DAIMON Toolbox Fact Sheets:

Methods to Study the Impact of Dumped Munitions on Marine Biota

Assessment category: Biological Effects

Toolbox components: Disease/Pathology, Carcinogenicity

Fact Sheet 3.17: Fish liver histopathology (LH)

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What is it?

Studies on fish liver histopathology have frequently been applied to identify effects of contaminants at the cellular and tissue level (Malins at al. 1985 a,b, Hinton and Lauren 1990, Vethaak and ap Rheinallt 1992, Bucke and Feist 1993, Hinton 1994, Vethaak and Wester 1996, ICES 1997, Myers et al. 1998 a,b, Stehr et al. 1998, Lang 2002, Stentiford et al. 2003, Feist et al. 2004, Lang et al. 2006, Fricke et al. 2012, Faber 2014, Lang et al. 2017). 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.

Liver histopathology is amongst the techniques recommended for monitoring biological effects of contaminants (Feist et al. 2004, OSPAR 2007, Davies and Vethaak 2012) 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).

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) (ICES 1997, Feist et al. 2004). An international quality assurance programme (BEQUALM) has been established that addresses, amongst other biological effects techniques, also liver histopathology (www.bequalm.org).

What does it tell you?

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. (Vethaak & ap Rheinallt 1992, ICES 1997, Lang 2002, Feist et al. 2004, Lang et al. 2017).

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.

Fish liver histopathology is applicable in a screening or detailed study on pathological effects of conventional or chemical munitions and warfare agents on fish and has been studied in the

projects CHEMSEA and DAIMON. In particular neoplastic pathologies are a suitable indicator to identify and assess effects of carcinogenic munitions compounds. However, because the indicator may also respond to non-munitions hazardous compounds, it is not recommended to use it in isolation, but in concert with other biological effects indicators and chemical measurements.

Type of Indicator (tick box)

☐ non-specific stress indicator
☒ specific for groups of contaminants incl. CWA or explosives
☐ CWA-specific indicator
☐ specific for substances related to explosives (e.g. TNT)

How to measure it?

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) (ICES 1997, Feist et al. 2004, Lang et al. 2017).

Technical guidelines for measuring liver histopathology as part of biological effects monitoring are available from ICES publications and from the Coordinated Environmental Monitoring Programme (CEMP) and Joint Assessment and Monitoring Programme (JAMP) of the OSPAR Commission (Feist et al. 2004, OSPAR Commission 2007, Davies and Vethaak 2012). These standardised methods are applied routinely by countries bordering the Baltic Sea and North Sea as well as adjacent areas.

The method consists of sampling of liver tissue from a defined number of fish (e.g., 30-50 specimens per sampling area, Feist et al. 2004), 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.

General sampling requirements for fish liver histopathology are identical with those detailed in Fact Sheet 3.16 for externally visible fish diseases and Fact Sheet 3.25 for macroscopic liver neoplasms.

***Species*:** Methodologies and diagnostic criteria involved in the monitoring of liver histopathology and for macroscopic liver neoplasms have largely been developed based on studies with flatfish species, in Europe mainly the flatfish species common dab (*Limanda limanda*) and European flounder (*Platichthys flesus*), but can also be adapted to other flatfish species, e.g., plaice (*Pleuronectes platessa*), and also to bottom-dwelling roundfish species, such as cod (*Gadus morhua*) (Faber 2014) or eelpout (*Zoarces viviparus*) (Fricke et al. 2012). In North American monitoring programmes, flatfish species such as winter flounder (*Pleuronectes americanus*), English sole (*Pleuronectes vetulus*), starry flounder (*Platichthys stellatus*), and rock sole (*Lepidopsetta bilineata*) have been widely used for biological effects studies due to their susceptibility to contaminants and their propensity to develop toxicopathic liver lesions (Myers *et al*. 1998b).

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

***Equipment*:** see DAIMON Fact Sheet 3.16 describing methods for studying externally visible fish diseases (EVFD) (Lang & Straumer 2019a) and Fact Sheet 3.25 for macroscopic liver neoplasms (Lang & Straumer 2019b). For dissection of the fish and tissue sampling for later histology, appropriate dissecting sets as well as fixative (preferably 10 % neutral buffered formalin), histological cassettes and appropriate storage containers are required.

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).

Tab. 1: 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 Phospholipidosis Fibrillar inclusions Peliosis and spongiosis hepatis	Indicator of early effects of various contaminants
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	Indicator reflecting endpoints of

	Pancreatic acinar cell carcinoma Mixed hepatobiliary carcinoma Mixed anguosarcoma/hepatocellular carcinoma Hemangiosarcoma Other	carcinogenesis caused by carcinogenic organic and inorganic contaminants

***Measurements and units*:** For general methods, see DAIMON Fact Sheet 3.16 on externally visible fish diseases (Lang & Straumer 2019a).

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 (Stentiford et al. 2010). 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.

It is recommended to combine the study of liver histopathology with studies on the occurrence of macroscopic liver neoplasms (MLN) (see DAIMON Fact Sheet 3.25, Lang & Straumer 2019b) and preferably also with externally visible fish diseases (EVFD) (see DAIMON Fact Sheet 3.16, Lang & Straumer 2019a), 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.

***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 (Bucke et al. 1996, Feist et al. 2004).

How to analyse and assess the data?

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. 1), the prevalence of these and differences in prevalence between samples can be calculated by using the methods detailed in DAIMON Fact Sheet 3.16 (Lang & Straumer 2019a) addressing externally visible fish diseases (EVFD).

The following statements by Feist et al (2004) are endorsed: "*For a multifactorial statistical analysis of disease data, multivariate tests based on logistic models (McCullagh and Nelder, 1989) have been applied successfully and are therefore recommended. In addition to enabling the identification of single host-specific and site-specific factors and their interaction with a significant relationship to the disease prevalence, these tests also allow for a quantification of their effects, thereby providing useful information on possible cause-effect relationships. Thorough descriptions*

for the design and application of such models are given in numerous studies (e.g., by Vethaak and Jol, 1996; Lang et al., 1999; Lang and Wosniok, 2000; Wosniok et al., 1999, 2000).”

For the assessment of liver histopathology data, the method by Lang et al. (2017) can be applied which is based on the definition of background assessment criteria (BAC), reflecting natural undisturbed and healthy conditions, and ecological assessment criteria (EAC), reflecting unacceptable contaminant effects. Specimens without lesions and/or with only non-specific lesions are assigned to the category FDI ≤ BAC, those with early toxicopathic non-neoplastic lesions and/or foci of cellular alteration to the category BAC < FDI ≤ EAC and those with benign or malignant tumours to the category FDI > EAC, analogous to the assessment of macroscopic liver neoplasms (MLN) (see DAIMON Fact Sheet 3.25, Lang & Straumer 2019b).

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