DAIMON Toolbox Fact Sheets:

Methods to Study the Impact of Dumped Munitions on Marine Biota

Assessment category: Biological effects

Toolbox component: Carcinogenicity

Fact Sheet 3.25: Macroscopic liver neoplasms (MLN)

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What is it?

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 (Lang 2002).

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).

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) (ICES 1989, Bucke et al. 1996, ICES 1997, Feist et al. 2004).

What does it tell you?

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

Since MLN is a 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.

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 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) (ICES 1989, Bucke et al. 1996, ICES 1997, Feist et al. 2004).

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 (ICES 1989, Bucke et al. 1996, Feist et al. 2004, OSPAR Commission 2007).

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.

The method consists of a visual examination of the liver surface of freshly collected fish belonging to defined size groups for the occurrence of liver nodules >2 mm in diameter (for examples see Annex 2) and other abnormalities. The macroscopic examination is followed by fixation of tissue samples for a subsequent histological confirmation of the neoplastic nature of the liver nodules. Basic methods have been standardised and intercalibrated repeatedly on an international level through ICES activities and the BEQUALM programme (Feist et al. 2004, http://www.bequalm.org).

However, 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. Information on methodologies for EVFD are provided in DAIMON Fact Sheet 3.16 (Lang & Straumer 2019a), Annex 1 provides information on macroscopic liver lesions/abnormalities to be recorded.

General sampling requirements for MLN are identical with those detailed in Fact Sheet 3.16 for externally visible fish diseases (EVFD) and Fact Sheet 3.17 for liver histopathology (LH) (Straumer & Lang 2019b).

***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*) (Faber 2014) or eelpout (*Zoarces viviparus*) (Fricke et al. 2012).

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

***Equipment*:** see Fact Sheet 3.16 (Lang & Straumer 2019a) describing methods for studying externally visible fish diseases (EVFD) and Fact Sheet 3.17 (Lang & Straumer 2019 b) for liver histopathology (LH). 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).

***Measurements and units*:** see Fact Sheet 3.16 (Lang & Straumer 2019a) describing methods for studying externally visible fish diseases (EVFD).

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 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 externally visible fish diseases (EVFD) (see Fact Sheet 3.16, Lang & Straumer 2019a) and on liver histopathology (see Fact Sheet 3.17, Lang & Straumer 2019b).

***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 (Bucke et al. 1996, Feist et al. 2004). Examples are given in Tab. 1.

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, Feist et al. 2004, www.bequalm.org; modified)

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

How to analyse and assess the data?

Based on the number of fish examined for MLN and the number of fish found to be affected by histologically confirmed cases of MLN, the prevalence of MLN 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).

For the assessment of MLN data, the method by Lang et al. (2017) is recommended. All fish without MLN represent natural background conditions and are, thus, rated as below the background assessment criterion (BAC). Specimens with MLN indicate an unacceptable contaminant effects and are, thus, rated as reaching or exceeding the environmental assessment criterion (EAC).

References

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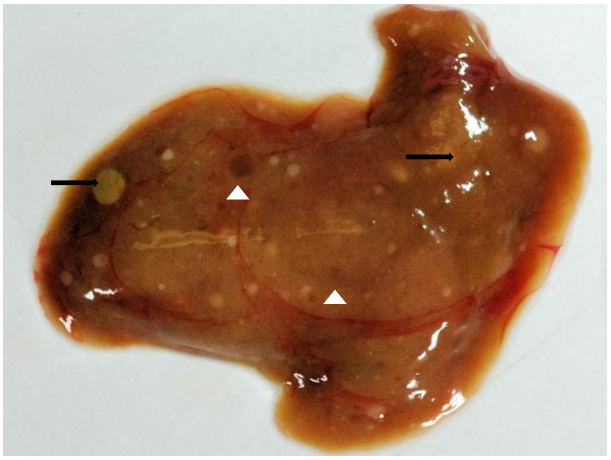
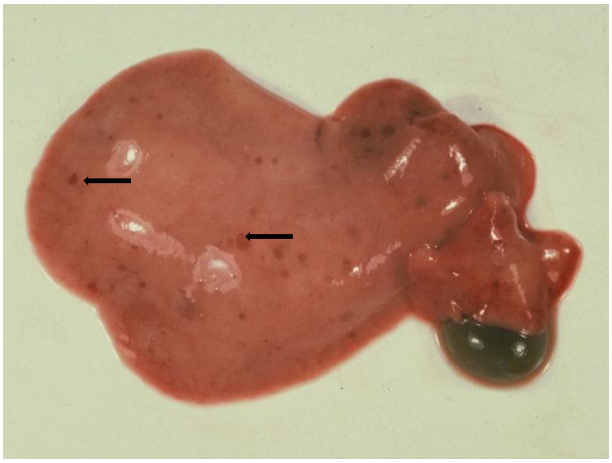
Annex 1: Macroscopic liver lesions/abnormalities to be recorded in Baltic Sea fish inspected for the presence of macroscopic liver neoplasms

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 *Ichthyophonus* sp. or *Glugea* 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 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

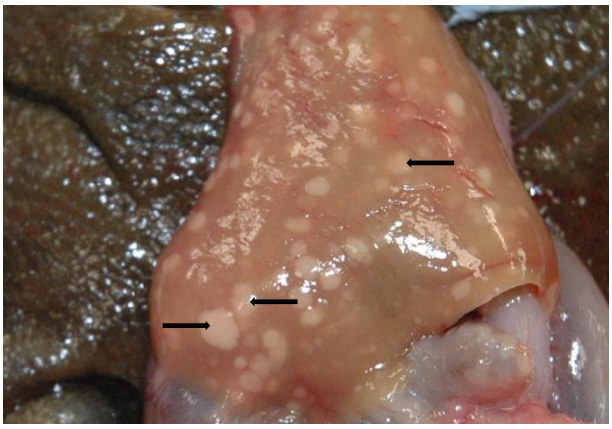
Annex 2: Common macroscopic liver nodules in dab (*Limanda limanda*) from the Baltic Sea and elsewhere (*copyright of all images: T. Lang, Thünen Institute*)



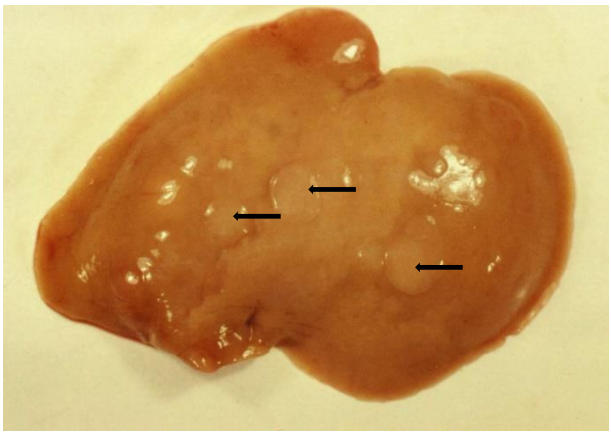
Multiple white (arrows) and transparent (glossy) (arrowheads) nodules of varying size, some >2 mm in diameter



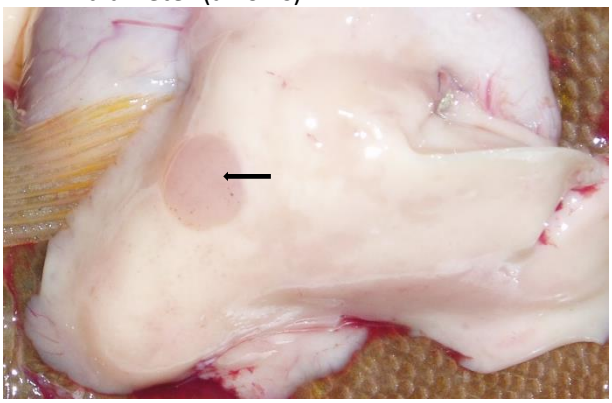
Multiple small transparent (glossy) nodules, only few >2 mm in diameter(arrows)



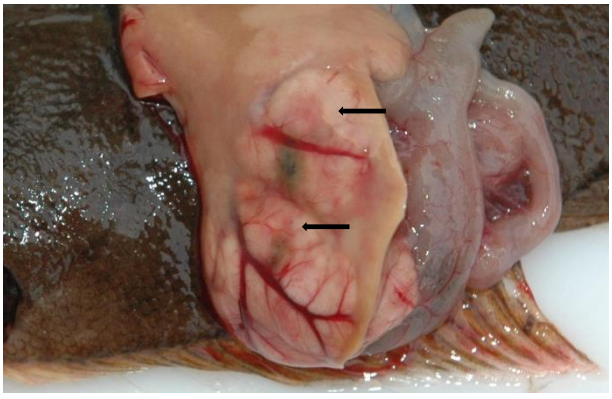
Multiple white nodules of varying site, some >2 mm in diameter (arrows)



Multiple nodules >2 mm in diameter (arrows), colour not different from normal liver tissue



Single transparent (glossy) nodule >5 mm in diameter (arrow)



Single large whitish nodule >10 mm in diameter with prominent vascularisation