

## Standard Operating Procedure (SOP)

### Aim of SOP (tick box)

- |   |   |
|---|---|
| <input type="checkbox"/> Munition detection or identification | <input type="checkbox"/> Toxicity                 |
| <input type="checkbox"/> Sampling                             | <input type="checkbox"/> In situ exposure studies |
| <input type="checkbox"/> Chemical analysis                    | <input type="checkbox"/> Bioassays                |
| <input checked="" type="checkbox"/> Bioindicators/biomarkers  |   |

### 26. Macroscopic liver neoplasms (MLN)

version 1.1

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### Scope

The present SOP addresses the examination of common Baltic Sea fish species for the occurrence of macroscopic liver neoplasms (MLN). The SOP is applicable in all Baltic Sea sub-regions where the following target fish species are present in sufficient numbers for a regular fish disease monitoring and assessment and where regular surveys take place, either specifically dedicated to fish disease monitoring or combined with other activities, e.g., as part of integrated monitoring concentrations of contaminants and their biological effects or fish stock assessment:

- Flounder (*Platichthys flesus*)
- Dab (*Limanda limanda*)
- Eelpout (*Zoarces viviparus*)
- Cod (*Gadus morhua*)

While flounder, cod and eelpout are wide-spread in most parts of the Baltic Sea, dab is restricted to the western Baltic Sea. Eelpout is largely restricted to coastal waters, whereas the other species can be sampled both in coastal and offshore areas.

The occurrence of MLN at a prevalence above natural background levels is considered as a contaminant-related indicator of habitat quality and environmental health, reflecting the impact of carcinogenic hazardous substances<sup>1-5</sup>.

Since MLN is regarded as a contaminant-specific indicator, it is applicable in a screening or detailed study on carcinogenic effects of conventional or chemical munitions and warfare agents on fish. Because the indicator may also respond to non-munitions carcinogenic compounds, it is not recommended to use it in isolation, but in concert with other biological effects indicators and chemical measurements.

### Summary of the method/SOP

The term macroscopic liver neoplasms (MLN) describes benign and malignant neoplastic liver changes (tumours) found in wild fish by means of visual inspection of the liver surface by naked eye, quantification of liver nodules >2 mm in diameter and subsequent histological confirmation and classification of the macroscopic nodules. MLN are frequently used as indicators of carcinogenic effects of hazardous substances in environmental monitoring programmes<sup>2</sup>.

Studies on MLN are amongst the techniques recommended for monitoring contaminant-specific biological effects of contaminants and are carried out under national and international monitoring programmes (EU Marine Strategy Framework Directive, OSPAR Coordinated Environmental Monitoring Programme, HELCOM Baltic Sea monitoring)<sup>6,7</sup>.

Technical guidelines addressing all steps involved, from sampling to data analysis, have been

developed and published, largely through activities of the International Council for the Exploration of the Sea (ICES)<sup>3, 5, 8, 9</sup>.

### Safety aspects

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. Gloves should be worn to protect the observer's skin. Protective clothes (rubber boots, oil skin trousers, gloves) should also be worn. If some samples should be taken, safety aspect depends on used chemicals. Depending on the chemicals to be tested, the corresponding risk assessments have to be considered before starting work. If further instructions are required, contact the local safety officer or the laboratory manager.

### Documentation

#### Equipment:

- Information on trapping method
- Information on sampling site

#### Fish:

- Record the fish species, size (cm, see table 1 below), total weight and sex
- Visual inspection of the liver: colour, existence of nodules, lesion, abnormalities, parasites or other changes
- Additional information on externally visible fish diseases (EVFD) and liver histopathology (LH) (see SOPs EVFD<sup>10</sup> and LH<sup>11</sup>)
- Detailed information on all liver lesion and abnormalities (see table 2)
- Information on taken samples

**Table 1: Fish species suitable for monitoring of macroscopic liver neoplasms in the Baltic Sea and sampling requirements (selection of sex, size ranges and sample sizes)** (Bucke et al. 1996<sup>8</sup>, Feist et al. 2004<sup>3</sup>, www.bequalm.org; modified)

Disease	Species	Sex	Size range (cm total length)	Sample size (no. specimens examined)
Macroscopic liver neoplasms >2 mm	Flounder ( <i>P. flesus</i> )	females + males	25-29	50
			30-34	50
	Dab ( <i>L. limanda</i> )	females + males	20-24	50
			≥25	50
	Cod ( <i>G. morhua</i> )	females + males	30-44	50
			45-60	50
	Eelpout ( <i>Z. viviparus</i> )	females + males	18-24	20
			≥25	20

**Table 2: Macroscopic liver lesions/abnormalities to be recorded in Baltic Sea fish inspected for the presence of macroscopic liver neoplasms**

Lesion/abnormality	Categories/severity grading	Comments
Liver nodules*  * Note: Histological confirmation of all nodules >2 mm is required	<2 mm in diameter 2-5 mm “ “ 6-9 “ “ ≥10 mm “ “	The number of nodules within each of the size categories can be recorded.  The colour of the nodules may be recorded, e.g., lighter or darker than liver colour, green, dark red, opaque appearance.
Parasites on the liver (nematodes, larval acanthocephaleans, parasite cysts/granulomas)	Number of parasites or severity grades; e.g., Grade 1: 1 parasite Grade 2: 2 parasites Grade 3: ≥3 parasites	For cysts occurring in high numbers (e.g., caused by <i>Ichthyophonus</i> sp. or <i>Glugea</i> sp. infections), a different grading has to be applied; e.g., Grade 1: 1-10 cysts Grade 2: 11-50 cysts Grade 3: ≥50 cysts
Liver colour	– Light – Medium – Dark red – Green – Heterogeneous colouration	The colour of the liver reflects the physiological state of the organisms. Light livers have a higher lipid content than dark livers.  A partial green discolouration of the liver is a sign of parasitic infection of the bile ducts, leading to a blockage (icterus). A zonal colour pattern may reflect general degenerative or regenerative change.
Black inclusions	-	Caused by increased number of macrophage aggregates
Textural changes	-	For instance, a marbled pattern may reflect general degenerative or regenerative change

## Methods

General sampling requirements for MLN are identical with those detailed in SOP for EVFD<sup>10</sup> and SOP LH<sup>11</sup>.

When inspecting fish, it is easy and, thus, recommended to record and quantify also the presence of other diseases. These are (a) externally visible fish diseases (EVFD) and (b) other types of macroscopic liver lesions/abnormalities (see information on methodologies for EVFD)

### Equipment:

- Research vessel, commercial fishing boat or sampling platforms (see SOP *Sampling of wild fish*<sup>12</sup> and EVFD<sup>10</sup>)
- Gill net, pelagic or standard gear for trawling (see SOP<sup>10,12</sup>)

- Fish tanks with fish and buckets for transporting live fish
- Running sea water
- PC, suitable software and other electronically equipment for documentation
- Protective clothes and thin gloves
- Dissection sets: scalpel and forceps
- Balance
- Sufficient working light for assessing colour, parasites and lesions
- Protocols and suitable pens (waterproof)
- Camera for documentation of disease symptoms and severity grades
- Appropriate: fixative (preferably 10 % neutral buffered formalin), histological cassettes and 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<sup>3</sup>.) see more SOP of Liver Histopathology

**Sample size:** Ideally, MLN should be recorded in 100 specimens per sampling site, divided into two pre-defined size groups consisting of 50 specimens each. The first group represents smaller fish, the second one larger fish<sup>3,8</sup>. Examples are given in Table 1.

**Species:** Methodologies and diagnostic criteria involved in the monitoring of macroscopic liver neoplasms and liver histopathology 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*)<sup>13</sup> or eelpout (*Zoarces viviparus*)<sup>14</sup>.

**Liver examination procedure and tissue sampling:** The specimens selected for examination should be inspected while fresh, i.e., shortly after they have landed on the ship or been taken from nets (not frozen or refrigerated). Fish should be kept alive in tanks with running seawater with physical parameters similar to the ambient water.

**Method's description** has been extracted from Bucke et al. (1996)<sup>8</sup> and Feist et al. (2004)<sup>3</sup> and has been modified slightly. Parts are cited from the SOP for the measurement of biological effects of contaminants in the Baltic Sea from BEAST-project<sup>15</sup>

#### Preparation of fish:

- Fish are either anaesthetised or stunned by a blow on the head and sacrificed by severing the spinal cord with a knife or scalpel.
- Record the size (cm below), total weight and sex of the fish.
- If applicable, examine fish for EVFD (details see SOP EVFD<sup>10</sup>)
- Individual fish are placed (flatfish underside downwards) on the table/dissection board.
- Carefully make an incision into the visceral cavity using a scalpel or scissors.
- Insert the blunt blade of the blunt/sharp scissors into the cavity and cut along (around for flatfish) the length of the visceral cavity, lifting the resulting flap of muscle away with blunt forceps.
- Examine the viscera and note any abnormalities.
- Hold the oesophagus gently with forceps and sever with scissors as close as possible to the pharynx.
- Gently lift out the viscera, severing any connective tissue with a scalpel/sharp scissor.
- When the mass is free of the cavity, sever the intestine at the anus with scissors.
- With the intestine on top, lift gently away from liver, gonad and spleen, severing adhesions to free the organs.
- Examine and sample the liver and spleen
  - o **If applicable**, record the gutted weight of the fish and – if possible - the weight of the liver, gonad and spleen for subsequent calculation of condition factors and

organosomatic indices.

- Remove the otoliths for age reading and store individually in Eppendorf tubes or cryo vial with the correct coding for the fish.

**Liver examination:**

- Most important visible anomalies/parasites/diseases are given in table 2
- Record the liver colour, e.g., by using a standardised colour plate.
- Inspect the liver for the presence of parasites. In most cases, these will be nematode larvae, coiled in spirals (diameter of the spirals approx. 3-5 mm), or acanthocephaleans on the surface of the liver. Other parasites (e.g., Glugea sp.) or granulomas associated with bacterial infections (e.g., caused by Francisella sp.) may be seen as white cysts on the organ surface. Record the number of parasites/cysts (see Table 2) and take a sample in 10 % NBF if appropriate.
- Record all other abnormalities, like discolorations (incl. green discoloration caused by parasites blocking the bile ducts), variation in colour pattern or texture etc. (see Table 2).
- It is recommended to take samples for histology from all lesions/abnormalities recorded.
- Inspect the liver for the presence of liver nodules (only those of non-parasitic origin). Record all liver nodules (rounded spot, normally raised above the surface of the normal liver, clearly demarcated from the surrounding tissue by its colour and texture) with a diameter > 2 mm and record data.

**Tissue sampling:**

- Take a tissue sample (nodule and surrounding normal tissue) for histological confirmation of the lesion by cutting a 3 mm thick slice longitudinally through the nodule and the adjacent normal tissue using a sharp scalpel. The use of disposal blades is recommended. If the nodule is small, the whole nodule with surrounding normal tissue should be taken.
- Place each liver tissue sample in a pre-labelled histological processing cassette.
- Place the cassettes with the samples in large sealed containers (ca. 1-1.5 L) with 10% neutral buffered formalin (NBF) (see recipe in Chapter 3.3) and with occasional agitation to ensure an even fixation of samples. The ratio between the volume of the samples and the volume of the fixative should be ca. 1:20.
- After 12-24 h, transfer the samples to 70 % ethanol for transportation and long-term storage (methylated ethanol is sufficient). For possible application of samples to immunohistochemical techniques, fixation in 10% NBF for more than 12–24 hours should be avoided. Storage in ethanol for many months is not recommended because of hardening of the tissue and potential problems in subsequent sectioning.

**Assessment:** 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<sup>16</sup>. 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 MLN. The best way is to do the ageing based on otolith reading. 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 MLN with studies on the occurrence of EVFD<sup>10</sup> and on liver histopathology<sup>11</sup>.

**Conclusions (if applicable)**

Methods for fish disease surveys, including studies on the occurrence of macroscopic liver neoplasms (MLN) and liver histopathology (LH), have largely been developed and repeatedly intercalibrated through ICES activities and through the fish disease component of the BEQUALM

programme ([www.bequalm.org](http://www.bequalm.org))<sup>3, 8, 9</sup>.

Technical guidelines for measuring MLN 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<sup>3, 8, 9, 17</sup>.

These standardised methods are applied routinely by a number of countries bordering the Baltic Sea and the North Sea as well as adjacent areas, e.g. as part of their monitoring requirements under the EU Marine Strategy Framework Directive (MSFD), OSPAR CEMP or HELCOM monitoring. Long-term data from national monitoring programmes carried out in the North Sea, Baltic Sea and adjacent areas are available from the ICES data portal DOME (Marine Environment) (<http://www.ices.dk/marine-data/data-portals/Pages/DOME.aspx>) and are updated on a regular basis. These data can be utilized for comparative purposes in the context of munitions-related assessments as required.

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#### Change history

- |     |                 |                 |
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