Standard Operating Procedure (SOP)

Aim of SOP (tick box)

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

24. Externally visible fish diseases (EVFD)

version 1.1

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Scope

The term externally visible fish disease (EVFD) describes defined pathological changes commonly found in wild marine fish and used as indicators in environmental monitoring programmes. EVFD can be identified by naked eye, by using standardized methods and criteria for examination and disease diagnosis[1,2,3,4,5]. These changes may have an infectious (e.g., viral, bacterial, parasitic) or non-infectious (various causes) aetiology.

Studies on EVFD are amongst the techniques recommended for monitoring 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) (see references above).

The occurrence of EVFD, or changes in their prevalence, respectively, are considered as a generic non-specific indicator of habitat quality and environmental stress, reflecting the well-being of fish and the status of their specific and non-specific immune system functioning [4-7].

Summary of the method/SOP

The EVFD is based on the examination of a fish species used for chemical/biomarker analysis. The method consists of a visual external examination of freshly collected fish for the occurrence of a defined set of externally visible diseases and pathomorphological changes with clinical signs and of parasitic infestations. The external examination focuses on the body surface including the fins as well as the mouth and gill cavities.

Safety aspects

Gloves should be worn to protect the observer's skin. If some samples should be taken, safety aspect depends on used chemicals. Additional safety when handling a scalpel at sea should be practiced.

Documentation

Methods for disease surveys in wild marine fish species have largely been developed and repeatedly intercalibrated through ICES activities and through the fish disease component of the BEQUALM programme (www.bequalm.org)[3-5,7]. Technical guidelines for measuring EVFD as part of general 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[2,8].

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.

Examination:
- To each fish should be reported the
  - Cruise number
  - Fishing area (geo-coordinates)
  - Exact date
  - Fish species
  - Length
  - Weight
  - Sex (at least for flatfish)
  - Each external disease and parasite including severity grade 1-3
  - Taken samples

It is well known that the presence and prevalence of fish diseases is influenced by a number of host-specific (size, weight, age, sex) and area-specific (water temperature, salinity, oxygen content, fish stock density) factors. These factors may affect disease susceptibility, immune responses, pathogen transmission and virulence. Therefore, these factors should preferably be measured and quantified in addition to the disease examination.

It is recommended to combine the study of EVFD with the study of condition factors (CF, either based on total weight or on gutted (somatic) weight) (see DAIMON SOP for CF, Lang & Straumer 2020) and hepatosomatic index (HSI, see DAIMON SOP HSI, Lang & Straumer 2020).

Results:

Based on the examination of single fish for the suite of target diseases, the prevalence of each disease in a sample can be calculated:

$$p = x/n$$

where p = prevalence, x = number of fish affected and n = number of fish examined.

The prevalence can either be expressed as a numeric value (p= $\leq$ 1.0) or as a percentage (p= $\leq$ 100 %). The latter is more common.

For a comparison of prevalence (p) recorded (e.g. between sampling sites or years), confidence intervals (C.I.) for p can be calculated. This offer the advantage of an easy-to-perform statistical comparison between samples: if the confidence intervals of means of two samples (e.g. sample A from a munitions dumpsite and sample B from a clean reference site) do not overlap, the mean prevalence are statistically different.

Because of the nature of the data, C.I. (described by the limits π_{upper} and π_{lower}) for binomial distributions are calculated, e.g. the 95 % C.I., according to the following formulas (for p > 0.0 and < 1.0):

$$\pi_{upper} = \frac{(x+1)F}{n - x + (x+1)F} \; with \, F_{(DF1=2(x+1), DF2=2(n-x))}$$

$$\pi_{\text{lower}} = \frac{x}{x + (n - x + 1)F} \; with \; F_{(DF1=2(n-x+1), DF2=2x)}$$

For p = 0.0 (none of the fish examined is affected) the following formula applies:

$$\pi_{\text{upper}} = \frac{F}{n + F} \; with \; F_{(DF1=2(x+1), DF2=2(n-x))}$$

For p = 1.0 (all of the fish examined are affected) the following formula applies:

$$\pi_{\text{lower}} = \frac{n}{n + F} \; with \; F_{(DF1=2, DF2=2n)}$$

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[3,4,9-12] (cited from [13]).

For the analysis and assessment of fish disease data (EVFD), ICES developed a Fish Disease Index (FDI), using data on diseases of the common dab (*Limanda limanda*) as a model[4,10,14]. The aim of this tool is to summarise information on the disease status of individual fish – based on a set of EVFD - into one robust and easy-to-understand and easy-to-communicate numeric figure per fish. By applying appropriate statistics and defined assessment criteria (Background Assessment Criteria, BAC; Environmental Assessment Criteria, EAC), the FDI can be used to assess the level and temporal changes in the health status of fish populations and can, thus, serve as a tool for the assessment of the ecosystem health of the marine environment, e.g. related to the effects of anthropogenic stressors such as contaminants (including munitions-related hazardous substances). Its design principle allows the FDI to be applied to other species with other sets of diseases (such as cod from the Baltic Sea, studied in DAIMON). Therefore, the FDI approach is applicable for wider geographical areas.

EVFD assessment criteria used in DAIMON were as follows:

BAC and EAC values for FDI$_{\text{EVFD}}$ in fish were defined on the basis of the lowest 10 % percentile (BAC) and the highest 10 % percentile (EAC) of all FDI$_{\text{EVFD}}$ values recorded in fish from reference areas.

Methods

Equipment:

- Fishing vessel and nets (pelagic, trawl or gill net)
- Fish tanks with running sea water – if available
- Tweezers, scissors, scalpels with disposable blades
- Length measure board
- scales
- Cleared table or bench
- Good lights
- gloves
- Computer including data entry software or paper protocols and suitable pens

- Camera for documentation
- Fish
- Measuring instruments for: salinity, water temperature, oxygen content

Preparation before examination: For visual disease examination, an area for working should be cleared either on board the vessel or in the lab, preferably a bench or table at standing height with good lighting and running water. Instruments required (measure boards, scissors, forceps, scalpels with disposable blades etc.) should be available in sufficient numbers and should be cleaned.

If computer data entry software is used instead of hand-written protocols, a PC with the software installed is required and should be checked. PCs used in the lab should be placed in a clean and dry environment. A camera (preferably digital) is useful for documentation of disease symptoms and severity grades.

Fish sampling and keeping: Studies on EVFD should be carried out onboard fisheries research vessels or commercial fishing vessels, because fish need to be examined whilst fresh, preferably being still alive. Direct after trawling, live fish should be sorted out from catches, transferred and kept alive in tanks containing running seawater of ambient temperature prior to examination.

If this is not possible, samples collected at sea have to be transported to the lab as quickly as possible, preferably being stored on ice, but not deep-frozen.

Examination and preparation of fish: Body surface of freshly collected whole fish, including fins, oral and gill cavities shall be examined.

EVFD can principally be measured in all fish species used for chemical/biomarker analysis. However, standard protocols for fish disease studies are so far available only for selected species:

- Atlantic cod (*Gadus morhua*) (see Annex 1)
- Common dab (*Limanda limanda*) (see Annex 2)
- Flounder (*Platichthys flesus*) (see Annex 3)

Fish should be examined for externally visible diseases and parasites after rinsing in clean water. It is recommended to wear thin gloves to protect the skin of the observer. Each fish should be length-measured (total length, commonly to the nearest 1.0 cm below) and sexed (at least flatfish species). If possible, the weight of the individual fish should be recorded (important for calculation of the condition factor – see SOP for Fulton's conditional factor) and, if feasible, prior to disease inspection.

The fish should be examined for the presence of a defined suite of EVFD. It is advised to record the presence/absence of each EVFD and a severity grade (usually 1-3) if a disease is present (definitions are available; see Annexes 1-3).

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(13)   Feist, S. W.; Lang, T.; Stentiford, G.; Köhler, A. Biological effects of contaminants: use of liver pathology of the European flatfish dab (Limanda limanda L.) and flounder (Platichthys flesus L.) for monitoring. **2004**.

(14)   ICES. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO): ICES WGPDMO report 2012. **2012**.

Change history

Version 1.0, 19.02.2020, First edition

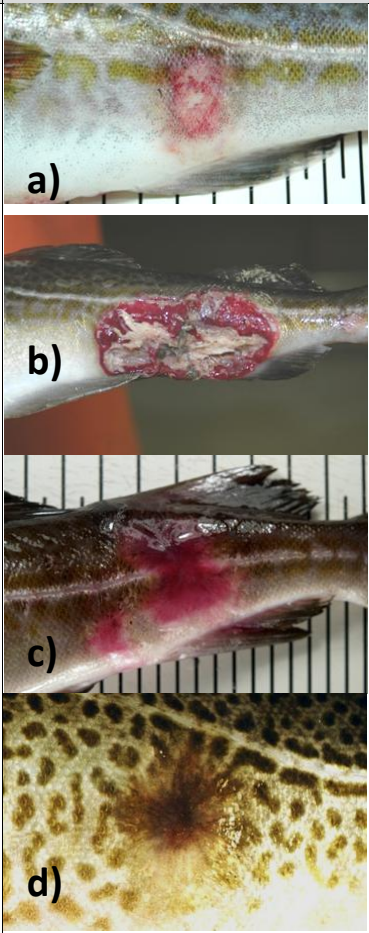Version 1.1, 30.10.2020, revised edition

List of authors
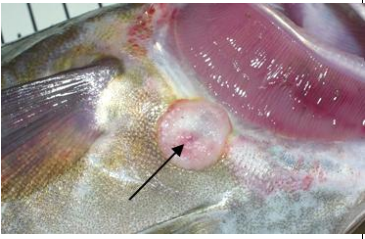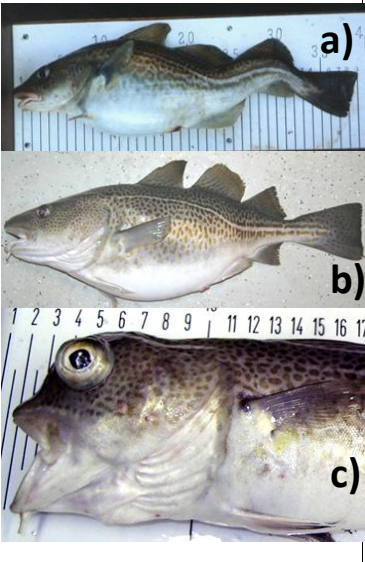
Thomas Lang, Katharina Straumer (1.0)

Daniel Koske (1.1)
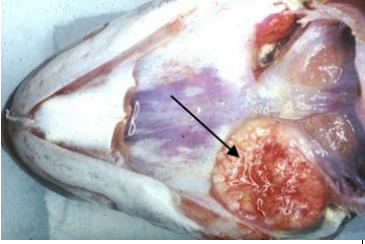
List of Reviewers

Aino Ahvo (1.0)

Annex 1: Externally visible diseases/parasites of cod (*Gadus morhua*) in the Baltic Sea recommended to be recorded in fish disease monitoring programmes (including information on identification and grading)

Disease	Clinical signs	Identification	Grade	Grading
Acute/healing/healed skin ulcers	a) b) c) d)	Red, open (or almost open) inflammatory lesions of the skin (a: acute stage); necrosis or excessive cell debris may be present (b: chronic stage); scar formation and melanin deposits may be visible at the periphery of the lesion (c: healing stage). Healed stages (d) are characterised by complete closure of lesion, scar formation and melanin deposition	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
Epidermal hyperplasia/papilloma		Lesions on the skin are slightly raised, smooth, opaque, from creamy white to slightly 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
Skeletal deformities		Lordosis/scoliosis (a) compression of the vertebral column (b) pug-headedness (c)	-	No grading, only presence recorded

Pseudo-branchial swelling/pseudo-tumour (X-cell disease)		Tumour-like swelling of the pseudobranches, uni- or bilateral, sometimes protruding into the gill tissue	-	No grading, only presence recorded
Lernaeocera branchialis		S-shaped red parasite (female copedpod) 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) (digeneanmetacercariae) 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
Nematode larvae (Anisakidae) on the liver surface		"Worms" on the liver surface; mainly larval Anisakidae, species *Contracaecumosculatum*	1	1-10 larvae
			2	11-20 larvae
			3	>20 larvae
			3	>50 cysts
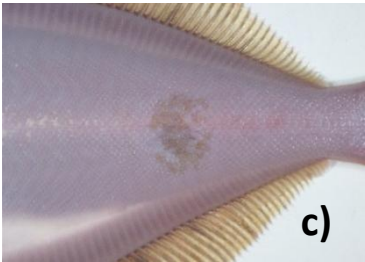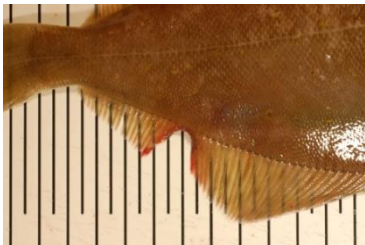
			Grade	Grading
Loma morhua		Small white cysts (microspora) in the gills	1	1-10 cysts
			2	11-50 cysts
			3	>50 cysts

Annex 2: Externally visible diseases/parasites of common dab (*Limanda limanda*) in the Baltic Sea recommended to be recorded in fish disease monitoring programmes (including information on identification and grading)
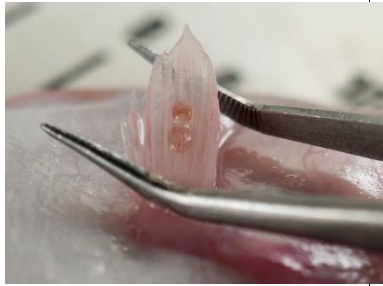
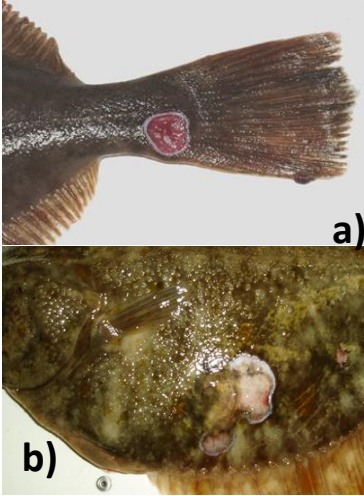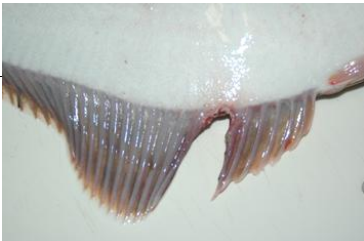| Disease	Clinical signs	Identification	Grade	Grading
Lymphocystis		Clusters of hard nodules (enlarged connective tissue cells) on the body surface (seldom in inner organs)	1	2-10 single nodules that may be grouped in a cluster (the area affected up to 10 mm in diameter) or may be distributed as single 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
Epidermal Hyperplasia/Papilloma		Lesions on the skin are slightly raised, smooth, opaque, from creamy white to slightly 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/healed	a)	Red, open (or almost open) inflammatory	1	Total area affected up to 10 mm in diameter

9

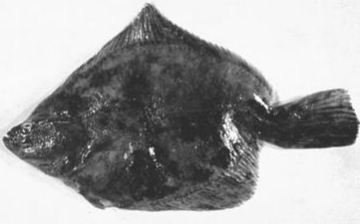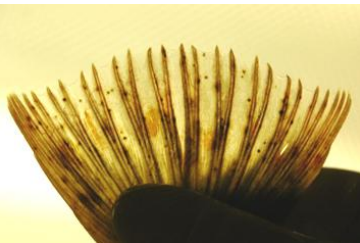| skin ulcers *		lesions of the skin (a: acute stage); scar formation and melanin deposits may be visible at the periphery of the lesion (b: healing stage); scar formation with melanin deposits are completed (c: healed stage).	2	Total area affected larger than 10 mm but smaller than twice the area of the spread-out caudal fin
			3	Total area affected larger than twice the area of the spread-out caudal fin ***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
	c)			
Acute/healing fin rot/erosion		Red open inflammatory lesions affecting the fins; healing processes may be present.	-	No grading, only presence recorded
Hyperpigmentation		Green to black discolouration of the skin,	1	Total area affected up to 10 mm in diameter

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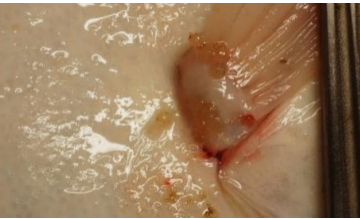|				
		caused by hyperplasia of pigment cells in the skin; causes so far unknown	2	Total area affected larger than 10 mm but smaller than twice the area of the spread-out caudal fin
			3	Total area affected larger than twice the area of the spread-out caudal fin
Skeletal deformities		Compression or lordosis/scoliosis of the vertebral column, pug-headedness	-	No grading, only presence recorded
Stephanostomum baccatum		Small white cysts (*digenean metacarcariae*) (approx. 1 mm in diameter) in the skin of the lower body side including the fins	1	1-10 cysts
			2	11-50 cysts
			3	>50 cysts
Acanthochondria		Parasitic copepods attached to the fins	1	1 parasite

			2	2 parasites
cornuta			3	3 or more parasites
Lepeophtheirus pectoralis		Parasitic copepods, either attached to the skin under the pectoral fins or on the skin surface of other parts of the body	1	1 parasite on the entire body surface
			2	2 parasites on the entire body surface
			3	3 or more parasites on the entire body surface
Cryptocotyle lingua		Small black cysts (*digeneanmetacarcariae*) (<1 mm in diameter) on the body surface (in the skin) including the fins; best to be seen in front of a light source	1	1-10 cysts on the skin (incl. fins)
			2	11-50 cysts on the skin (incl. fins)
			3	> 50 cysts on the skin (incl. fins)

Annex 3: Externally visible diseases/parasites of European flounder (*Platichthysflesus*) in the Baltic Sea recommended to be recorded in fish disease monitoring programmes (including information on identification and grading)

Disease	Clinical signs	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 (a: 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 (b: 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 ***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.
Actute/healing fin rot/erosion		Red open inflammatory lesions affecting the fins; healing processes may be	-	No grading, only presence recorded

		present.		
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 rays of the caudal fin
			3	> 50 cysts between the rays of the caudal fin
Lepeophtheirus pectoralis		Parasitic copepods, either attached to the skin under the pectoral fins or on the skin	1	1 parasite on the entire body surface

		surface of other parts of the body	2	2 parasites on the entire body surface
			3	3 or more parasites on the entire body surface