DAIMON Toolbox Fact Sheets:

Methods to Study the Impact of Dumped Munitions on Marine Biota

Assessment category: Biological Effects

Toolbox component: Disease/Pathology

Fact Sheet 3.16: Externally visible fish diseases (EVFD)

Authors: Thomas Lang and Katharina Straumer, Thünen Institute of Fisheries Ecology

What is it?

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 (ICES 1989, Bucke et al.1996, Lang and Mellergaard 1999, Lang et al. 2017a,b). 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).

What does it tell you?

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 (Vethaak & ap Rheinallt 1992, Lang 2002, Lang et al. 2017a,b).

Changes in the prevalence of EVFD may be caused by a variety of natural and/or anthropogenic stressors, including exposure to hazardous substances (Vethaak & ap Rheinallt 1992, Lang 2002), and may have consequences at the population level, such as increased mortality of different life stages, changes in natural behavior as well as a decrease in growth, reproduction and recruitment (Lang 2002).

Since EVFD is a generic stress indicator, it is applicable in a screening or detailed study on effects of conventional or chemical munitions and warfare agents on fish, but only in concert with selected more munitions-specific biological indicators (biomarkers) and targeted chemical contaminant analyses. Because of the non-specificity of the indicator, it

is not recommended to use it in isolation, unless the aim is solely to obtain an overview of the general health status of a certain fish population.

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 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) (ICES 1989, Lang and Mellergaard 1999, Lang 2002, Lang et al. 2017 a, b). 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 (Anonymous 1989, Bucke et al. 1996, 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 orHELCOM 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 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.

General requirements to be taken into account when designing a sampling strategy/programme are outlined in DAIMON Fact Sheet 3.1 addressing method for sampling wild fish (Lang 2019).

**Species****:** 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 (_Gadusmorhua_) (see Annex 1)
  − Common dab (_Limandalimanda_) (see Annex 2)
  − Flounder (_Platichthysflesus_) (see Annex 3)

Matrix: Body surface of freshly collected whole fish, including fins, oral and gill cavities

Equipment: Ideally, studies on EVFD are carried out onboard fisheries research vessels or commercial fishing vessels, because fish need to be examined whilst fresh, preferably being still alive. 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.

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. Sample containers and fixatives (ethanol, formalin) should be prepared in case that tissue samples need to be taken for subsequent histological assessment. Suitable wishing cloths should be available. Paper protocols, boards and suitable pens (waterproof) for hand-written protocols and for labeling sample containers should be available.

If computer data entry software is used instead of hand-written protocols, a PC with the software installed is required and should have been checked. PCs used in the lab should be placed in a clean and dry environment.

If the individual fish weight (total weight and/or organ weight) is to be recorded at sea on unstable platforms (e.g., onboard research vessels), special balances (scales) are required (e.g. https://marel.com/fish-processing/systems-and-equipment/on-board/surimi/receiving--handling/weighing/marine-scales/303?prdct=1) which are able to integrate over fluctuating values. These need to be calibrated at least once per day.

A camera (preferably digital) is useful for documentation of disease symptoms and severity grades.

Measurements and units: 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 Fact Sheet 3.5, Lang & Straumer 2019a) 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).

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= ≤ 1.0) or as a percentage (p= ≤ 100 %). The latter is more common.

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 Fact Sheet 3.5, Lang & Straumer 2019a) and hepatosomatic index (HIS, see DAIMON Fact Sheet 3.7, Lang 2019). For CF based on somatic weight and for HSI, a certain number of fish examined for EVFD (e.g. 100 specimens) should be dissected and further processed as described in DAIMON Fact Sheets3.5(Lang & Straumer 2019a) and 3.7 (Lang 2019).

It is further recommended to combine the study of EVFD with studies on the occurrence of macroscopic liver neoplasms (tumours) (see DAIMON Fact Sheet 3.25, Lang & Straumer 2019b) and on liver histopathology (see DAIMON Fact Sheet 3.16, Lang & Straumer 2019c).

**Sample size****:** Ideally, EVFD should be recorded in 500 randomly collected specimens per sampling site. The minimum requirement is 250 fish, since this number allows for the detection of a disease prevalence of at least 1.5 % with 95 % confidence intervals (Bucke et al. 1996). The size distribution of the fish examined should be representative for the demographic composition of the population.

The fish used for disease examination should be the same fish that are used for recording the condition factor (CF) and hepatosomatic index (HIS) (see above).

How to analyse and assess the data?

Based on the number of fish examined for EVFD and the number of fish found to be affected, the prevalence (p) of the single diseases can be calculated (see above).

For a comparison of prevalences (p) recorded (e.g. between sampling sites or years), confidence intervals (C.I.) for p can be calculated. These 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 prevalences 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):

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

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

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 (e.g., by Vethaak and Jol, 1996; Lang et al., 1999; Lang and Wosniok, 2000; Wosniok et al., 1999, 2000) (cited from Feist et al. 2004).

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 (Lang and Wosniok 2008, ICES 2012, Lang et al. 2017a). 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$_{EVFD}$ in cod were defined on the basis of the lowest xx % percentile (BAC) and the highest xx % percentile (EAC) of all FDI$_{EVFD}$ values recorded in fish from reference areas. The following criteria were used:

- Cod (*Gadus morhua*): BAC: $FDI_{EVFD} \leq x.xx$; EAC: $FDI_{EVFD} \geq x.xx$
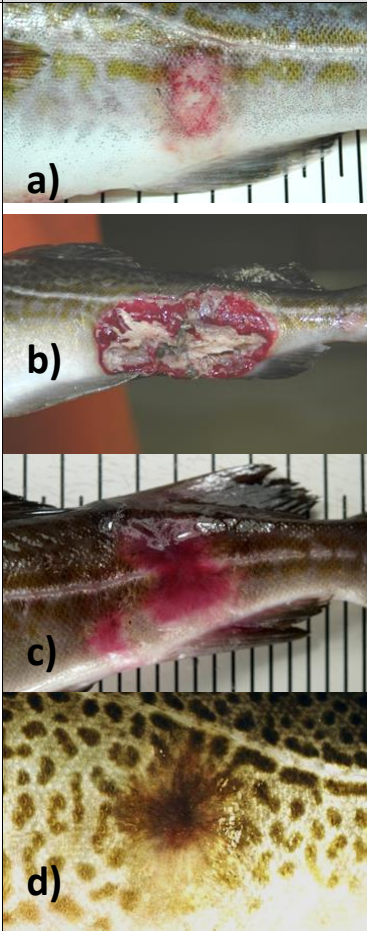- Dab (*Limandalimanda*): BAC: $FDI_{EVFD} \leq x.xx$; EAC: $FDI_{EVFD} \geq x.xx$

References

Bucke D, Vethaak AD, Lang T, Mellergaard S (1996). Common diseases and parasites of fish in the North Atlantic: Training guide for identification. ICES Techniques in Marine Environmental Sciences 19, 27 pp.

Dethlefsen V, Egidius E, McVicar AH (1986). Methodology of fish disease surveys. Report of an ICES Sea-going Workshop held on RV 'Anton Dohrn' 3-12 January 1984. ICES Cooperative Research Report 140, 33 pp.

Feist SW, Lang T, Stentiford GD, Köhler A (2004). Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limandalimanda* L.) and flounder (*Platichthysflesus* L.) for monitoring. ICES Techniques in Marine Environmental Sciences 38, 42 pp.

ICES (1989). Methodology of fish disease surveys. Report ofan ICES Sea-going Workshop held on RV U/F 'Argos'16–23 April 1988. (ICES) Cooperative Research Report,166. 33 pp.

ICES (2012). Report of the Working Group on Pathology and Diseases of Marine Organisms. ICES CM 2012/SSGHIE:03, pp. 48e61.

Lang T (2002). Fish disease surveys in environmental monitoring: the role of ICES. ICES Marine Science Symposia 215: 202-212.

Lang T (2019). DAIMON Ecotox Toolbox Fact Sheet 3.1: Sampling of wild fish. Interreg Project DAIMON.

Lang T, Mellergaard S (1999). The BMB/ICES Sea-Going Workshop 'Fish Diseases and Parasites in the Baltic Sea' – introduction and conclusions. ICES Journal of Marine Science 56: 129-133.

Lang T, Straumer K (2019a). DAIMON Ecotox Toolbox Fact Sheet 3.5: Fulton's Condition Factor (CF) in Fish. Interreg Project DAIMON.

Lang T, Straumer K (2019b). DAIMON Ecotox Toolbox Fact Sheet 3.25: Macroscopic liver neoplasms (MLN). Interreg Project DAIMON.

Lang T, Straumer K (2019c). DAIMON Ecotox Toolbox Fact Sheet 3.17: Fish liver histopathology (LH). Interreg Project DAIMON.

Lang T, Wosniok W (2008). The Fish Disease Index: a method to assess wild fish disease data in the context of marine environmental monitoring. ICES CM 2008/D:01, 13 pp. Lang, T. (2002).

Lang T, Feist SW, Stentiford GD, Bignell JP, Vethaak AD, Wosniok W (2017a). Diseases of dab (*Limanda limanda*): analysis and assessment of data on externally visible diseases, macroscopic liver neoplasms and liver histopathology in the North Sea, Baltic Sea and off Iceland. Marine Environmental Research 124: 61-69.

Lang T, Kotwicki L, Czub,M, Grzelak K, Weirup L, Straumer K (2017b). The health status of fish and benthos communities in chemical munitions dumpsites in the Baltic Sea. In: Towards the Monitoring of Dumped Munitions Threat (MODUM). J. Beldowski, R. Been. E.K. Turmus (eds.) NATO Science for Peace and Security Series – C: Environmental Security. Springer. p. 129-152.
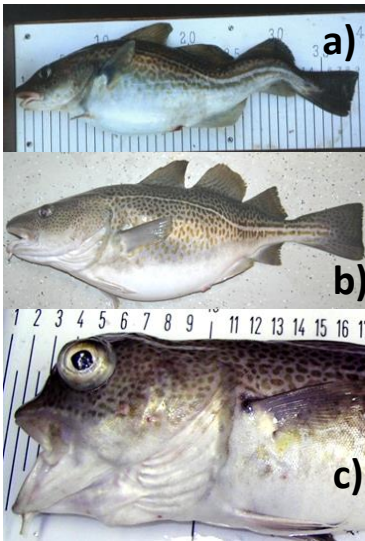
Lang T, Wosniok W (2000). ICES data for a holistic analysis of fish disease prevalence. *In* Report of the ICES Advisory Committee on the Marine Environment, 1999. ICESCooperative Research Report, 239:193–209.

McCullagh P, Nelder JA (1989). Generalized linear models. 2nd edition, Chapman &Hall, London.

OSPAR Commission (2007) JAMP Guidelines for General Biological Effects Monitoring (OSPAR Agreement 1997-7, revised in 2007) Technical Annex 7 (https://www.ospar.org/work-areas/cross-cutting-issues/cemp).

Vethaak AD, Jol JG (1996). Diseases of flounder *Platichthysflesus*in Dutch coastal andestuarine waters, with particular reference to environmental stress factors. I.Epizootiology of gross lesions. Diseases of Aquatic Organisms, 26: 81–97.

Vethaak AD, ap Rheinallt T (1992). Fish disease as a monitor for marine pollution: the case of the North Sea. Reviews in Fish Biology and Fisheries 2: 1-32.Lang et al. (2017)

Wosniok W, Lang T, Dethlefsen V, Feist SW, McVicar AH, Mellergaard S, Vethaak AD(2000). Analysis of ICES long-term data on diseases of North Sea dab (*Limanda limanda*) in relation to contaminants and other environmental factors. ICESCM 2000/S:12. 15 pp.

Wosniok W, Lang T, Vethaak AD(1999). Statistical analysis of fish disease prevalence data from the ICES Environmental Data Centre. *In* Report of the ICES Advisory Committee on the Marine Environment, 1998. ICES Cooperative Research Report, 233:297-327
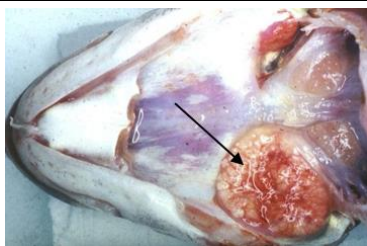
Annex 1: Externally visible diseases/parasites of cod (*Gadus morhua*) in the Baltic Sea recommended to be recorded in fish disease monitoring programmes (including information on identification and grading)

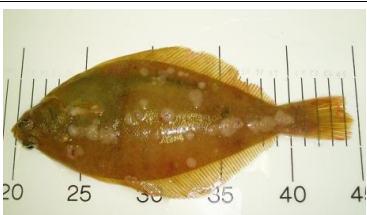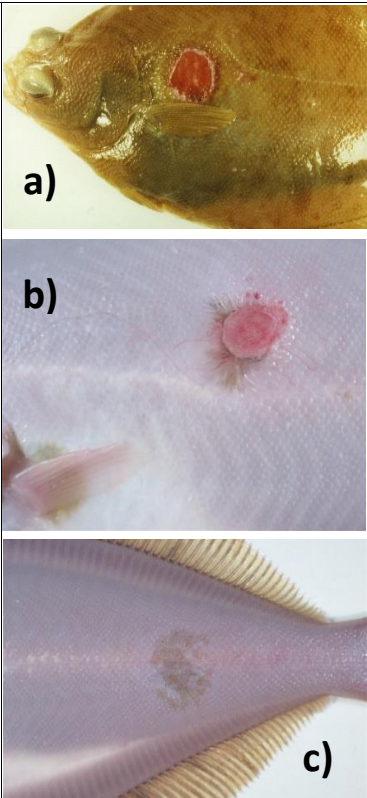| Disease	Clinical signs	Identification	Grade	Grading
Actue/healing/healed skin ulcers	a) b) c) d)	Red, open (or almost open) inflammatory lesions of the skin (a: acute stage); necrosis or excessive cell debris may be present (b: chronic stage); scar formation and melanin deposits may be visible at the 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

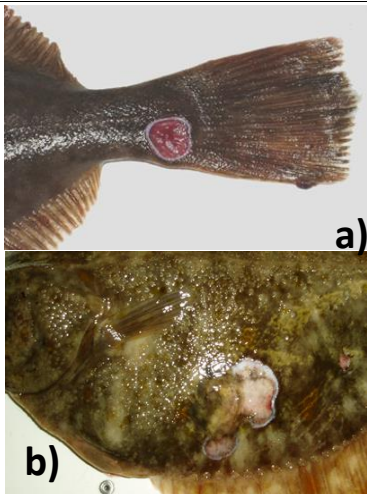| Actute/healing/healed skin ulcers *	a) b) c)	Red, open (or almost open) inflammatory lesions of the skin (a: acute stage); scar formation and melanin deposits may be visible at the periphery of the lesion (b: healing stage); scar formation with melanin deposits are completed (c: healed stage).	1	Total 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
Acute/healing fin rot/erosion		Red open inflammatory lesions affecting the fins; healing processes may be present.	-	No grading, only presence recorded

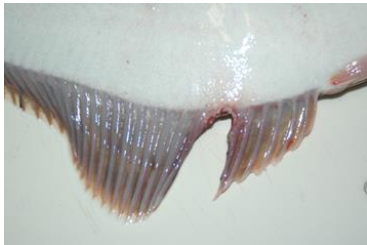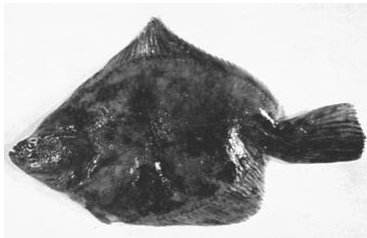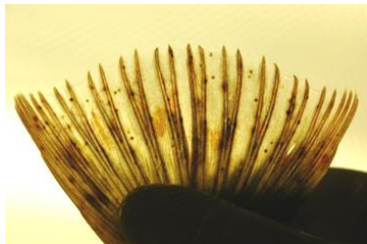| Hyperpigmentation | | Green to black discolouration of the skin, caused by hyperplasia of pigment cells in the skin; causes so far unknown | 1 | Total area affected up to 10 mm in diameter |
			2	Total area affected larger than 10 mm but smaller than twice the area of the spread-out caudal fin
			3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

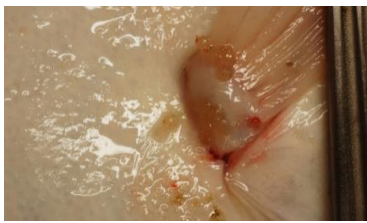| *Acanthochondria cornuta* | | Parasitic copepods attached to the fins | 1 | 1 parasite |
			2	2 parasites
			3	3 or more parasites
Lepeophtheirus pectoralis		Parasitic copepods, either attached to the skin under the pectoral fins or on the skin surface of other parts of the body	1	1 parasite on the 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 *	a) b)	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.

Acute/healing fin rot/erosion		Red open inflammatory lesions affecting the fins; healing processes may be present.	-	No grading, only presence recorded
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 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