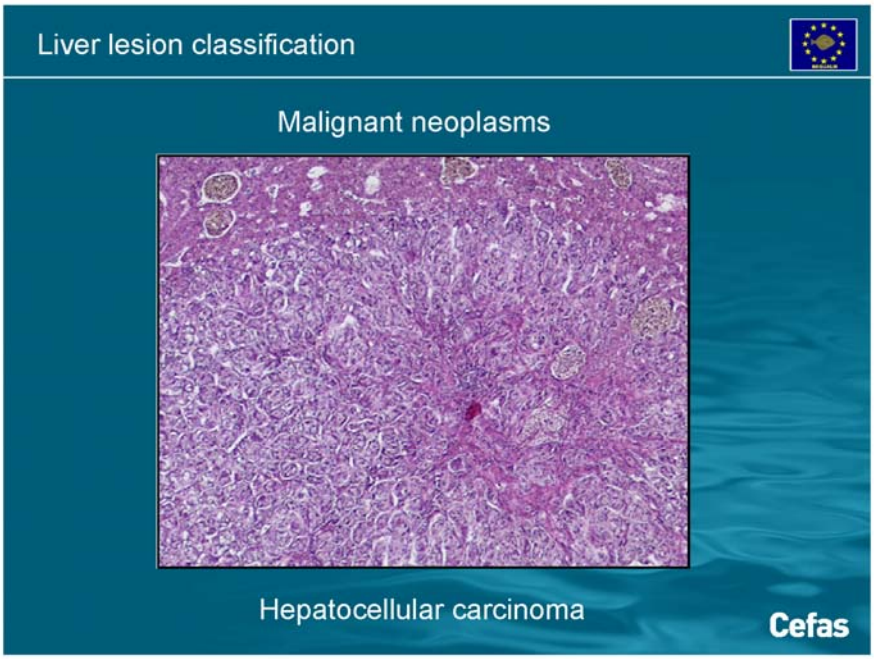

Liver lesion classification
Foci of cellular alteration
• Basophilic FCA
• Normal hepatic architecture.
• Eosinophilic FCA
• Normal hepatic architecture.
Cefas

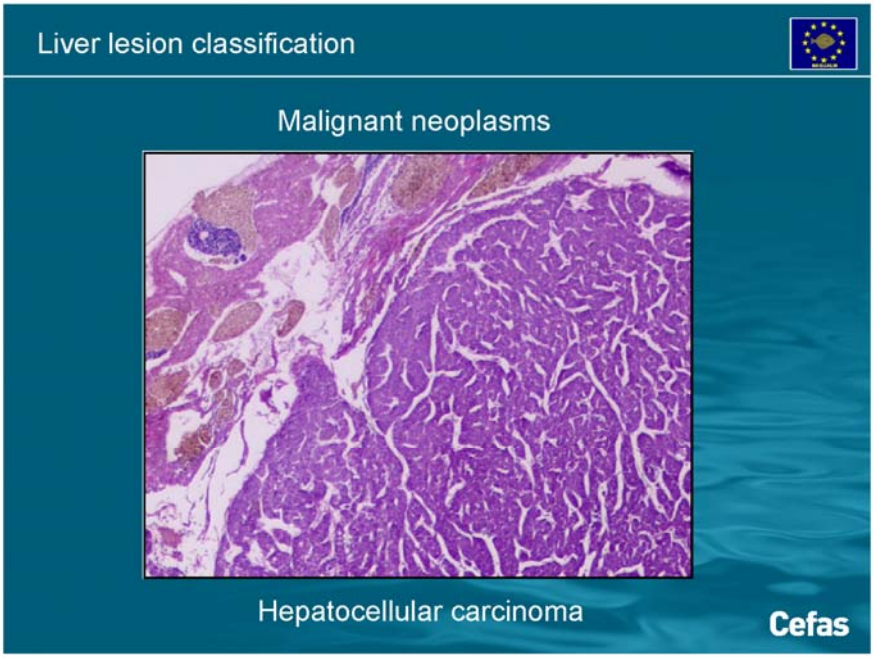

Liver lesion classification
Foci of cellular alteration
Cefas

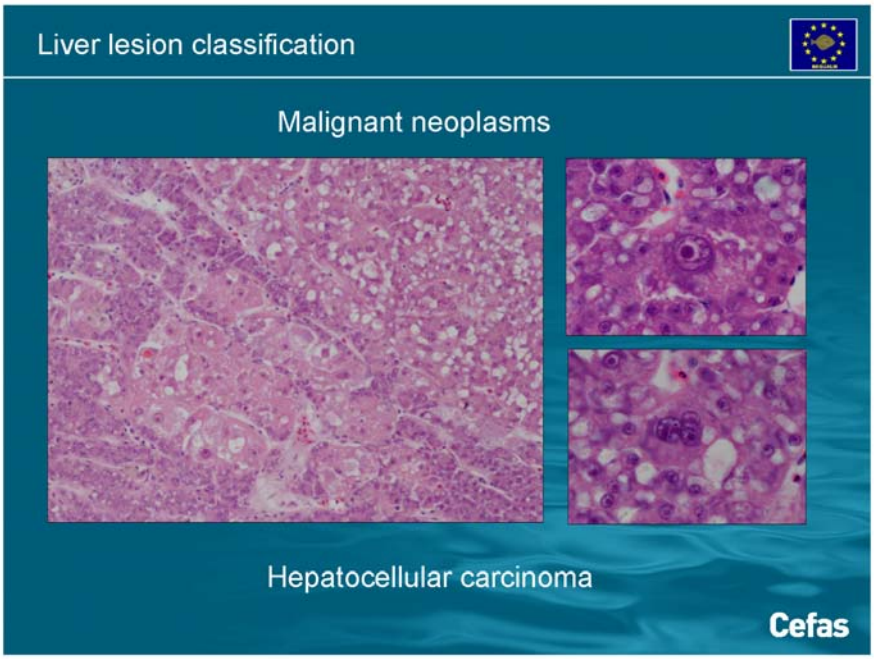

Liver lesion classification
Benign neoplasms
Basophilic hepatocellular adenoma
Cefas

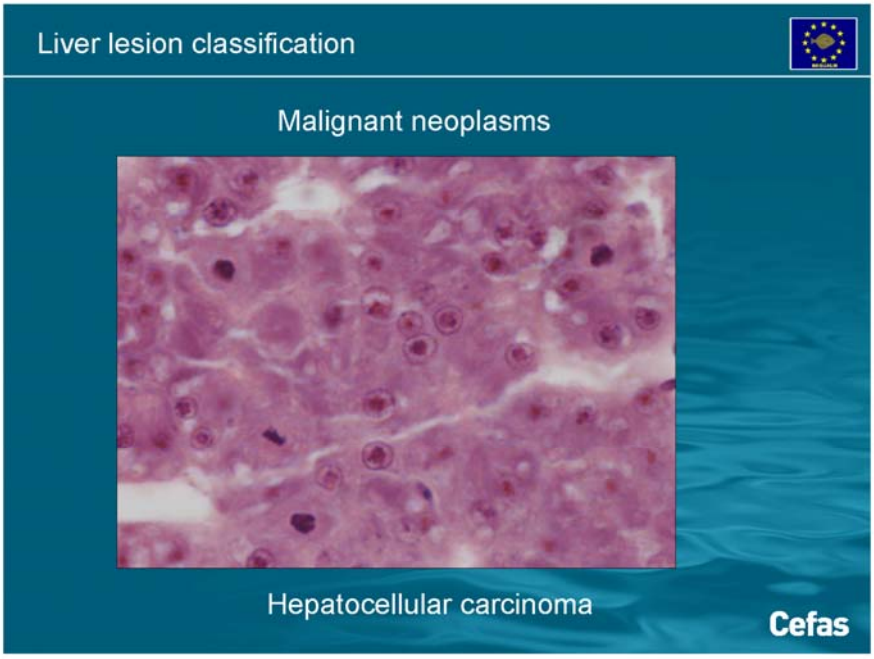

Liver lesion classification
Benign neoplasms
Hepatocellular adenoma
Cefas

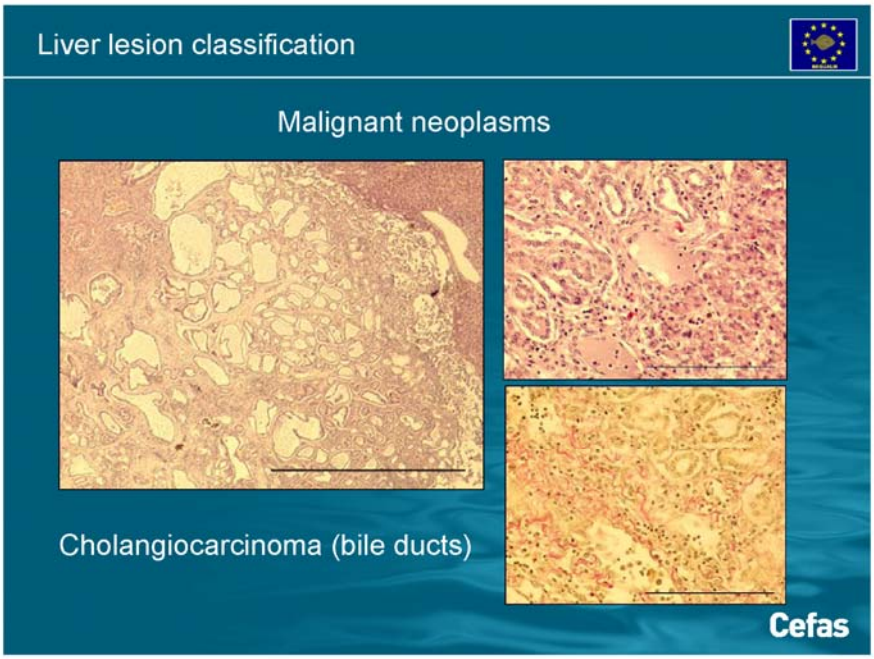

Liver lesion classification
Benign neoplasms
Hepatocellular adenoma
Cefas

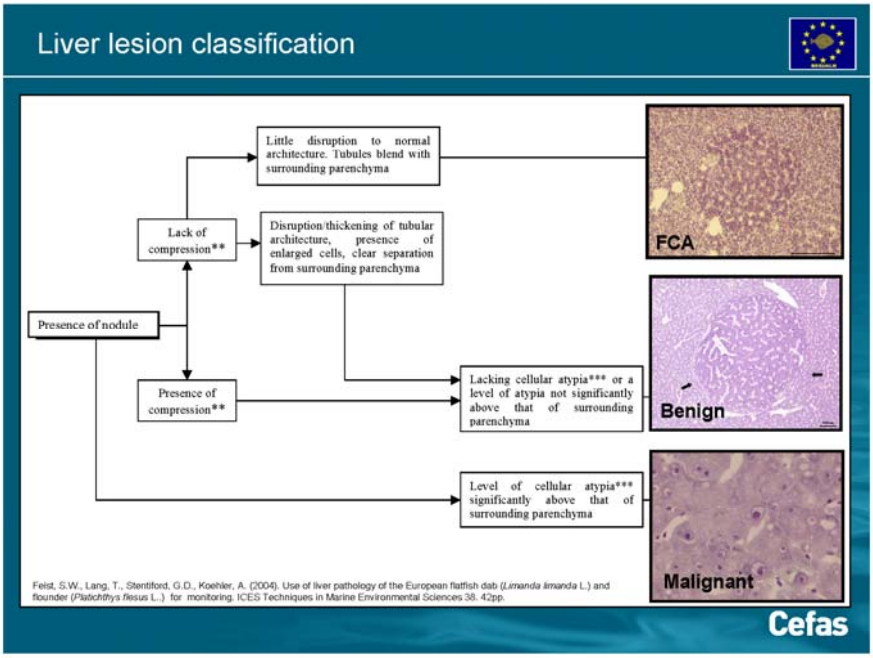

Liver lesion classification
Malignant neoplasms
Hepatocellular carcinoma
Cefas


Liver lesion classification
Malignant neoplasms
Hepatocellular carcinoma
Cefas

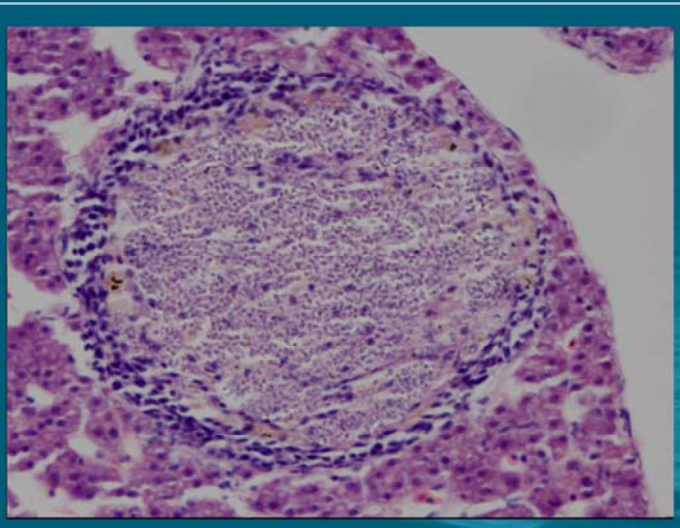

Liver lesion classification
Malignant neoplasms
Hepatocellular carcinoma
Cefas


Liver lesion classification
Malignant neoplasms
Hepatocellular carcinoma
Cefas


Liver lesion classification
Malignant neoplasms
Cholangiocarcinoma (bile ducts)
Cefas


Liver lesion classification
Little disruption to normal architecture. Tubules blend with surrounding parenchyma
Lack of compression**
Disruption/thickening of tubular architecture, presence of enlarged cells, clear separation from surrounding parenchyma
Presence of nodule
Presence of compression**
Lacking cellular atypia*** or a level of atypia not significantly above that of surrounding parenchyma
Level of cellular atypia*** significantly above that of surrounding parenchyma
FCA
Benign
Malignant
Feist, S.W., Lang, T., Stentiford, G.D., Koehler, A. (2004). Use of liver pathology of the European flatfish dab (Limanda limanda L.) and flounder (Platichthys flesus L.) for monitoring. ICES Techniques in Marine Environmental Sciences 38. 42pp.
Cefas

Liver lesion classification



Cefas

Liver histopathology - summary

- Liver neoplasia is significantly more common in wild flatfish inhabiting European waters than in human populations.
- Diagnostic criteria for flatfish liver histopathology is largely based upon description of lesions in mammals.
- Significant progress toward quality assurance in lesion diagnosis (in dab and flounder) under the BEQUALM Programme (more partners required!).
- More effort is required to directly compare the lesions observed in the fish liver with those seen in human patients.
- Molecular based approaches (e.g. via genomics, proteomics and metabolomics) are enhancing our understanding of tumour pathogenesis in flatfish in relation to environmental conditions.

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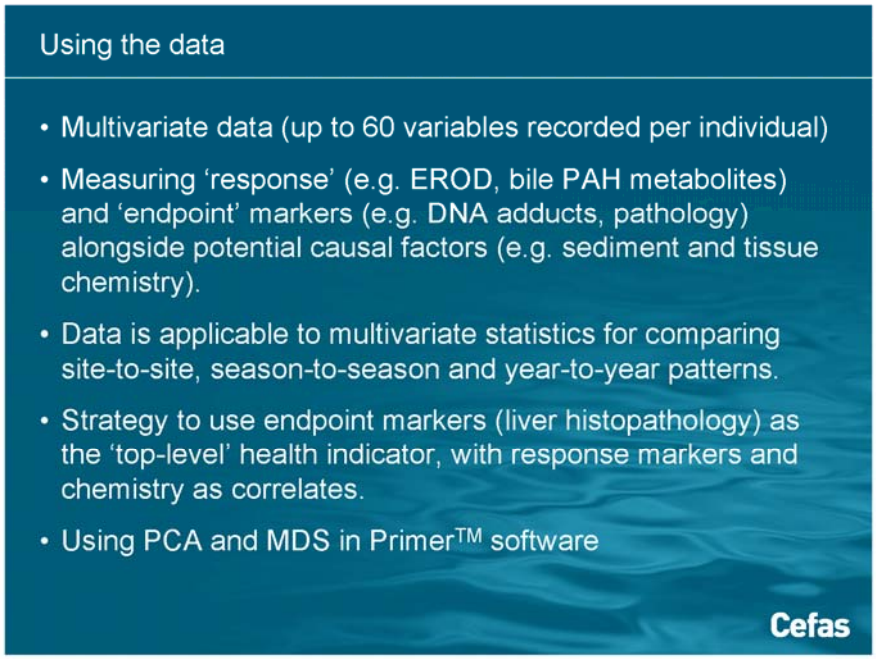
Using the data
• Multivariate data (up to 60 variables recorded per individual)
• Measuring 'response' (e.g. EROD, bile PAH metabolites) and 'endpoint' markers (e.g. DNA adducts, pathology) alongside potential causal factors (e.g. sediment and tissue chemistry).
• Data is applicable to multivariate statistics for comparing site-to-site, season-to-season and year-to-year patterns.
• Strategy to use endpoint markers (liver histopathology) as the 'top-level' health indicator, with response markers and chemistry as correlates.
• Using PCA and MDS in Primer™ software
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Annex 5: Handling and analysis of fish disease data

by

W. Wosniok

Introduction

The monitoring of fish diseases is part of a cyclic process consisting of data collection – data analysis – drawing of conclusions – revision of the data collection and the strategy –collection of new data. The main result of the process are the conclusions drawn. Their nature determines the kind of data to be collected, the sampling strategy and the way of data analysis. These activities require a way of handling the data technically which is appropriate for the intended analysis. In the following sections, the main considerations and methods are summarised from a presentation given by W. Wosniok.

Aims of data analysis

There are three main activities with regard to the analysis of fish disease data:

- calculation of the fish disease prevalence, including a statement on the precision of the value obtained,
- comparing the disease prevalence over sites or against a background level for a fixed point in time (spatial comparison),
- comparing the disease prevalence over time at a given site (temporal comparison).

Each of these aims requires its specific statistical methods. Rules for the data collection with regard to sample size and sampling strategy can be derived after the aim of the analysis (and consequently the statistical method) plus the precision requirement on the expected results have been defined.

Methods for the analysis of fish disease data

Calculation of disease prevalence and its precision

The prevalence p of a disease is calculated from the number r of diseased and the number n of examined fish as $p = r / n$. As in monitoring situations observations are always made within a sample and never on the complete population of all existing fish, this value is only an estimate of the true value, which is the one valid for the whole population. If a second (independent) sample were examined, very likely a different prevalence would be found. Neither the first nor the second result can be assumed to represent the exact true value, instead both of them deviate from the true value by some random amount called the sampling error. The size of the sampling error in the result from a specific sample remains unknown. However, it is possible to calculate a range of values which contains the true value with a pre-specified probability. Such a range, called the confidence interval, allows to compare two prevalences in the sense that a difference which is a consequence of random fluctuation (the sampling error) can be separated from a difference due to a difference in the underlying two population prevalences. Also, the sample size (n) required to achieve a pre-specified precision of the prevalence estimate can be derived from the confidence interval.

For observations $0 < r < n$, the lower and upper limits of the confidence interval for the prevalence estimate p are calculated as

$$CI_{lower} = \frac{r}{r + (n - r + 1)F_{lower}} \qquad \text{and} \qquad CI_{upper} = \frac{(r+1)F_{upper}}{n - r + (r+1)F_{upper}}$$

where

$$F_{lower} = F(2(n - r + 1); 2r; 1 - \alpha/2) \qquad \text{and} \quad F_{upper} = F(2(r+1); 2(n-r); 1 - \alpha/2)$$

are quantiles of the inverse of the cumulative F distribution and α is the probability that the true value of p lies outside of the calculated interval. The F quantiles can be calculated, e.g., using the FINV function of Microsoft Excel. If r = 0 then $CI_{lower} = 0$ and CI_{upper} is calculated as above; if r = n then CI_{lower} as above and $CI_{upper} = 1$.

Figure A5.1 shows the 95% confidence interval obtained by setting α = 0.05, corresponding to a probability of 5% that the true prevalence lies outside the interval, equivalent to a probability of 0.95 (or 95%) of the true value being contained in the interval. It can be seen that the width of the interval depends on the observed prevalence and that the width has its maximum at p = 0.5. It can also be seen that an observation of r = 0 (no diseased fish seen) in a sample does not imply that the true prevalence is zero, instead it is compatible with a true prevalence of up to 0.071 (for a sample of size 50). A corresponding statement holds for an observation of r = n (all observed fish diseased).

Figure A5.1 also shows the dependence of the confidence interval width on the sample size: the larger the sample size n, the smaller the confidence interval width for a fixed observed prevalence r/n.

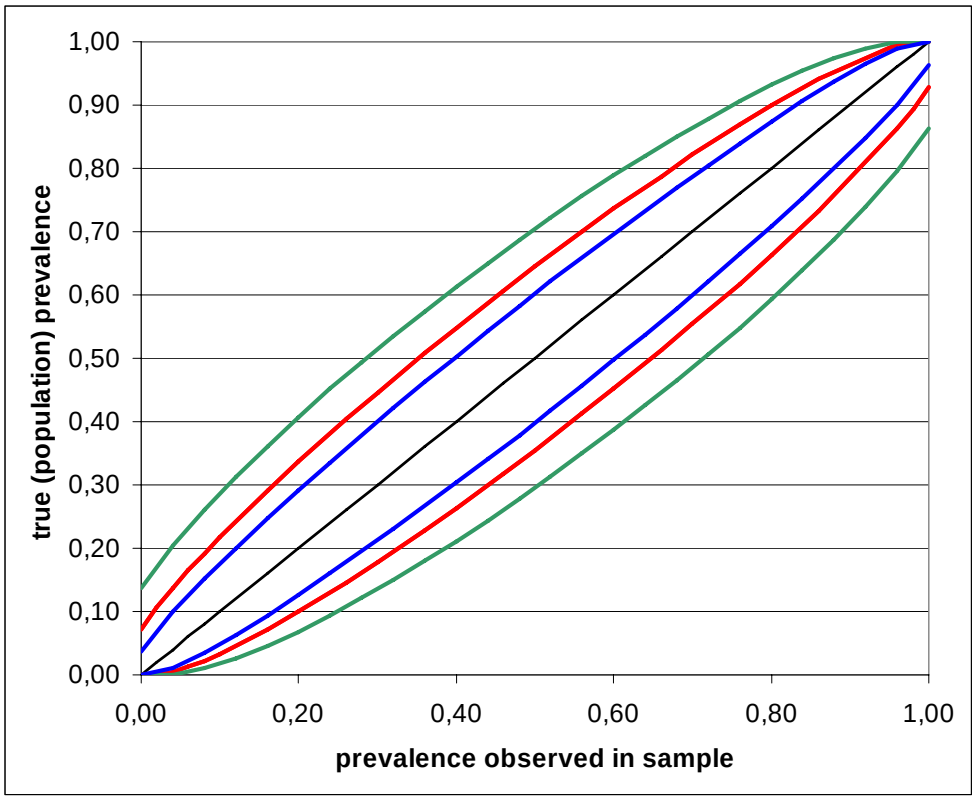

Figure A5.1: 95% confidence intervals for observed prevalence for various sample size 25 (blue), 50 (red) and 100 (green).

Sample size to determine a prevalence with specified precision

The sample size needed to determine a prevalence with specified precision can be determined by finding the smallest number n of fish for which the confidence interval width (= CI_{upper} – CI_{lower}) is equal to or smaller than the specified precision. This operation can be done by calculating the confidence interval for $p_0 = 0.5$ for a list of candidate n values, using a spreadsheet software.

Testing an observed prevalence against a reference value

The reference value p_0 is assumed to be known without any error. To test if the difference between reference and observed prevalence can be considered as purely random, it has to be checked if the confidence interval of the observed prevalence contains the reference value p_0. If so, the difference between both is considered as random, hence negligible, otherwise the test prevalence is considered as significantly different from the reference (with significance level α).

Sample size for comparing a prevalence to a reference value

Figure A5.2 indicates how confidence intervals can be used for sample size calculation. Here the simplest possible situation is assumed in which there is a fixed reference prevalence p_0, assumed to be known without any error, and the task is to check whether this reference value holds in the study situation or is exceeded. It is further assumed that p_0 is a background level so that an observed prevalence can only deviate in upward direction. Then the aim of the sample size calculation is to determine the minimal sample size, which would allow to detect a upward deviation from p_0 of at least an amount of δ with a safety of 1 – ß, if such a deviation existed in reality. If in reality no deviation existed, the probability to declare it erroneously as

existing should not exceed a value of α. Then the necessary sample size is calculated as follows:

- Fix α (the probability of a "false alarm"), ß (the probability of not to detect an existing relevant deviation) and δ (the size of the relevant deviation). These quantities are in general chosen according to the severity of the consequences of wrong decisions. Typical values for α and ß are α = 0.05 and ß = 0.80. No typical value exists for δ. Fix an initial guess for the sample size n.
- For the initial n, calculate the upper limit of the 100·(1-2α)% confidence interval around p0 and the lower limit of the 100·(1-2ß)% confidence interval around p0 + δ. Use r = n·p0 and r = n·(p0 + δ), respectively, in these calculations (rounded to integers, where necessary). Check if the first limit is smaller than or equal to the second one. If no, increase n and re-calculate the confidence interval limits. If yes, decrease n and re-calculate. The procedure is finished, when a value of n is found which cannot be reduced without violating the separation condition above. The n found in this way is the minimal sample size which fulfils the defined precision requirements.

Comparing two empirical prevalences

Testing the hypothesis that two empirical prevalences $p_1 = r_1/n_1$ and $p_2 = r_2/n_2$ differ only by a purely random amount means to compare two quantities which both contain a sampling error. This is different from the situation discussed above in section 0, in which an observed prevalence (with sampling error) was compared to a fixed reference (without sampling error). For this reason, a different statistical test is needed.

As a first orientation, the 100·(1-α)% confidence intervals for p_1 and p_2 can be checked for overlap. If they do not overlap, then there is a significant difference between the two prevalence values. Such a test can easily be done graphically, however, this procedure does

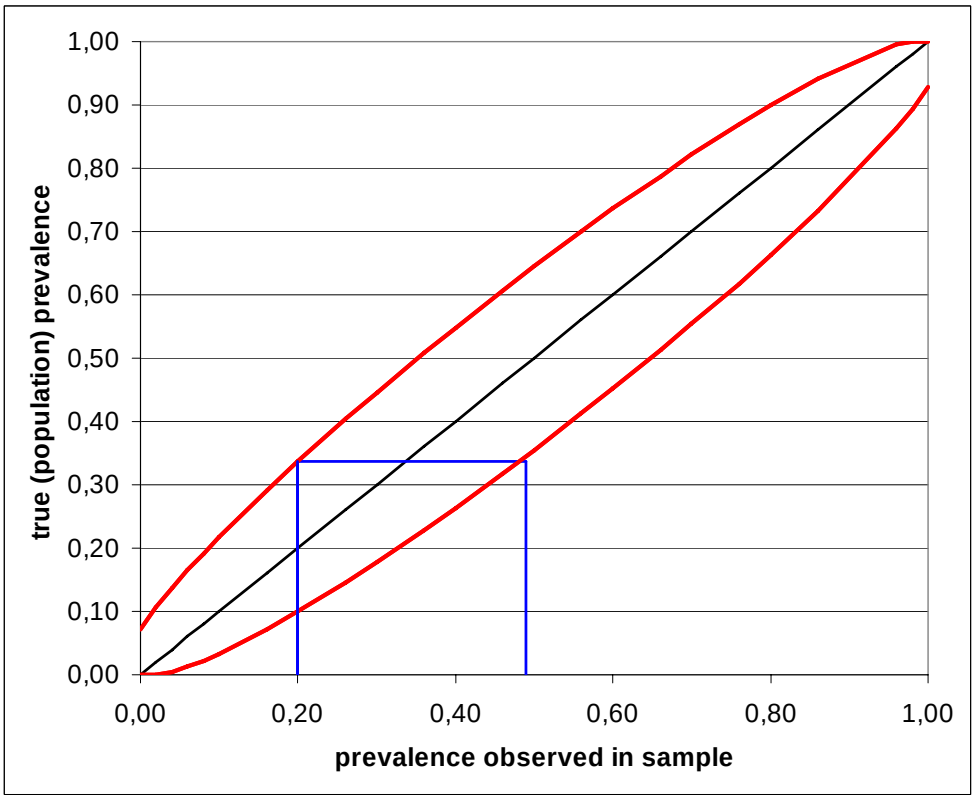

Figure A5.2: Use of the 95 % confidence interval for n = 50 to determine the δ value that can be detected, if the reference value is p = 0.2. A value of 0.49 corresponding to δ = 0.29 could be detected with error probabilities α=β=0.05.

not exactly attain the required significance level α. A simple alternative which attains the required significance level (exception see below) is the χ^2 test. For this test, the observed counts r_1, $d_1 = n_1 - r_1$, r_2, $d_2 = n_2 - r_2$ are arranged in a cross-tabulation:

Table A5.1: Input data for the χ2 test.

	Sample 1	Sample2	row sums
diseased	r_1	r_2	$r = r_1 + r_2$
non-diseased	d_1	d_2	$d = d_1 + d_2$
examined	$n_1 = r_1 + d_1$	$n_2 = r_2 + d_2$	$n = n_1 + n_2$

The χ^2 value is computed from the entries in Table A5.1 as

$$\chi^2 = \frac{n(r_1 \cdot d_2 - r_2 \cdot d_1)^2}{n_1 \cdot n_2 \cdot r \cdot d}.$$

A χ^2 value of at least 3.84 indicates a significant difference between the two observed prevalence values, where the significance level α is 0.05 (5%). Critical values for other significance values can be calculated from the cumulative χ^2 distribution function with one degree of freedom. This function is, e.g. available in Microsoft Excel as function CHIINV. Alternatively, the p level corresponding to the calculated χ^2 value can be obtained directly via the Excel function CHIVERT. A p level equal to or smaller then 0.05 indicates indicates a significant difference.

The χ^2 test relies on the assumption that the counts involved in the test are large, more precisely, that the expected count in each cell is larger than 5. Table A5.2 shows how expected counts are calculated from the row and column sums in Table A5.1.

Table A5.2: Calculation of expected counts for the χ^2 test.

	Sample 1	Sample2	row sums
diseased	$e_{11}=r \cdot n_1/n$	$e_{12}=r \cdot n_2/n$	$r = r_1 + r_2$
non-diseased	$e_{21}=d \cdot n_1/n$	$e_{22}=d \cdot n_2/n$	$d = d_1 + d_2$
examined	$n_1 = r_1 + d_1$	$n_2 = r_2 + d_2$	$n = n_1 + n_2$

The result of the χ^2 test becomes unreliable if there is an expected count smaller than 5. In such a case, an exact test like Fisher's exact test should be used. This test, however, is cumbersome to perform and therefore the use of appropriate software is advisable (see section Data recording and processing).

Sample size to detect differences between two empirical prevalences

Assuming that the χ^2 test will be used to perform the test on significant difference between two prevalence levels, the required number of fish is calculated by

$$n_1 = n_2 = \left(\frac{z_\alpha + z_\beta}{\delta} \right)^2 \cdot p_1 \cdot (1 - p_1),$$

where α and β are the error probabilities as described in section 0, p_1 is the prevalence in sample 1, δ is the difference to be detected between the two prevalences. If p_1 is completely unknown, $p_1 = 0.5$ is used. The quantities z_α and z_β are the α and β quantiles, respectively, of the cumulative standard normal distribution. They can be calculated, e.g., via the Excel function NORMINV. Typical values are $z_\alpha = -1.645$ (for $\alpha = 0.05$) and $z_\beta = -0.842$ (for $\beta = 0.20$). The formula shows that the required total sample size increases with decreasing error probabilities and decreasing relevant difference δ, and that the required sample size has its maximum if one of the prevalences involved has the value 0.5. Additionally it should be noted that the χ^2 test has highest power to detect an existing difference if the samples to compare have identical size, as it is assumed in the sample size formula above.

Assessing time series of disease prevalence

The long-term monitoring of fish diseases generates time series of prevalence data, which allow the investigation of changes over long periods. As an initial method, a generalisation of the χ^2 test from section 0 could be used to test the hypothesis that the prevalence experienced only purely random variation over time, but no substantial change. However, this approach is inappropriate only if no seasonal fluctuation is present in the data. Also, such a test could only indicate that substantial changes exist, but not the nature of these changes. In general it must be assumed that a prevalence time series contains (i) a long-term trend of unknown shape, (ii) periodic seasonal fluctuations of unknown shape, and (iii) random variation. An approach to identify these components is to use a Generalized Additive Model (GAM) with three components:

- a smooth curve s(t), e.g. a spline or a local regression function, for the long-term change,
- a factor component for the seasonal variation,
- a binomial error term for the random fluctuation.

The mathematical representation of such a model is

$$p(t) = 1/\left[1 + \exp(u(t))\right]$$

$$u(t) = s(t) + \sum_{i=2}^{12} b_i \cdot 1_{\text{month}(t)=i}$$

where t is the calendar date of the observation, p(t) is the modelled prevalence at time t, s(t) is the contribution of the long-term trend, the b_i coefficients describe the seasonal component, and the expression $1_{\text{month(t)} = I}$ denotes the indicator function which has value 1, if the month of the observation date t is equal to I (I = 1: January, …). Figure A5.3 gives an example result for the fit of a GAM model to a disease prevalence time series. It shows empirical and predicted prevalence, the estimated long term trend s(t) and its confidence interval. The estimated long-term trend does not contain seasonal fluctuations. The insert in Figure A5.3 shows the estimated seasonal variation, which was used to calculate predictions per sampling date. Seasonal components were determined only for such months for which data was available. The approach can be used not only with incomplete seasonal data, but also for time series, in which the seasonal pattern of observations varies over time, as it was the case in the example.

Fitting a GAM requires appropriate software like SAS, Splus, Statistica (a non-exhaustive list of commercial products) or R, a free software.

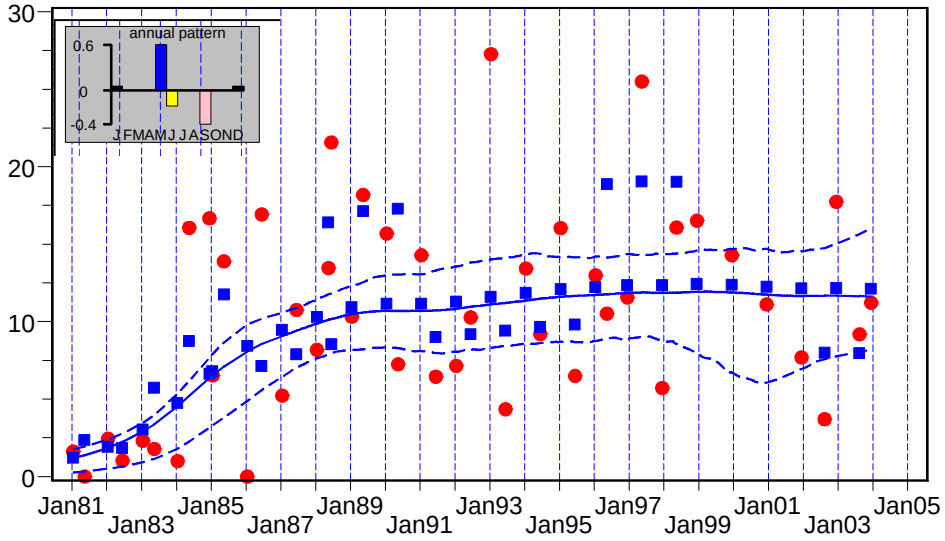

Figure A5.3: An example for an estimated long-term trend, fitted by a Generalized Additive model. Red dots: observed prevalence, blue squares: predicted prevalence, solid blue line: season-adjusted long-term trend, dashed blue line: 95% confidence interval for the long-term-trend. The insert in the upper left shows the estimated seasonal pattern.

Investigating the relation between disease prevalence and suspected explaining factors

In order to investigate the relation between disease prevalence and suspected explaining factors (age, gender, condition; nutrients available, water temperature, salinity, contaminant concentration, …), a logistic regression is the appropriate method. It has the form

$$p = 1/\left[1 + \exp(a_0 + a_1 \cdot \text{age} + a_2 \cdot \text{condition} + \ldots)\right]$$

where p is the prevalence predicted by the model and a_1, a_2, … are coefficients which express the impact of the associated variable on the disease prevalence. These significance of these coefficients can be tested, which means that the relevance of the associated variables for the prevalence can be assessed. The mathematical model above can also be used to calculate

scenarios: what is the expected prevalence if some of the explaining quantities take another value?

Fitting a logistic regression can be done by software like SAS, Splus, Statistica (a non-exhaustive list of commercial products) or R, a free software.

Statistical requirements for fish disease monitoring

The selection of sampling sites is a matter of biological interests and only to a smaller extent a consequence of statistical consideration. However, the sites used for disease monitoring should be "important" in the sense that the species under study is regularly present. The totality of sites selected should provide a "representative" picture of the area, which means that not only highly polluted or background level areas should be visited. The sites should be visited when the fish is in a stable situation, which means (among others) out of the spawning season. The samples taken should have a size that allows the detection of relevant changes (see the consideration on sample sizes above and the relevant ICES guidelines.). Also, no pre-selection should take place, which could happen if fish were taken from regular commercial catches. The researcher should be prepared for the fact that a change in prevalence will not always be detectable by just comparing consecutive prevalence values, instead, a more sophisticated way of analysis like the GAM analysis above might be necessary. This will be particularly be the case if interfering seasonal effects are present.

Data recording and processing

When sampling fish disease data, all potential supporting information (fish species, gender, length, hydrography, …) should be collected and stored as well as the core disease data. All data must have a proper identification (e.g. date and time and location of collection, and haul and fish species and fish number) so that information from different compartments can be merged in an unambiguous way for later analysis. Data should be recorded on individual basis, e.g. individually for each fish. Summaries can always be generated from individual records, while the opposite way is not possible.

A universal way of data storage is to arrange the information in a rectangular array, e.g. in a spreadsheet, where the rows correspond to individual observations (i.e. one row contains information on one fish) and columns correspond to variables (identification, measured parameters). The first row of this structure should contain a (short) label which describes the contents of the respective column. Such a structure can serve as input to standard software. It can also be used to prepare data submission to the ICES databank. The ICES Data Centre holds instructions for reporting fish disease data (see http://www.ices.dk/datacentre/data_intro.asp).

All data recording and processing needs appropriate software. Data recording can be done by using a spreadsheet product (e.g. Microsoft Excel or a corresponding free software product). Also some data analysis and sample size planning can be done within Excel, as indicated in the previous sections. More sophisticated analyses need specialised software, where either commercial products like SAS, Splus, Statistica can be used, or the R software, which is a free product available via http://www.r-project.org/.

Experience with the analysis of ICES data with regard to fish diseases and environmental factors

The ICES Data Centre holds a wealth of marine data which had been submitted by researchers from the ICES member countries. The site http://www.ices.dk/env/index.htm contains the starting form for searches in the environmental database. Data requests can also be started from there. The procedure of a data request involves to define the parameter for which data is sought as well as the geographical region and the time window of interest. The search request

produces an initial overview on the available data on the basis of which the user may decide to start the actual data request or modify his search.

In an earlier study (Wosniok *et al*., 2000), data from the ICES Data Centre were used to investigate the relation between diseases of dab (*Limanda limanda*) in selected areas of the North Sea. Parameters from various compartments were considered as potential explaining quantities, among them contaminants in water, biota and sediment, nutrients and host factors. Even though the data available was irregularly distributed over time and space, various relationships could be identified, among them also such between fish disease and contaminants.

Reference

Wosniok, W., Lang, T., Dethlefsen, V., Feist, S.W., McVicar, A.H., Mellergaard, S., Vethaak, A.D. 2000. Analysis of ICES long-term data on diseases of North Sea dab (*Limanda limanda*) in relation to contaminants and other environmental factors. ICES CM 2000/S:12, 15 pp.

Annex 6: Guidelines for fish disease monitoring in the Baltic Sea

by T. Lang and G. Rodjuk

Introduction

In the following, guidelines are provided that should be used for fish disease monitoring in the Baltic Sea. These are largely based on guidelines already developed through ICES activities and within the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM) programme and on experience made during the ICES/BSRP Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM) (Bucke *et al*., 1996; Dethlefsen *et al*., 1986; Feist *et al*., 2004; ICES, 1989; ICES, 1997; Lang, 1996; Lang *et al*., 1999).

Target fish species

Fish species considered to be particularly suitable for monitoring of diseases and parasites in the Baltic Sea because of their abundance and wide geographical distribution are flounder (*Platichthys flesus*), cod (*Gadus morhua*) and herring (*Clupea harengus*). For these species, regular stock assessment surveys are carried out by Baltic Sea countries and the disease monitoring may be incorporated in such surveys if feasible. Other common species might also be appropriate, such as dab (*Limanda limanda*), whiting (*Merlangius merlangus*), viviparous blenny (= eelpout) (*Zoarces viviparus*), 4-bearded rockling (*Rhinonemus cimbrius*) or perch (*Perca fluviatilis*). However, these species are more locally restricted (either to certain parts of the Baltic Sea or to coastal areas) and there is less information available on diseases and parasites than for flounder, cod or herring.

Sampling

Sampling sites

The selection of sampling sites has to be based on the objectives of the monitoring. For instance, if environmental effects of a known point source of pollution are to be monitored and assessed, sampling sites may be arranged on a contaminant gradient. If the ecosystem health of a region known or suspected to be affected by anthropogenic stressors without point source impacts is monitored and assessed, a different strategy with a number of sampling sites at places representing different habitats may be considered feasible.

Selection of sampling sites should in any case take into account information on fish species availability, age/length structure of the population, temporal and spatial migration patterns, disease occurrence and stressors affecting fish health. Areas with mixed stocks of the same species should be avoided, because there might be genetic differences in disease susceptibility or stress reactions.

Basically, there are two strategies for sampling: either sampling is carried out on a fixed nominated latitude and longitude or in a box with a nominated latitude and longitude at each corner of the box. Within the box, sampling positions may be randomised. Sampling should preferably be based on multiple samples in order to reduce sampling variation (haul-to haul variation, patchiness).

Sampling gear

Sampling on a long-term basis should preferably be conducted using identical equipment (ship, gear) and conditions (e.g., towing time and speed in case of trawling) in order to reduce sampling variation. Changes in sampling gear and sampling conditions might change the catch

composition and even the disease prevalence because there might be differences in behaviour and catchability between healthy and diseases fish.

In offshore regions, bottom trawling with standard gears (e.g. those used in internationally coordinated stock assessment surveys) is the method of choice, particularly if flatfish or other demersal species (e.g., cod) are targeted. For pelagic species (such as herring), pelagic trawls or other standard gears for fishing schooling fish may be selected. In coastal or shallow waters not suitable for trawling, fike or entangling nets may be more appropriate. Gill nets should only be used if fish are examined very rapidly after catching.

For trawling, the towing time should be between 30 and 60 min, depending on the abundance of fish. Prolonged trawling would lead to too much superficial damage of the fish and thus problems to identify externally visible diseases/parasites. It, furthermore, would increase stress in fish caught which should be avoided if, e.g., biomarker samples (e.g. for enzymatic or immunological measurements) are taken in addition to the disease examination. When using a standard bottom trawl, the average trawling speed is 3–4 knots.

Sampling frequency and season

Sampling should be carried out in a period of the year when the fish species to be examined is in its stationary phase. The spawning season is not advisable because of partly considerable geographical migrations between spawning and feeding grounds and because of potential interference with spawning stress.

The frequency of sampling depends on the objectives of the monitoring and on resources available. However, it is recommended to sample once a year, always in the same narrow time window (because many diseases show a clear seasonality).

Target diseases appropriate for monitoring and assessment

Diseases appropriate for monitoring purposes should fulfil some major requirements:

- they should occur commonly in the selected fish species,
- they should, with a certain degree of training, be easily detectable and quantifiable,
- they should respond to environmental stressors, either in a non-specific (as general stress indicator) or in a stressor-specific way (e.g., as indicator of effects of specific contaminants).

Tables A6.1–A6.4 provide information on externally visible diseases/parasites of dab (available only in the western Baltic Sea), flounder, cod and herring considered to be useful for monitoring purposes in the Baltic Sea. Information is given on diseases identification and grading. For flounder, the grading scheme is, with one exception, according to the BEQUALM guidelines. For cod and herring a BEQUALM scheme does not yet exist and, therefore, a new scheme is suggested here.

Table A6.1: Diseases/parasites of dab (*Limanda limanda*) recommended to be recorded in fish disease monitoring programmes in the Baltic sea (including information on identification and grading) (after Bucke *et al.*, 1996 and BEQUALM guidelines; with modifications)

DISEASE	IDENTIFICATION	GRADE	GRADING
Lymphocystis	Clusters of white to redish hard nodules (enlarged connective tissue cells) on the body surface (seldom in inner organs)	1	2-10 single nodules that may be grouped in a cluster (the area affected up to 10 mm in diameter) or may be distributed as single enlarged cells over the whole body (including upper, lower side and fins)
		2	More than 10 nodules; total area affected larger than 10 mm but smaller than twice the area of the spread-out caudal fin
		3	Total area affected larger than twice the area of the spread-out caudal fin
Epidermal hyperplasia/ papilloma *	Lesions on the skin are slightly raised, smooth, opaque, from creamy white to slighly pink, partly associated with brown pigmentation; lesions easily slough off.	1	Total area affected up to 10 mm in diameter
		2	Total area affected larger than 10 mm but smaller than twice the area of the spread-out caudal fin
		3	Total area affected larger than twice the area of the spread-out caudal fin
Actute/healing skin ulcers **	Red, open (or almost open) inflammatory lesions of the skin (acute stage); necrosis or excessive cell debris may be present (chronic stage); scar formation and melanin deposits may be visible at the periphery of the lesion (healing stage)	1	Total area affected up to 10 mm in diameter
		2	Total area affected larger than 10 mm but smaller than twice the area of the spread-out caudal fin
		3	Total area affected larger than twice the area of the spread-out caudal fin
Acute/healing fin rot/erosion	Red open inflammatory lesions affecting the fins; healing processes may be present.	-	No grading, only presence recorded
X-cell gill disease	Creamy white to light pink swollen gill lamellae, opercula slightly raised.	-	No grading, only presence recorded
Skeletal deformities	Compression or lordosis/scoliosis of the vertebral column, pug-headedness	-	No grading, only presence recorded
***Cryptocotyle* spp.**	Small cysts (< 1 mm in diameter) on the body surface (in the skin) including the fins, best to be seen between the fin ray in front of a light source	1	1-10 cysts between the rays of the caudal fin
		2	11-50 cysts between the ray of the caudal fin
		3	> 50 cysts between the ray of the caudal fin
Lepeophtheirus pectoralis	Parasitic copepod (size up to 8 mm) under the pectoral fins or on the skin	1	1 parasite
		2	2 parasites
		3	3 or more parasites

***NOTE 1: In the BEQUALM guidelines, a different grading of epidermal hyperplasia/papilloma has been suggested: grade 1: at least one but less than four lesions between 2 mm and 10 mm in diameter; grade 2: more than four lesions between 2 mm and 10 mm in diameter; grade 3: presence of lesions over 1cm in diameter. However, the BEQUALM grading to differentiate bezween grad 2 and grade 3 was felt to be contradictory. Therefore, a different grading system which is more coherent with the system used for the other diseases is suggested here.**

****NOTE 2: In the BEQUALM guidelines, a different grading of skin ulcers has been suggested: grade 1: acute; grade 2: healing; grade 3: healed. However, the BEQUALM grading does not provide information on severity of the disease, only on consecutive developmental stages. Therefore, a different grading system which is more coherent with the system used for the other diseases is suggested here.**

Table A6.1 (cont.): Diseases/parasites of dab (*Limanda limanda*) recommended to be recorded in fish disease monitoring programmes in the Baltic Sea (including information on identification and grading) (after Bucke *et al.,* 1996 and BEQUALM guidelines; with modifications)

DISEASE	IDENTIFICATION	GRADE	GRADING
Liver nodules > 2 mm	Macroscopic nodular lesions in the liver tissue larger than 2 mm in diameter, often raised above the surface and different in colour from the surrounding non-affected tissue. **Note:** these lesions need subsequent histological confirmation since only neoplastic lesions (benign and malignant liver tumours) are to be recorded	-	Number, size and colour of liver nodules may be recorded.
Liver histopathology	5 categories of lesions: - Non-specific lesions - Early toxicopathic non-neoplastic lesions - Pre-neoplastic lesions (foci of cellular alteration) - Benign liver tumours - Malignant liver tumours	-	-

Table A6.2: Diseases/parasites of Baltic flounder (*Platichthys flesus*) recommended to be recorded in fish disease monitoring programmes (including information on identification and grading) (after Bucke *et al.*, 1996 and BEQUALM guidelines; with modifications)

DISEASE	IDENTIFICATION	GRADE	GRADING
Lymphocystis	Clusters of hard nodules (enlarged connective tissue cells) on the body surface (seldom in inner organs)	1	2–10 single nodules that may be grouped in a cluster (the area affected up to 10 mm in diameter) or may be distributed as single enlarged cells over the whole body (including upper, lower side and fins)
		2	More than 10 nodules; total area affected larger than 10 mm but smaller than twice the area of the spread-out caudal fin
		3	Total area affected larger than twice the area of the spread-out caudal fin
Actute/healing skin ulcers *	Red, open (or almost open) inflammatory lesions of the skin (acute stage); necrosis or excessive cell debris may be present (chronic stage); scar formation and melanin deposits may be visible at the periphery of the lesion (healing stage)	1	Total area affected up to 10 mm in diameter
		2	Total area affected larger than 10 mm but smaller than twice the area of the spread-out caudal fin
		3	Total area affected larger than twice the area of the spread-out caudal fin
Acute/healing fin rot/erosion	Red open inflammatory lesions affecting the fins; healing processes may be present.	-	No grading, only presence recorded
Skeletal deformities	Compression or lordosis/scoliosis of the vertebral column, pug-headedness	-	No grading, only presence recorded
***Cryptocotyle* spp.**	Small cysts (< 1 mm in diameter) on the body surface (in the skin) including the fins, best to be seen between the fin ray in front of a light source	1	1–10 cysts between the rays of the caudal fin
		2	11–50 cysts between the ray of the caudal fin
		3	> 50 cysts between the ray of the caudal fin
Liver nodules > 2 mm	Macroscopic nodular lesions in the liver tissue larger than 2 mm in diameter, often raised above the surface and different in colour from the surrounding non-affected tissue. **Note:** these lesions need subsequent histological confirmation since only neoplastic lesions (benign and malignant liver tumours) are to be recorded	-	Number, size and colour of liver nodules may be recorded.
Liver histopathology	5 categories of lesions: - Non-specific lesions - Early toxicopathic non-neoplastic lesions - Pre-neoplastic lesions (foci of cellular alteration) - Benign liver tumours - Malignant liver tumours	-	-

NOTE: In the BEQUALM guidelines, a different grading of skin ulcers has been suggested: grade 1: acute; grade 2: healing; grade 3: healed. However, the BEQUALM grading does not provide information on severity of the disease, only on consecutive developmental stages. Therefore, a different grading system which is more coherent with the system used for the other diseases is suggested here.

Table A6.3: Diseases/parasites of Baltic cod (*Gadus morhua*) recommended to be recorded in fish disease monitoring programmes (including information on identification and grading)

DISEASE	IDENTIFICATION	GRADE	GRADING
Actute/healing skin ulcers	Red, open (or almost open) inflammatory lesions of the skin (acute stage); necrosis or excessive cell debris may be present (chronic stage); scar formation and melanin deposits may be visible at the periphery of the lesion (healing stage)	1	Total area affected up to 10 mm in diameter
		2	Total area affected larger than 10 mm but smaller than twice the area of the spread-out caudal fin
		3	Total area affected larger than twice the area of the spread-out caudal fin
Acute/healing fin rot/erosion	Red open inflammatory lesions affecting the fins; healing processes may be present.	-	No grading, only presence recorded
Skeletal deformities	Compression or lordosis/scoliosis of the vertebral column, pug-headedness	-	No grading, only presence recorded
Pseudobranchial swelling (X-cell disease)	Tumour-like swelling of the pseudobranches, uni- or bilateral, somwtimes protruding into the gill tissue	-	No grading, only presence recorded
Lernaeocera branchialis	S-shaped red parasite in the gill chamber, size up to 2 cm	1	1 parasite
		2	2 parasites
		3	3 or more parasites
Cryptocotyle lingua	Small black cysts (< 1 mm in diameter) on the body surface (in the skin) including the fins	1	1-10 cysts between the rays of the caudal fin
		2	11-50 cysts between the ray of the caudal fin
		3	> 50 cysts between the ray of the caudal fin

Table A6.4: Diseases/parasites of herring (*Clupea harengus*) in the Baltic Sea recommended to be recorded in fish disease monitoring programmes (including information on identification and grading)

DISEASE	IDENTIFICATION	GRADE	GRADING
Lymphocystis	Clusters of hard nodules (enlarged connective tissue cells) on the body surface (seldom in inner organs)	1	2–10 single nodules that may be grouped in a cluster (the area affected up to 10 mm in diameter) or may be distributed as single enlarged cells over the whole body (including upper, lower side and fins)
		2	More than 10 nodules; total area affected larger than 10 mm but smaller than twice the area of the spread-out caudal fin
		3	Total area affected larger than twice the area of the spread-out caudal fin
Skeletal deformities	Compression or lordosis/scoliosis of the vertebral column, pug-headedness	-	No grading, only presence recorded
***Anisakis simplex* larvae**	Larval helical nematodes in the body cavity, diameter of helix approx. 5 mm.	1	1–10 nematodes
		2	11–20 nematodes
		3	≥ 20 nematodes
Ichthyophonus hoferi	White nodules (granulomas) in the heart tissue	-	No grading, only presence recorded

Disease examination procedures

Disease examination and sampling for subsequent analyses should be carried out by trained experts following a strict protocol with standard operating procedures (SOPs). If new staff have to be trained, this should be done by the experts and the results should be intercalibrated internally on a recurrent basis.

After each haul, the fish species to be examined should immediately be sorted from the catches (either from the total catch or from representative sub-samples). The sample weight and the length-frequency distribution (total length rounded to the nearest cm below) of the fish should be recorded, the latter for males and females separately. Measured fish should either be completely examined for diseases or should be sorted into the length classes recommended (see above) prior to examination.

The specimens selected for examination should be inspected while fresh, i.e., shortly after they have been landed on the ship or taken from nets (not frozen or refrigerated). Ideally, fish should be kept alive in appropriate tanks with seawater supply with the temperature being similar to the ambient water.

An area form working should be cleared, preferably a bench or table at standing height with good lighting and running water.

At least two people are needed for examining a large number of fish: one conducting the examination and the other one for recording the data either onto special paper forms or directly onto a computer keyboard if a special data entry software is used. These positions should be interchangeable, so that both workers know how to take the measurements and how to transcribe the data.

Externally visible diseases

Fish should be examined for externally visible diseases and parasites after rinsing it in clean water. It is recommended to wear thin gloves to protect the skin of the observer. Each fish should be length-measured and sexed and, if feasible, weighed prior to disease inspection. Externally visible diseases and parasites detected on the body surface, including the upper and lower body side, the spread-out fins and the gill and mouth cavities, should be recorded. In addition to information on the presence of a disease condition, its severity grade should be recorded (see Tables A6.1–A6.3).

Macroscopic liver anomalies

For internal examination of flatfish for liver anomalies (liver nodules > 2 mm), the fish should be anaesthetised and killed and be placed on an appropriate board underside downwards, and an incision should be made on the upper side with a sharp blade (preferably with a scalpel with disposal blades) from the pectoral fin to the outer edge of the abdominal cavity. The intestine should be pulled out carefully and the liver will be clearly visible. The liver should be removed from the intestine by using the scalpel or a pair of scissors and should be inspected from both sides. Any nodule > 2 mm (rounded spot, normally raised above the surface of the normal liver, clearly demarcated from the surrounding tissue by its colour and texture) should be recorded together with information on its maximum diameter, its colour and its texture. In addition, the colour of the normal liver (as an indicator of the general physiological ste of the fish) and the presence of parasites on the liver (e.g. nematodes, acanthocephaleans, microspora etc.) may be recorded.

It is advised that all liver nodules detected are examined histologically in order to confirm the neoplastic nature of the lesion. The nodule and some normal, adjacent tissue should therefore be carefully dissected (up to 5 mm thick pieces only) and placed (either directly or transferred

into pre-labelled histological cassettes) in a jar of 10% neutral buffered formalin (or Bouin's fluid) for preservation. This should be done as soon as possible after killing and examining the fish in order to avoid post mortem degradation of the tissue.

Liver histopathology

For the monitoring of liver histopathology in a random set of livers from flatfish (see Tables A6.1 and A6.2), a slice (preferably not thicker than 2 mm) from the central part of the liver should be taken by using a sharp scalpel blade and should be placed (either directly or transferred into pre-labelled histological cassettes) in a jar of 10% neutral buffered formalin (or Bouin's fluid) for preservation. A transfer to 70% ethanol after 12–24 hours is recommended if immunohistochemical studies are to be carried out. However, a long time storage in ethanol is not recommended because of hardening of the tissue and potential problems in subsequent sectioning.

Further histological processing should be done according to the scheme given in Table A6.4. Comprehensive information on all histological procedures to be applied, including recipes for fixation and staining etc. are provided by Feist *et al*. (2004).

Table A6.4: Histological processing of fish liver tissue samples (after Feist *et al.*, 2004, details in there)

STEPS IN HISTOLOGY	
Fixation	10% neutral buffered formalin
Dehydration	Increasing ethanol concentrations
Clearing	Xylene or less toxic substitute
Embedding	Paraffin wax
Sectioning	4–5 µm
Drying	On a hotplate
Clearing	Xylene or less toxic substitute, followed by 100% ethanol
Staining	Haematoxylin & Eosin
Dehydration	Increasing ethanol concentrations
Clearing	Xylene or less toxic substitute
Mounting	Synthetic mountant

Feist *et al*. (2004) also provide guidelines on diagnostic criteria for the following categories of histopathological liver lesion (see Table 3 in section 4.2 of the present report):

- Early toxicopathic non-neoplastic lesions,
- Putative pre-neoplastic lesions (foci of cellular alteration),
- Benign tumours, and
- Malignant tumours.

Diagnostic criteria for common non-specific liver lesions (e.g. inflammatory lesions, storage cell disorders, melanomacrophage aggregates etc.) are provided in the BEQUALM guidelines.

Size ranges and sample sizes

According to the ICES guidelines (later taken on board by BEQUALM), a size-stratified sampling should be conducted for flounder, dab and cod (Table A6.5). For other species relevant for fish disease monitoring in the Baltic Sea, no such recommendations had existed so far. Therefore, a suggestion is made in Table A6.5 for herring.

Table A6.5: Fish species suitable for fish disease monitoring and selection of gender, size ranges and sample sizes

DISEASE	SPECIES	GENDER	SIZE RANGE (CM TOTAL LENGTH)	SAMPLE SIZE
Externally visible diseases	Dab (*L. limanda*)	females + males	15–19 20–24 ≥ 25	100 100 50
	Flounder (*P. flesus*)	females + males	20–24 25–29 ≥ 30	100 100 50
	Cod (*G. morhua*)	females + males	< 29 30–44 ≥ 45	100 100 50
(for herring also internal parasites)	Herring (*C. harengus*)	females + males	15–19 20–24 ≥ 25	100 100 50
Liver nodules > 2 mm	Dab (*L. limanda*)	females+ males	20-24 cm ≥ 25	(50) 50
	Flounder (*P. flesus*)	females+ males	25–29 ≥ 30	(50) 50
Liver histopathology	Dab (*L. limanda*)	females	20–24	30–50
	Flounder (*P. flesus*)	females	25–29	30–50

The size ranges were defined based on long-term experience regarding the availability of fish of certain size groups and were implemented in order to enable regional comparisons even between fish population with different size structure.

The minimum sample size of 250 specimens per species and sampling site recommended for external examination is based on statistical requirements because this sample size allows for the detection of a disease prevalence of at least 1.5% with 95% confidence intervals (Bucke *et al.* 1996). The internal examination of flatfish for the presence of liver nodules > 2 mm in diameter is to be carried out in less specimens and only those belonging to the largest size group (dab: ≥ 25 cm; flounder: ≥ 30 cm) because in large (= older) fish there is a much higher likelihood for liver tumours to occur than in small (= younger) fish. If fish of the largest size group are not available in sufficient numbers, smaller fish may be added.

Experience has shown that the minimum requirements in terms of the sample size and size ranges can often not be met. Another disadvantage of size-stratified sampling is that the data generated do not allow for conclusions about the health status of a total population at a given site because a stratified sampling is not representative of the size structure of the population. Furthermore, size-stratified sampling ignores possible age effects on the diseases prevalence or grade. Since growth rates may differ considerably between sampling sites (at least for those that are far apart from each other) it might be that fish of the same size from different sites differ significantly in age and, therefore, the probability of a disease to occur may be quite different. This may possibly lead to misinterpretations of disease data.

Therefore, another sampling strategy is to take non-stratified samples (preferably larger than 250 specimens) representative of the demographic composition of population and, for regional comparisons, to compensate for size, age and gender effects by applying appropriate mathematical demographic standardisation models (e.g. used by Lang *et al.*, 1999).

Additional measurements

Most present environmental monitoring programmes have already evolved or are in the process of evolving into more integrated monitoring and assessment programmes, encompassing a wider range of measurements related to biotic and abiotic parameters that have an impact on the marine fauna.

For diseases of marine fish, key host- and site-specific factors involved in the disease aetiology or with an influence on the disease pathogenesis, ideally to be measured in an integrated fashion, are, e.g. :

- age,
- gender,
- population density and demography,
- nutritional status,
- responses of other biomarkers,
- presence of intermediate hosts,
- contaminants,
- nutrients,
- hydrographical factors (e.g., temperature, salinity, oxygen),
- anthropogenic disturbances (e.g., fishing, sand and gravel extraction, offshore installations).

Reporting and statistical analysis of fish disease data

Data generated in a standardised programme meeting quality assurance requirements should be submitted to the ICES Data Centre (see at http://www.ices.dk/datacentre/data_intro.asp) on an annual basis. Methodologies, validation processes and standard formats for data submission have been developed by ICES and are in place.

Assessments of the data submitted to ICES are carried out by the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) (Wosniok *et al.*, 1999, 2000) and guidelines for statistical techniques to be applied have been developed (Wosniok *et al.*, 2006) (see also section 4.3 and Annex 5 of the present report).

Quality assurance

All steps involved in the monitoring of fish diseases should be conducted according to standard guidelines and methods and results should be intercalibrated repeatedly. The BEQUALM programme provides a framework for all relevant components and it is, therefore, strongly recommended that institutes involved in fish disease monitoring in the Baltic Sea participate in the BEQUALM programme (http://www.bequalm.org). As regards external quality assurance, interlaboratory performance assessments including intercalibration exercises are being carried out as part of the remits of the BEQUALM programme.

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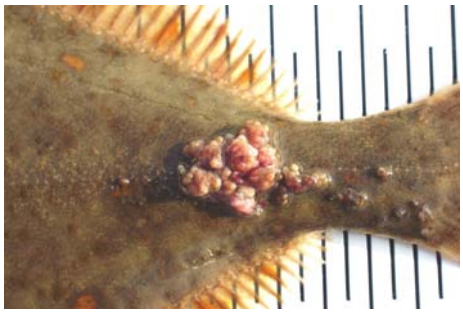
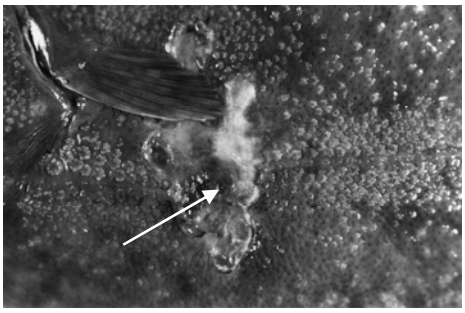
Plate A6.I: Common diseases/parasites of flounder (*Platichthys flesus*) in the Baltic Sea recorded for monitoring purposes

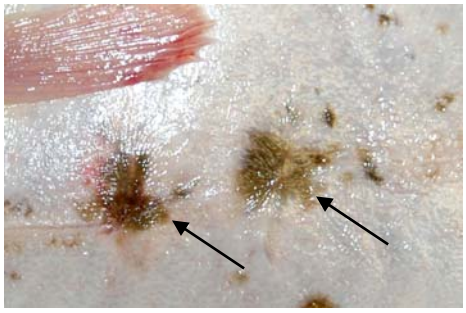


Lymphocystis (upper body side)

Acute stage of skin ulceration (upper body side)

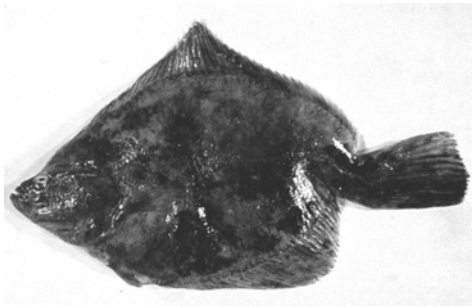


Healing stage of skin ulceration
(scar formation started)

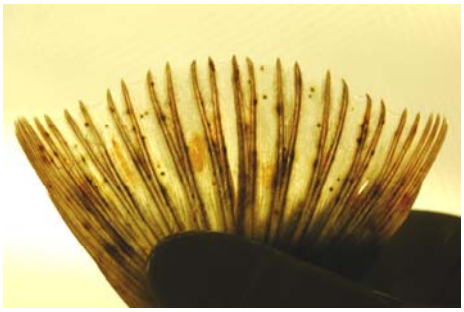


Healed stage of skin ulceration
(scar formation completed, pigment inclusions)

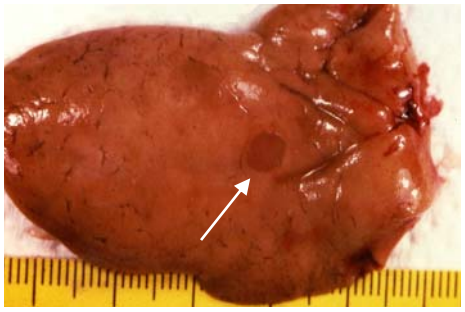
Acute stage of skin rot/erosion



Skeletal deformity



Cysts (metacercariae) of *Cryptocotyle* spp.
between the rays of the caudal fin

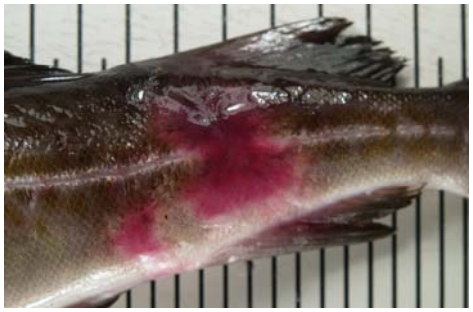


Liver nodule > 2 mm in diameter

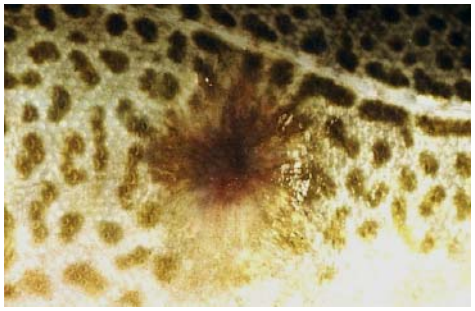
Plate A6.II: Common diseases/parasites of cod (*Gadus morhua*) in the Baltic Sea recorded for monitoring purposes

Early acute stage of skin ulceration
(skin not yet completely eroded)

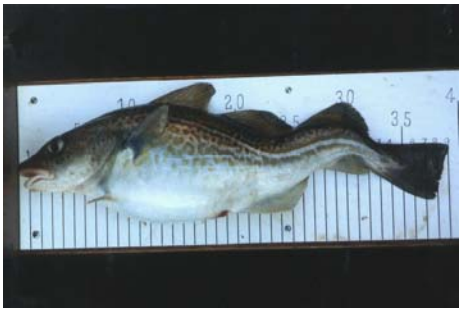
Chronic stage of skin ulceration
(necrotic tissue and tissue debris present)



Healing stage of skin ulceration
(scar formation started)

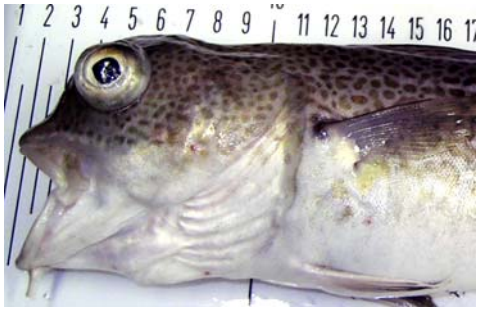


Healed stage of skin ulceration
(scar formation completed, pigment inclusion)

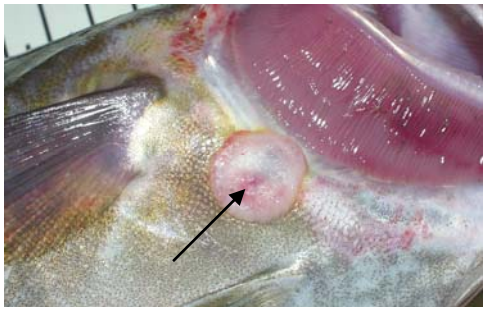


Skeletal deformity (lordosis)

Skeletal deformity (vertebral compression)

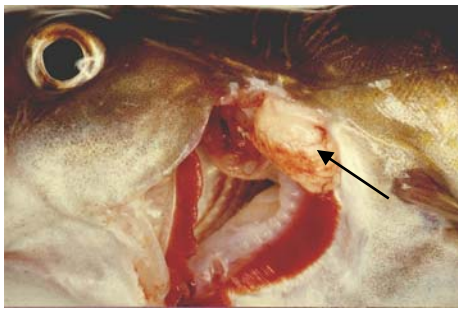


Skeletal deformity ('pug-head')

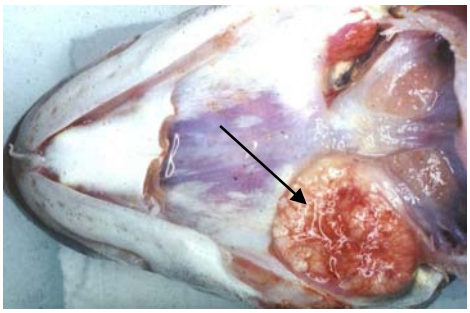


Epidermal hyperplasia/papilloma

Plate A6.2 (cont.): Common diseases/parasites of cod (*Gadus morhua*) in the Baltic Sea recorded for monitoring purposes (cont.)

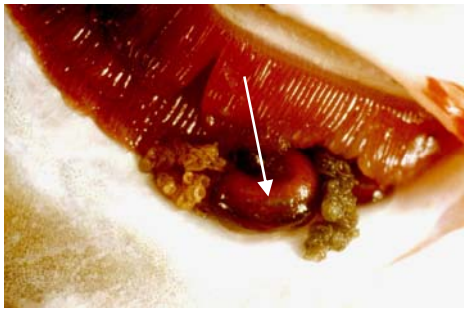


Pseudobranchial swelling ('x-cell disease'), lateral view



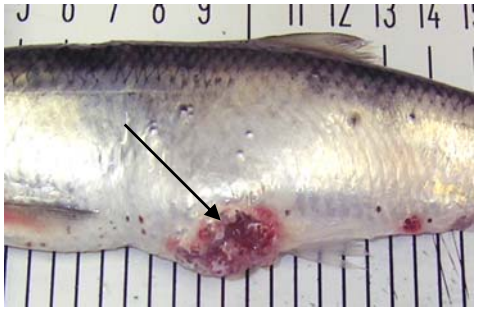
Pseudobranchial swelling ('x-cell disease'), lower jar removed

Black cysts (metacercariae) of *Cryptocotyle lingua*



Lernaeocera branchialis in the gill chamber

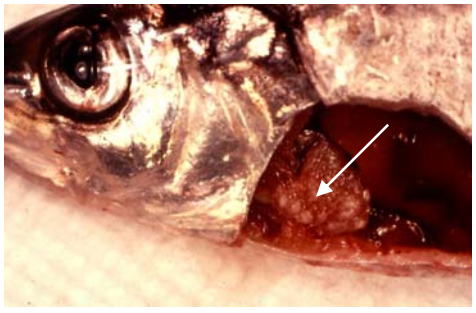
Plate A6.3: Common diseases/parasites of herring (*Clupea harengus*) in the Baltic Sea recorded for monitoring purposes



Lymphocystis

Skeletal deformity (vertebral compression) (photo by courtesy of M. Wyszynski)

Larval nematodes (*Anisakis simplex*) in the body cavity



White cysts (granulomas) in the heart caused by *Ichthyophonus* sp.

Annex 7: Recommendations

RECOMMENDATION	ACTION
1. ICES to communicate the WKFDM report and recommendations to relevant national ministries/agencies responsible for monitoring the environmental status of the Baltic Sea and to international organisations (HELCOM, OSPAR, EU, EEA);	ICES
2. Baltic Sea countries use fish diseases as a 'top-level indicators' of health in national integrated chemical and biological effects monitoring programmes;	Baltic Sea countries
3. Baltic Sea countries conducting fish disease studies should apply the guidelines developed by ICES and through the BEQUALM programme with the amendments proposed by WKFDM summarised in the present report as Annex 6.	Baltic Sea countries
4. Baltic Sea institutes involved in fish disease monitoring in the Baltic Sea participate in the Biological Effects Quality Assurance in Monitoring Programme (BEQUALM);	Baltic Sea countries
5. ICES/BSRP organise a land-based workshop on methodologies for coastal fish disease monitoring. The workshop could be held in 2006 or 2007 at the AtlantNIRO, Kaliningrad, Russia, or at the Estonian Marine Institute, Tallinn;	ICES SGEH, WGPDMO
6. Baltic Sea countries harmonise the components of their national marine monitoring and assessment programmes in order to implement an integrated programme on contaminants (and other anthropogenic stressors) and their biological effects;	Baltic Sea countries
7. Baltic Sea countries and HELCOM investigate the potential for an internationally coordinated integrated monitoring programme in the Baltic Sea, encompassing joint sampling campaigns and the involvement of appointed expert laboratories in the Baltic countries responsible for the conduct of specific analytical measurements;	Baltic Sea countries, ICES SGEH, HELCOM
8. ICES/BSRP, HELCOM and Baltic Sea countries consider to organise an international demonstration project in 2007 or 2008 on the ecosystem health of the Gulf of Finland, providing baseline data and assessing the feasibility of coordinated sample collection and analysis;	ICES SGEH, Baltic Sea countries
9. The ICES Working Group on Pathology and Diseases (WGPDMO) takes note of the proposals made by the WKFDM regarding the Fish Health Index and conducts work on its further development based on the conclusions made by WKFDM.	ICES WGPDMO