Standard Operating Procedure (SOP)

Aim of SOP (tick box)

☐ Munition detection or identification	☐ Toxicity
☐ Sampling	☐ In situ exposure studies
☐ Chemical analysis	☐ Bioassays
☒ Bioindicators/biomarkers	

25. Fish liver histopathology (LH)

version 1.1

Thomas Lang and Katharina Straumer

Thünen Institute of Fisheries Ecology

Scope

Studies on fish liver histopathology have frequently been applied to identify effects of contaminants at the cellular and tissue level [1-18]. Since the liver is the main metabolic organ and is involved in detoxification of environmental contaminants, it is particularly suitable as target organ for histopathological studies.

Summary of the method/SOP

Liver histopathology is amongst the techniques recommended for monitoring biological effects of contaminants [11, 19, 20] and studies are carried out under national and international monitoring programmes (EU Marine Strategy Framework Directive, OSPAR Coordinated Environmental Monitoring Programme, HELCOM Baltic Sea monitoring).

The occurrence of fish liver pathology is considered as an indicator of habitat quality and environmental health, reflecting the impact of stressors, including hazardous substances, on fish health. [3, 7, 8, 11, 17, 18].

Some of the known and well-documented liver pathologies are regarded as contaminant-specific indicators. For instance, the occurrence of neoplastic liver lesions (liver tumours and their pre-stages) are a well-documented indicator of exposure to carcinogenic contaminants. Other types of liver lesions are non-specific (e.g., inflammatory lesions) and reflect general stress conditions if they occur at a prevalence higher than normal.

Technical guidelines and quality assurance procedures for studying fish liver histopathology in the context of monitoring have been published and implemented, largely through activities of the International Council for the Exploration of the Sea (ICES) [11, 17]. An international quality assurance programme (BEQUALM) has been established that addresses, amongst other biological effects techniques, also liver histopathology (www.bequalm.org).

Safety aspects

Gloves should be worn to protect the observer's skin. If some samples should be taken, safety aspect depends on used chemicals (e.g. Fixatives (formalin and other fixatives used, ethanol). Chemicals should be stored adequately and should be used according to specific safety instructions and, preferably under a fume cupboard or fume hood, wearing gloves, protective

clothes and preferably safety glasses).
Since the work is largely carried out onboard of unstable platforms (e.g., research vessels) under partly bad weather conditions, specific safety regulations apply and relevant staff training needs to be carried out. Equipment and fish tanks etc. need to be fixed properly. Dissecting fish and taking tissue samples involves handling of sharp knives and scalpels and these should be used carefully in order to avoid injuries.

Documentation

Equipment:

- Information in sampling fish and liver samples (see details SOP sampling fish[21], MLN[22] and EVFD[23]

Samples:

- Detailed information on fish
  - Sex, Length, weight,
  - age based on otolith reading → If age cannot be determined, total length may be used as surrogate
- detailed information on liver: see SOP for MLN[22]

Methods

Methods for fish disease surveys, including studies on liver histopathology, have largely been developed and repeatedly intercalibrated through ICES activities and through the fish disease component of the BEQUALM programme (www.bequalm.org) [7, 11, 17]. The method consists of sampling of liver tissue from a defined number of fish (e.g., 30-50 specimens per sampling area, [11]), fixation in 10 % neutral buffered formalin, histological processing (embedding, cutting, staining etc.) and microscopic analysis. Detailed information addressing all relevant methods is provided by Feist et al. (2004). This includes guidelines for lesion diagnosis (diagnostic key) and a categorisation of lesions according to the groups listed in Tab. 1.

For detailed information, see also SOP describing methods for externally visible fish diseases (EVFD)[23] and SOP for macroscopic liver neoplasms (MLN)[22].

It is recommended to combine the study of liver histopathology with studies on the occurrence of MLN[22] and preferably also with EVFD[23], because a combination of all three techniques provides the best overview to assess the health status of fish and the possible impact of toxic dumped munitions compounds.

Equipment:

- All equipment for fishing and storing fish (see details in SOPs Sampling of wild fish[21], EVFD[23] and MLN[22])
- Dissection sets: scalpel and forceps
- Histological cassettes
- Pencils for writing protocols and for labelling
- Protocols for recording data
- fixative (preferably 10 % neutral buffered formalin), histological cassettes and storage containers are required
- 70 % Ethanol
- Camera for documentation of disease symptoms and severity grades
- for subsequent histological processing and diagnosis, a fully equipped histology lab and a high quality light microscope are needed (see details in Feist et al. 2004[11]).

***Matrix*:** Liver tissue of freshly collected and dissected fish.

***Sample size*:** Ideally, liver histopathology should be recorded in a minimum of 30 specimens per sampling site belonging to a defined size group. For instance, for dab and flounder, the standard guidelines recommend the size groups 20-24 cm and 25-35 cm, respectively [11, 24].

Method:

Taking samples:

Figure 1 show the sampling procedure for dissecting samples. If there is a nodule/tumour, make sure that the sample (slice) is taken in a way that it contains both normal and tumour tissue.
In cases where there are no grossly visible lesions, a 3 mm slice is cut longitudinally through the central axis of the liver using a sharp blade (e.g., scalpel blade No. 24). If visible anomalies are present, a section should also be taken through the entire depth of the affected area(s), including, where possible, adjacent normal tissue. Place the sample in a labelled histological cassette. Close the cassette and put it into the bin/bottle with neutral buffered formalin solution. Carefully agitate the bottle to obtain a uniform distribution of the formalin solution around the sample. If the cassettes are not labelled, write a number on it with a lead pencil (don't use a marker/felt-tip pen), because this will keep in formalin. Mark the cassettes with a "T" if a tumour-/nodule-sample is inside. At this juncture, it is important that accurate notes are taken to describe the gross features of the lesion so that it may be confidently related to its microscopic appearance after the sample has been processed in the laboratory. Relevant photographs may also be taken to assist with the identification of small lesions during embedding. In all cases, it is essential that care be taken to avoid crushing or ripping the tissue with forceps or other dissection instruments.

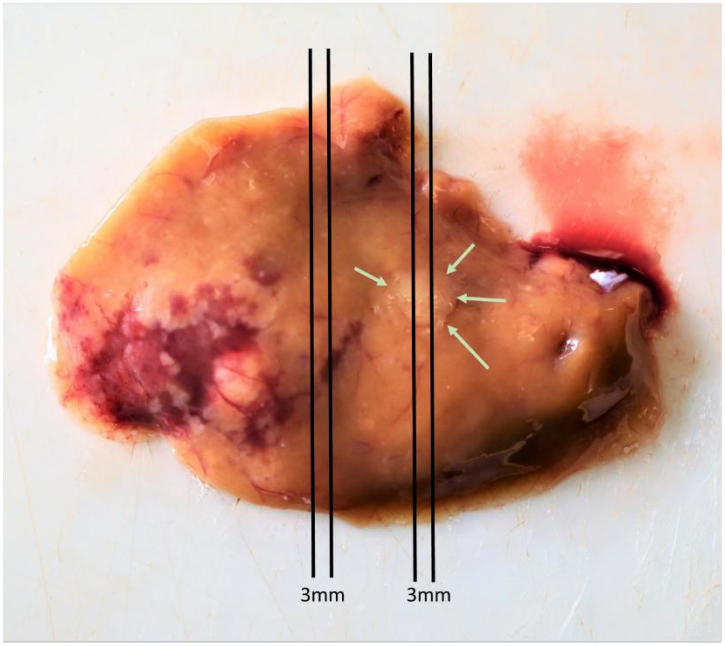

Figure 1: Showing sampling procedure for dissecting histological samples for a obviously healthy part of liver tissue (left) and a nodule (arrows), possibly a tumour.

Fixation is allowed to proceed for 12–24 hours with occasional agitation to ensure the even fixation of samples. Samples may then be transferred to 70% ethanol for transportation and long-term storage.

Comprehensive information on all histological procedures to be applied, including recipes for fixation and staining and protocols for automated processing etc., are provided by Feist et al. (2004)[11]. A sort summary of routine procedure involved is given in Tab.1

Table 1: Histological processing of liver tissue samples (after Feist et al. 2004, details there)

Steps in histology	
Fixation	10% neutral buffered formalin (recipe in Chapter 3.3)
Dehydration	Increasing ethanol concentrations
Clearing	Xylene or less toxic substitute
Embedding	Paraffin wax
Sectioning	4–5 µm, using a rotary microtome
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

Histological assessment and confirmation:

Histological confirmation of liver nodules and diagnosis of lesions is essential and should be carried out as described in Feist et al. (2004)[11]. Confirmation is required because only prevalence data on histologically confirmed cases of macroscopic liver neoplasms >2 mm (benign and malignant tumours) are reported according to ICES and BEQUALM guidelines and are used as indicator of PAH-specific biological effects, e.g., in the OSPAR Coordinated Environmental Monitoring Programme (CEMP).

Tab. 2: Categories and types of histopathological liver lesions commonly found in fish and recommended for monitoring (Feist et al. 2004, with modifications)

Lesion category	Lesion types	Remarks
Non-specific lesions	Lymphocytic infiltration Granulomatosis Atrophy Necrosis Apoptosis Increased number/size of macrophage aggregates Regeneration Micro-/macrosteatosis	Non-specific indicator of effects of natural (e.g., infection, malnutrition) and anthropogenic (e.g., contaminants) stressors
Early toxicopathic non-neoplastic lesions	Hepatocellular and nuclear pleomorphism Hydropic vacuolation of biliary epithelial cells and/or hepatocytes	Indicator of early effects of various contaminants

	Phospholipidosis Fibrillar inclusions Peliosis and spongiosis hepatis	
Pre-neoplastic lesions	Foci of cellular alteration (FCA) (clear cell, vacuolated, eosinophilic, basophilic, mixed cell foci)	Indicator of early carcinogenesis caused by carcinogenic organic and inorganic contaminants
Benign liver tumours	Hepatocellular adenoma Cholangioma Hemangioma Pancreatic acinar cell adenoma	Indicator of carcinogenesis caused by carcinogenic organic and inorganic contaminants
Malignant liver tumours	Hepatocellular carcinoma Cholangiocarcinoma Pancreatic acinar cell carcinoma Mixed hepatobiliary carcinoma Mixed anguosarcoma/hepatocellular carcinoma Hemangiosarcoma Other	Indicator reflecting endpoints of carcinogenesis caused by carcinogenic organic and inorganic contaminants

It is well known that the presence and prevalence of neoplastic liver lesions in fish are influenced by host-specific factors, in particular by age [25]. For neoplastic lesions, age is a key variable to be taken into account, because age is a risk factor for the onset of tumour diseases. It is, thus, very important to determine the age of fish examined for liver histopathology. The best way is to do the ageing based on otolith reading, applying standard fish stock assessment methodologies. If age cannot be determined, total length may be used as surrogate, which is, however, less reliable than age, because the growth of fish may differ between study areas.

Based on the number of fish examined for liver histopathology and the number of fish found to be affected by specific histopathological lesion types or lesion categories (see Tab. 2), the prevalence of these and differences in prevalence between samples can be calculated by using the methods detailed in SOP EVFD[23].

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List of authors

Thomas Lang, Katharina Straumer (1.0)

Katharina Straumer (1.1)

List of Reviewers

Aino Ahvo (1.0)