



# The use of Atlantic hagfish (*Myxine glutinosa*) as a bioindicator species for studies on effects of dumped chemical warfare agents in the Skagerrak. 1: Liver histopathology

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## ABSTRACT

Within the framework of the international project DAIMON (Decision Aid for Marine Munitions), the impact of dumped chemical munitions on fish health was investigated. The Skagerrak Strait (North Sea, at 600 m depth) contains munitions with chemical warfare agents (CWA), scuttled after the end of World War II. Studies of liver histopathology in Atlantic hagfish (*Myxine glutinosa*) were carried out at three sampling sites: at a wreck with CWA in the Skagerrak (n = 82), a Skagerrak reference site considered to be free of CWA (n = 14) and at a reference site in the northern North Sea outside the Skagerrak (n = 17). Liver lesions were diagnosed and categorized according to standardized ICES and BEQUALM protocols and OSPAR guidelines.

Non-specific liver lesions were found in 87.6% of 113 hagfish examined. The prevalence of pre-neoplastic lesions was 7.1% and of neoplastic lesions 6.2%. There was no statistically significant difference in prevalence between hagfish samples from the wreck site and from the reference site near the wrecks. However, at the reference site in the northern North Sea, the prevalence of non-specific lesions was low and neither pre-neoplastic nor neoplastic lesions were observed.

## 1. Introduction

After World War II, chemical and conventional weapons were disposed of by sea dumping. Furthermore, 36 ships loaded with estimated 168,000 tons of chemical warfare ammunition were scuttled intentionally in the Skagerrak and 40,000 tons free chemical warfare agents (CWA) disposed of in Bornholm Deep, Baltic Sea (Knobloch et al., 2013; Tørnes et al., 2002, 2006; Missiaen and Henriot, 2002; Missiaen et al., 2010, Hansen et al., 2019). In the Skagerrak, shipwrecks are now in different states of deterioration and chemical ammunition lies on the seafloor, perforated by corrosion and releasing chemicals into the water column and sediment, but their environmental risk potential is unknown. The international projects MERCW (Modelling of ecological risks related to sea-dumped chemical weapons: 2005–2009), CHEMSEA (Chemical munition in the sea: 2011–2014; <http://chemsea.eu>),

MODUM (NATO-project: towards the monitoring of dumped munitions threat: 2013–2016; Beldowski et al., 2017) and, recently, DAIMON (2016–2019; <https://www.daimonproject.com>) have advanced chemical analysis and research of sediment samples in dumping areas. Sulphur mustard and arsenic-containing compounds were detected in sediment samples (Tørnes et al., 2006; Paka & Spiridonov, 2001). During the CHEMSEA project, a focus was on chemical analysis of marine biota, and Clark I and Adamsite were for the first time quantified in experimentally exposed blue mussels (*Mytilus trossulus*) (Turja et al., 2014; Höher et al., 2019). An aim of the project DAIMON (2016–2019) was to develop specific and sensitive methods to assess the fate and impact of toxic CWAs on marine biota in order to improve risk assessment ([www.daimonproject.com](http://www.daimonproject.com)). It is well known that arsenic accumulates in aquatic organisms, is toxic and can induce neoplastic lesions (Mason et al., 2000; Sharma and Sohn, 2009), and arsenic CWA-related

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compounds were detected in marine biota samples collected near a CWA dumpsite (Niemikoski et al., 2017).

In order to assess the biological effect of exposure to anthropogenic contaminants, the use of liver histopathology in various fish species as a biomarker in marine monitoring programmes is well established (Bucke and Feist, 1993; Stentiford et al., 2005, 2014; Wolf and Wolfe, 2005; Lang et al., 2006, 2017; Fricke et al., 2012). The liver is the most important organ in uptake, storage and redistribution of nutrients, storage, biotransformation and detoxification of xenobiotics and formation and excretion of bile (Hinton et al., 2001). It is also one of the most affected organs by contaminants in water (Stentiford et al., 2003; Au, 2004; Myers et al., 1991) and, as a consequence, undergoes different levels of cellular and tissue damage (Hinton et al., 2001). For the examination of macroscopic neoplastic liver lesions and liver histopathology, guidelines have been developed by the International Council for the Exploration of the Sea (ICES) and the Biological Effects Quality Assurance in Monitoring programme (BEQUALM) (Feist et al., 2004; BEQUALM, 2005; Ospar, 2003, 2004). These are routinely applied in marine monitoring (Lang, 2002; Fricke et al., 2012; Wosniok et al., 2000).

The Atlantic hagfish (*Myxine glutinosa*) is a common sediment-dwelling agnathan fish species and can be found on muddy ground, hiding in the sediment, down to a water depth of more than 1000 m. It is an eel-like scavenger, completely blind, with a specialised olfactory sense. It has a peculiar defensive mechanism producing large quantities of slime from mucus sacs near the abdomen, in disproportionate quantities to its size (Jørgensen et al., 1998). The structure of its liver has been described by Mugnaini and Harboe (1967). Hepatomas and other neoplasms in *M. glutinosa* have been described associated to exposure with chemical contaminants (Falkmer et al., 1977).

The aim of the present study was to compare data on the prevalence of histopathological liver lesions in Atlantic hagfish between three

different sampling sites: 1. Skagerrak wreck no. 13, contaminated by chemical warfare agents; 2. Skagerrak reference, near a contaminated area (21 km away) and 3. a CWA-free reference site in the North Sea over 400 km away from any dumped munitions.

In addition to the histopathological studies of hagfish liver, parallel investigations of established biomarkers were undertaken by Ahvo et al. (2020). The results of this study can be found in the corresponding paper (Ahvo et al., 2020.).

## 2. Material and methods

A total of 113 hagfish were examined. Hagfish were collected at three different sampling sites: Skagerrak at wreck no. 13, Skagerrak reference site known to contain no wrecks or munitions and at a reference site in the northern North Sea free from any munitions. Samples were taken during three campaigns, the cruises of RV IMOR (June 2017), H.U. Sverdrup II (January 2018) and RV Walther Herwig III (October 2018). The locations of the Skagerrak sampling sites are shown in Fig. 1, geographical coordinates of all sampling sites and numbers of hagfish collected are given in Table 1.

The hagfish were collected using two different procedures, one for the two Skagerrak sites and another for the North Sea reference site. Hagfish near the wreck and from the Skagerrak reference site were captured using baited traps. Plastic soda bottles turned upside down, with the top cut off and a piece of mackerel inside as bait, were prepared and used exclusively to trap the hagfish. Each trap was lowered to the sea floor with a concrete weight and left on the sea bottom for approximately 12 h. After the set time, the trap was liberated by an electronic release transponder and lifted to the surface by a number of floats. Live fish were removed from the traps and were processed on board the vessel. At the North Sea reference site, hagfish were captured as by-catch by fishing with a standard bottom trawl used for stock

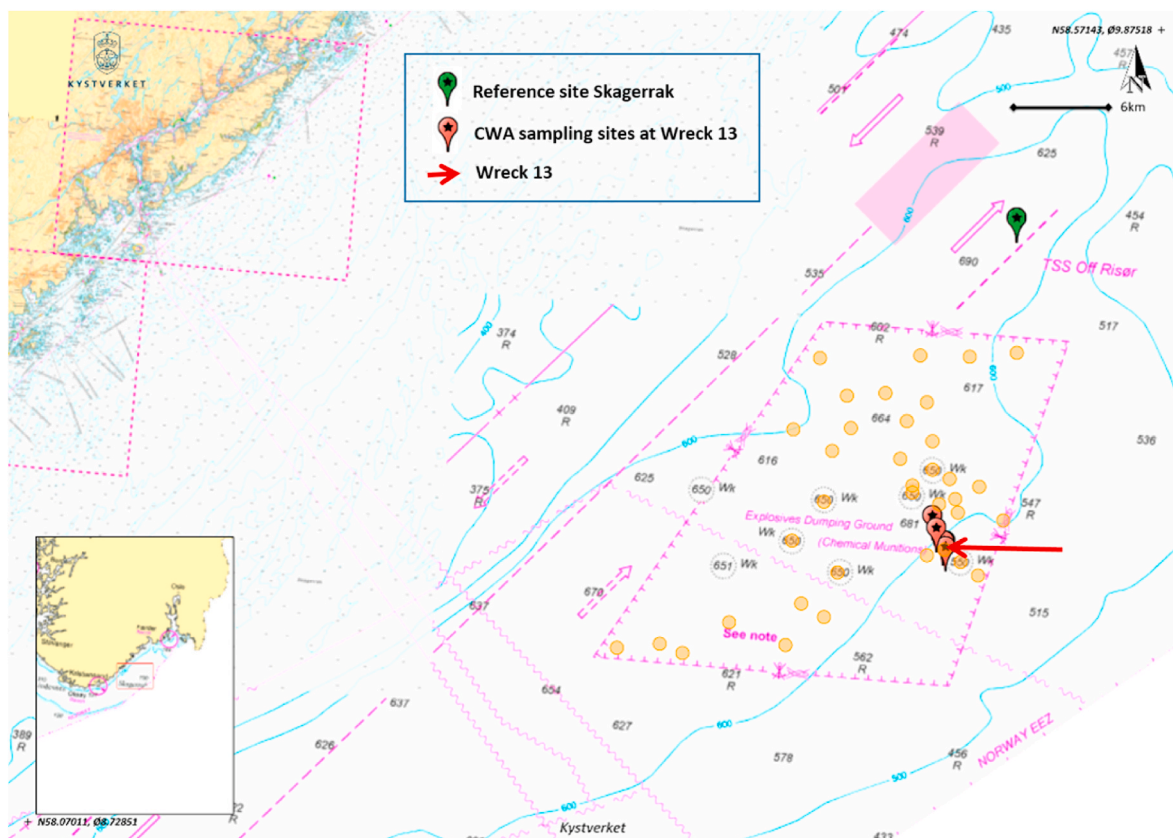


Fig. 1. Sea chart with five sampling points near wreck 13 in the Skagerrak (red dots) and the reference site (green dot), 21 km away from wreck 13. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 1**Number of fish examined and mean length of Atlantic hagfish (*Myxine glutinosa*) by sampling site, sampling coordinates and campaign.

Area	Station Coordinates	Campaign			
		RV IMOR	H.U. Sverdrup II	RV Walther Herwig III	
		June 2017	January 2018	October 2018 (WH 418)	
Wreck 13; Skagerrak contaminated by CWA	58° 15.9677 N - 58° 16.9913 N 9° 41.0057 E - 9° 40,00 E	N	82	–	–
		Mean length ± SD	29.29 ± 5.34	–	–
Skagerrak reference site	58° 27.302 N - 9° 44.052 E	N	–	14	–
		Mean length ± SD	–	29.00 ± 3.84	–
North Sea reference site	58° 40.93 N - 58° 48.0102 N 2° 25.3689 E - 2° 41.1198 E	N	–	–	17
		Mean length ± SD	–	–	26.12 ± 5.03

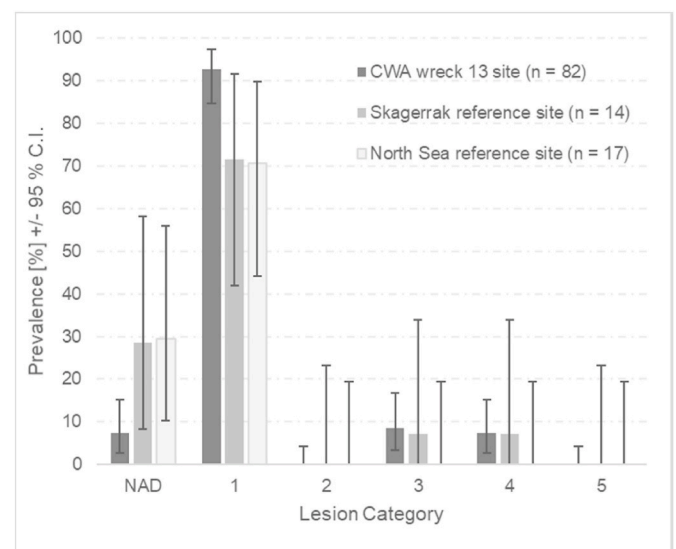
assessment purposes (GOV, grande ouverture vertical); towing time was 30 min with a towing speed of 3–4 knots. Live hagfish were sorted from the catches and transferred to tanks containing seawater prior to examination and tissue sampling.

Hagfish were measured (length), sacrificed by decapitation and dissected. The livers were removed and liver tissue samples were fixed in 10% neutral buffered formalin. Taking samples and histological processing was performed using standard protocols, including embedding in paraffin wax, microtomy and staining with haematoxylin and eosin (H&E) (Feist et al., 2004; BEQUALM, 2005). Histological examination of tissue sections was either done using an Aperio Turbo slide scanner (Leica) and reading with Aperio Image Scope software (Leica) or by light microscopy (Nikon Eclipse 80i).

The diagnosis of histopathological liver lesions in the Skagerrak and North Sea samples was done according to standard diagnostic guidelines for histopathology in flatfish species (Feist et al., 2004) and the BEQUALM guidelines (BEQUALM, 2005). In the present study, the five basic categories established for flatfish, namely non-specific lesions, early toxicopathic non-neoplastic lesions, pre-neoplastic lesions, benign neoplasms and malignant neoplasms were used in consideration of the anatomy of hagfish liver (Mugnaini and Harboe, 1967; Jørgensen et al., 1998) and with some modifications for the specific lesions identified. For all non-specific liver lesions a semi-quantitative assessment was used (0 = no, 1 = mild; 2 = moderate; 3 = extensive). The quantity and extend were decisive for the classification of the pre-neoplastic lesion FCA. Significant differences between sampling sites were analysed by calculating the means and the 95% confidence intervals for a binomial distribution (see Fig. 2) and by comparing these between areas mean. Additionally, Fisher's exact test was used for calculating significances of single non-specific lesions.

### 3. Results

During internal inspection, 32 hagfish displayed a green liver discoloration and 14 livers were dark and red-copper coloured. The rest of livers showed a normal light-brown colour. Macroscopically, at the wreck site, in one liver a white nodule  $\leq 2$  mm and in a second liver two 2–5 mm nodules, one white and one brown, were visible. A variety of pathological liver changes (Table 2) recorded according to the guidelines above were seen and are described in the following sections. Data about semi-quantitative evaluation of non-specific lesions are given in Table 3. The liver histopathological analysis of all Skagerrak and North Sea samples revealed that 86.7% of all specimens with lesions harboured non-specific lesions, 7.1% pre-neoplastic lesions and 6.2% benign tumours (for calculation of prevalence, only the most severe lesion type was considered). In the latter cases ( $n = 2$ ), one or more pre-neoplastic lesions were also present. Some of the samples with non-specific lesions



**Fig. 2.** Prevalence (with 95% confidence intervals for binomial distribution\*) of histopathological liver lesions in Atlantic hagfish (*Myxine glutinosa*) according to lesion categories (NAD: no anomaly detected; 1: non-specific lesions; 2: early toxicopathic non-neoplastic lesion; 3: pre-neoplastic lesion; 4: benign neoplasms; 5: malignant neoplasms) and sampling areas (CWA wreck 13 site: dumping site in the Skagerrak; Skagerrak reference site: reference site in Skagerrak and North Sea reference site outside the Skagerrak.) \*non-overlapping confidence intervals indicate a statistically significant difference at the  $p < 0.05$  level, overlapping confidence intervals indicate the absence of significant differences.

also exhibited lesions assigned to other categories. Malignant tumours were not detected.

Overall, the non-specific lesions do not differ specifically at all sites. A closer look at the non-specific lesions (Table 2) showed a significant difference with an increased fibrosis as well as necrosis at the CWA wreck site compared to the Skagerrak reference site.

Hagfish from the reference sites showed a lower prevalence of non-specific lesions than hagfish from the CWA wreck site (Fig. 2). The hagfish from the North Sea had the highest number of non-affected livers (29.4% vs. 28.6% and 7.3%, respectively). However, the overlapping confidence intervals of the prevalences show that there were no significant differences in lesion prevalence between sites. Neither pre-neoplastic lesions nor tumours could be detected in fish from the North Sea reference site. Here, healthy livers with typical structures shown in Fig. 3 were most frequently found. A thin membrane encloses

**Table 2**

Prevalence (%) and corresponding n-numbers of liver lesion types detected. Note that individual fish may have more than one lesion type present. (NAD: no abnormalities detected; FCA: Focus of cellular alteration; MA: Macrophage aggregate); (\* indicate a statistical significance  $p < 0.05$ ).

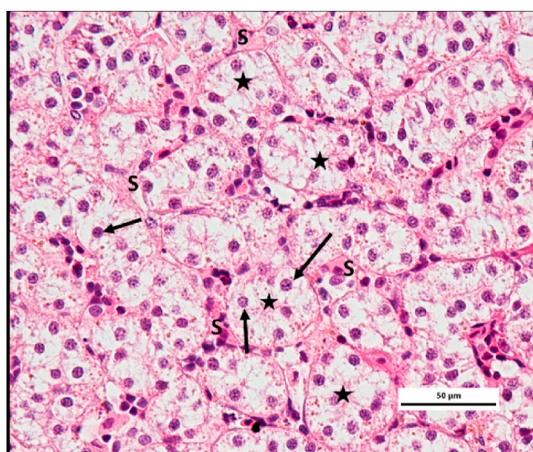
Sampling site	N	Lesion type (%)								
		NAD	FCA	Adenoma	Necrosis	MA	Granuloma	Mononuclear infiltration	Fibrosis	Vacuolization
<b>CWA wreck 13</b>	82	7.3	8.5	7.3	40.2*	63.4	4.9	37.8	23.2*	98.8
<b>N</b>		6	7	6	33	52	4	30	19	81
<b>Skagerrak reference</b>	14	28.6	7.1	7.1	0.0*	64.3	0.0	21.4	0.0*	100
<b>N</b>		4	1	1	0	9	0	3	0	14
<b>North Sea reference</b>	17	29.4	0.0	0.0	23.5	47.1	0.0	17.6	5.9	100
<b>N</b>		5	0	0	4	8	0	3	1	17

**Table 3**

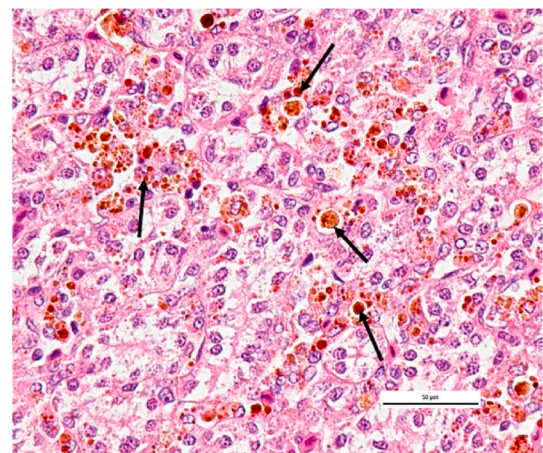
Distribution of the semi-quantified non-specific liver lesions over three degrees of severity (prevalence and corresponding n-number). Percentages refer to the absolute found of lesions. (+mild; ++ moderate; +++ extensive).

Sampling site	Lesion type [%]														
	Vacuolization			MA			Fibrosis			Necrosis			Mononuclear infiltration		
	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
<b>CWA wreck 13</b>	22.2	45.7	32.1	51.9	15.4	32.7	15.8	68.4	15.8	42.4	33.3	24.2	23.3	60.0	16.7
<b>N (82)</b>	18	37	26	27	8	17	3	13	3	14	11	8	7	18	5
<b>Skagerrak reference</b>	35.7	50.0	14.3	33.3	22.2	44.4	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
<b>N (14)</b>	5	7	2	3	2	4	0	0	0	0	0	0	3	0	0
<b>North Sea reference</b>	52.9	17.6	29.4	50.0	12.5	37.5	100.0	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0
<b>N (17)</b>	9	3	5	4	1	3	1	0	0	4	0	0	3	0	0

liver tubules, which show cell groups of hepatocytes in cross section. Blood vessels, containing blood cells, surround these. In a cell that is not or only slightly vacuolated, the round nucleus lies in the centre. Similarly, the lowest prevalence of non-specific lesions (Figs. 4–8) was found at the North Sea reference site. Only in the Skagerrak, pre-neoplastic lesions (foci of cellular alterations, FCA (Fig. 9)) were recorded. Eosinophilic foci were detected in six samples (according to all three sampling sites: prevalence range 0.0–10.5%), and only two samples showed clear cell foci. In addition, three of the samples with eosinophilic foci also exhibited a hepatocellular adenoma. Additionally, all samples with a tumour or an FCA exhibited a moderate to high prevalence of macrophage aggregates in liver tissue outside the FCA and the tumour, respectively, as shown in Fig. 10. In cases where benign neoplasms were detected, hepatocellular adenomas were present in six cases and as



**Fig. 3.** Low power view of healthy hagfish liver. Liver tubules are surrounded by a thin membrane and by liver sinusoids (S) containing blood cells. Asterisk point to the lumen of tubules. The liver cells had a round dark nucleus (arrows), usually located centrally and cells exhibit a low degree of vacuolation. (bar = 50 μm; H&E).

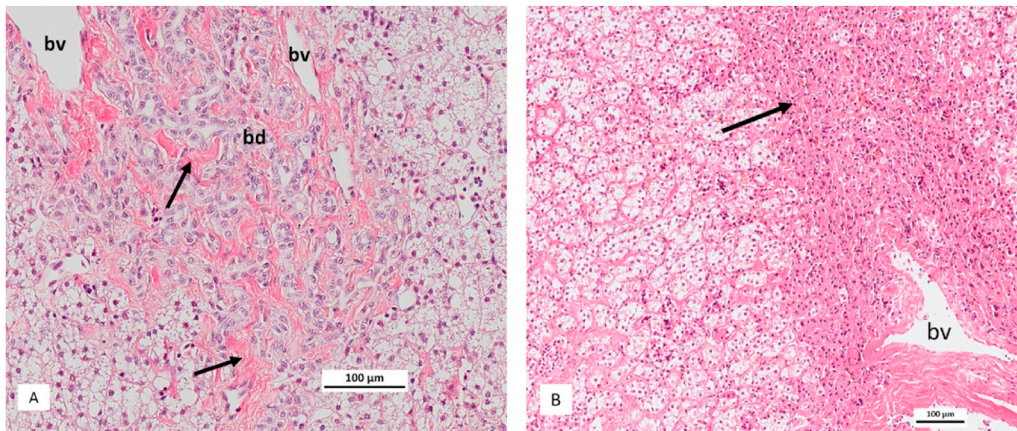


**Fig. 4.** H&E stained liver sections of normal liver tissue, but a high degree of infiltration by pigmented macrophages and aggregates (MA) (arrows); (bar = 50 μm).

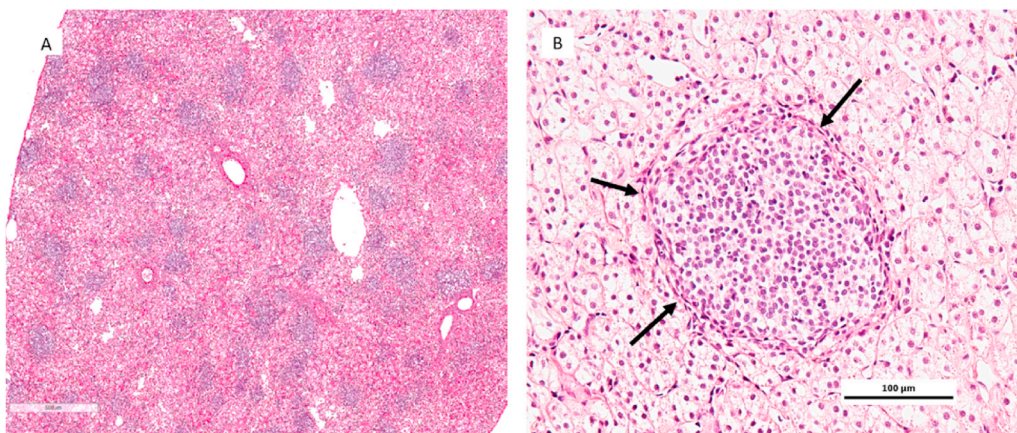
choangiocellular adenoma (Fig. 11) in one sample. The macroscopic 2–5 mm large nodule was diagnosed histopathologically as a hepatocellular adenoma. The smaller nodule ( $\leq 2$  mm), however, was not. A semi-quantitative assessment of pre-neoplastic lesion showed that the liver at the Skagerrak reference site had six FCA of eosinophilic type and one hepatocellular adenoma. Likewise, three samples at the CWA wreck site showed a hepatocellular adenoma in addition to multiple FCA. A moderate form of FCA was identified in two samples and an extensive accumulation of FCA in two samples.

**3.1. Healthy liver – no anomalies detected**

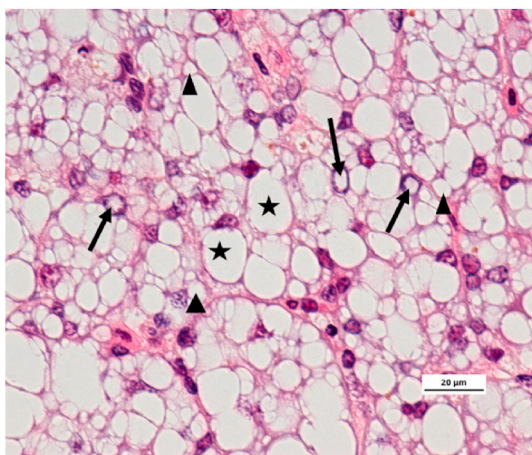
Almost all samples (92.7%) from the Skagerrak CWA wreck site as well as the nearby reference site (71.4%) showed non-specific lesions of varying degrees. Since it is assumed that mild degenerative or regenerative changes reflect the normal cellular variability range and



**Fig. 5.** Fibrotic changes appear at varying degrees. A) Fibrosis (arrow) was frequently found around the walls of larger blood vessels (bv) associated with proliferation of irregular bile ducts (bd) (bar = 100 µm; H&E). B) Normal vacuolated liver tissue on the left side with apparent fibrosis of liver tissue around blood vessels (bv) and increased fibrotic alteration (arrow) (bar = 100 µm; H&E).



**Fig. 6.** A) Low power view of a sample with multiple foci of inflammatory cells (bar = 600 µm); B) Granuloma with incipient capsule formation and surrounding fibrotic layer (arrows) (bar = 100 µm).



**Fig. 7.** High power view of a highly vacuolated liver tissue. Hepatocytes display a mixture of micro- (arrow heads) and macrovesicular (asterisk) vacuolation of an extensive lipid-type with some vacuolated nuclei (arrow) (bar = 20 µm; H&E).

probably no physiological impairment is to be expected, such fish were classified as healthy (NAD – no anomalies detected: prevalence range: 7.3–29.4%). This classification scheme was also applied to the North Sea

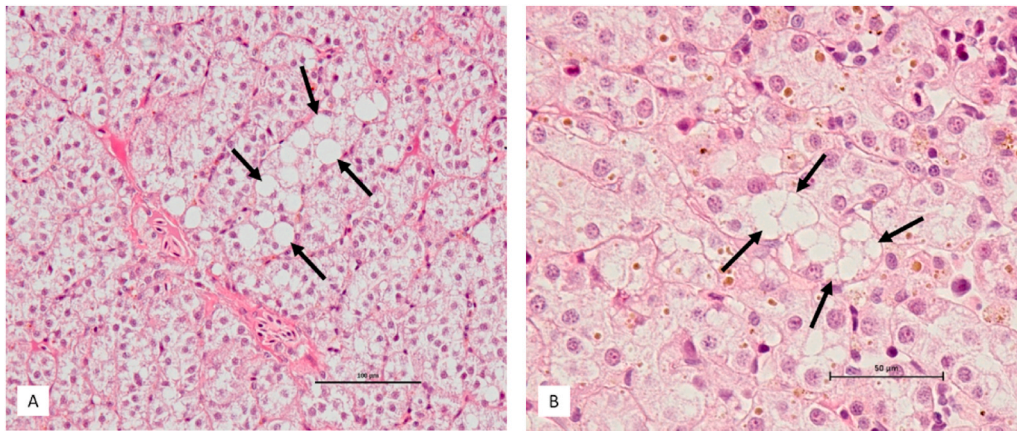
fish.

### 3.2. Non-specific lesions

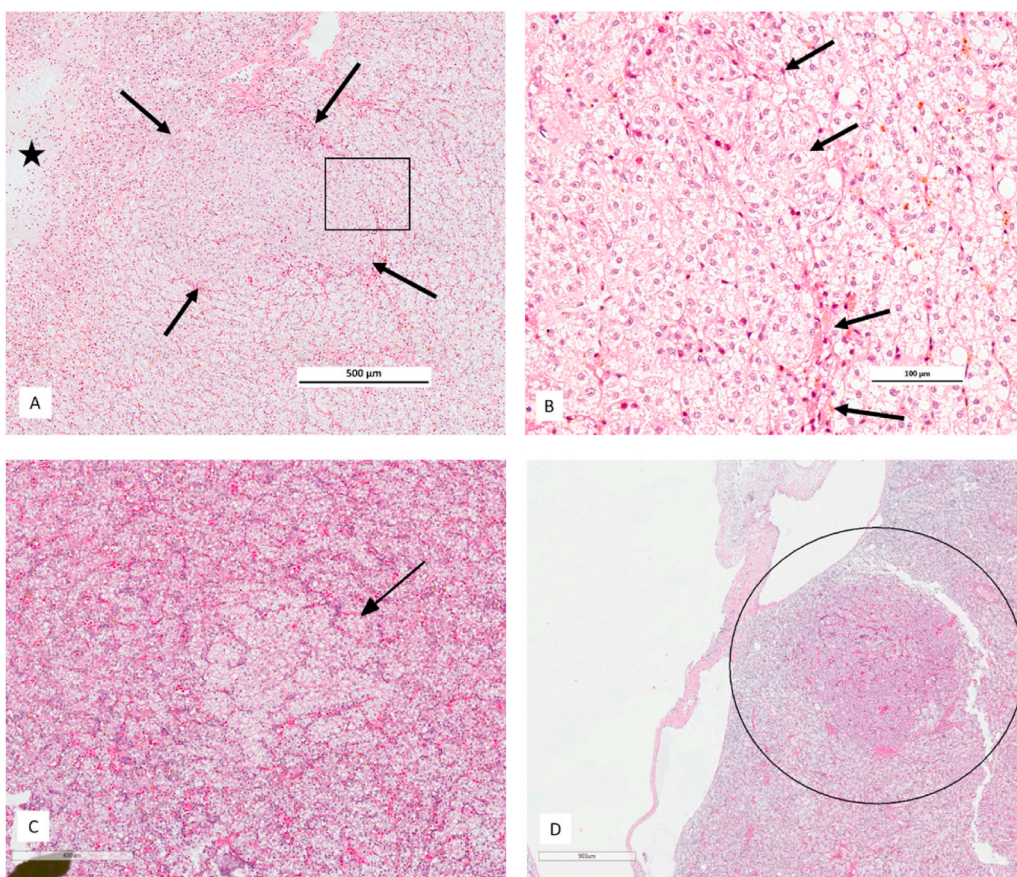
Macrophage aggregates (MA) (Fig. 4) were present in the hagfish livers in varying degrees in more than 60% of the samples. They varied in size and were surrounded by a layer of connective tissue or lymphocytes infiltrating the MAs. In 6.2%, a high degree of large MAs was accompanied by a higher rate of infiltration by lymphocytes.

Fibrosis (Fig. 5) was frequently found around vascular walls of larger blood vessels or, less frequently, associated with bile duct proliferation. The fibrotic changes occurred at varying frequencies (prevalence range: 0.0–23.3%). Mild or extensive fibrotic changes were detected in 15% of all positive samples and medium degrees in 70%. For fibrosis, a significant difference ( $p < 0.05$ ) was recorded between CWA wreck site and the Skagerrak reference site.

As shown in Fig. 6, inflammatory lesions were of varying character, from aggregates of mononuclear leucocytes to granulomas surrounded by a thin fibrous capsule. In some samples, single and small granulomas were found. Such granulomas or pronounced infiltration of mononuclear leucocytes were only detected at the Skagerrak wreck site. The granuloma in Fig. 6 B is probably of infectious cause although infectious agents such as bacteria, fungi or parasites were not identified using standard H&E or special stains (Luna, Feulgen, Gram and Ziehl Neelsen). In the North Sea reference site a mild form of inflammation was only



**Fig. 8.** A (bar = 100 µm) and B (bar = 50 µm) show a focal putative hydropic degeneration of hepatocytes (arrows).



**Fig. 9.** A) Low power view of an eosinophilic FCA and a necrotic lesion (asterisk) (HE; bar = 500 µm) B) Detail view of eosinophilic FCA seen in 9 A; the left side is the less vacuolated, eosinophilic tissue and on the right side more vacuolated and with many small MAs. The arrows show the border between the normal and the FCA tissue. C) Low power view of a clear cell focus (HE; bar = 400 µm). D) Low power view of a basophilic FCA (HE; bar = 900 µm).

recorded in three samples. In the Skagerrak CWA wreck site, 16.7% of inflammation cases was extensive, 60% moderate and 23.3% mild.

In Fig. 7, a mixture of lipid-type micro- and macrovesicular vacuolation of differing severity and irregularly distribution in the liver tissue is shown. Some of the nuclei of vacuolated hepatocytes were vacuolated. Severely vacuolated liver tissue was found in fish from the Skagerrak CWA site as well as from both reference sites. The distribution of the degrees of severity of the vacuolation over the three sampling sites is given in Table 3. The only one sample in the Skagerrak CWA site that was also diagnosed with an adenoma showed no vacuolation.

Fig. 8 A and B show a more specific and focal type of vacuolation of hepatocytes in a liver with hepatocytes generally lacking cytoplasmic vacuolation. The larger vacuoles were more separated. This lesion type resembles hydropic degeneration of hepatocytes found in flatfish livers and categorized as early toxicopathic non-neoplastic lesion (Feist et al., 2004). However, since it is not clear if this categorisation is also applicable to hagfish, lesions shown in Fig. 8 were assigned to non-specific vacuolation and not to the toxicopathic lesion category. With one exception at a mild degree in the North Sea reference site, this type of liver lesion was only found in eight samples from the CWA wreck site.

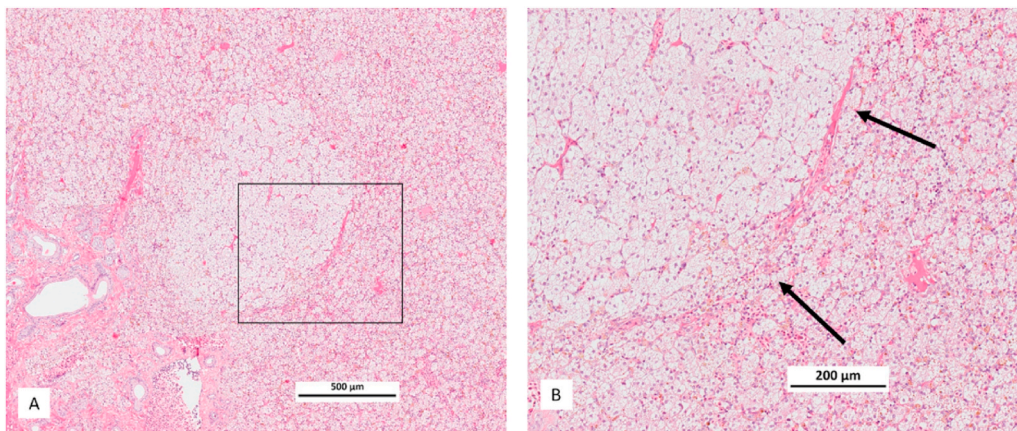


Fig. 10. A clear cell adenoma with enlarged hepatocytes and a compression of cells (arrow) at the border of the tumour (HE; A: bar = 500 µm; B: bar = 200 µm).

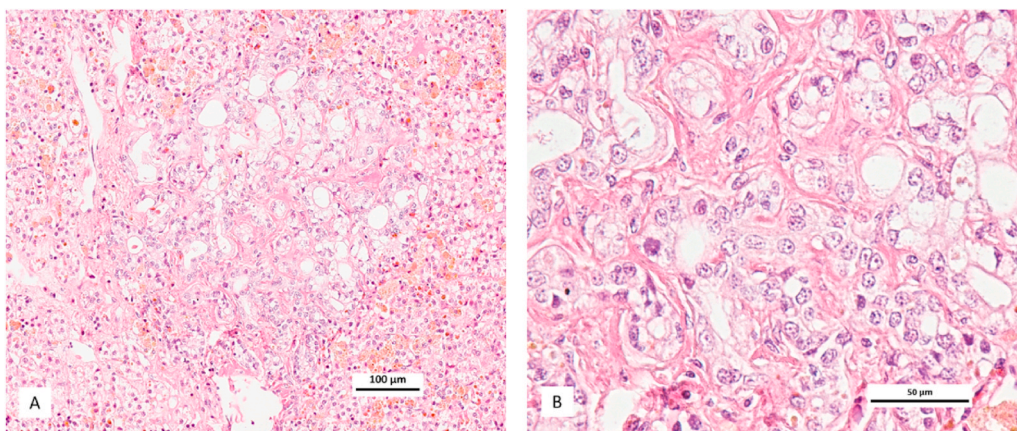


Fig. 11. A) Low power view of a cholangiocellular adenoma from the wreck site (HE; bar = 100 µm). B) High power view of the cholangiocellular adenoma (HE; bar = 50 µm).

### 3.3. Pre-neoplastic lesion

At the Skagerrak wreck site, pre-neoplastic lesions as shown in Fig. 9 were found in 8.5% of the samples in comparison to 7.1% at the Skagerrak reference site and 0.0% at the North Sea reference site. Common FCA identified were of the basophilic, eosinophilic and clear cell type.

### 3.4. Neoplasms

Three fish from the Skagerrak wreck site and two fish from the Skagerrak reference site were affected by benign neoplasms (a prevalence of 7.3% and 7.1%, respectively). At the munition-free reference site in the North Sea, none of the fish examined was affected by benign or malignant neoplasms. Six neoplasms were classified as hepatocellular adenoma and one as cholangiocellular adenoma.

Fig. 10 A and B exhibit a clear cell adenoma with compression of the surrounding tissue at the tumour margin due to the enlarged hepatocytes of neoplastic liver cells (bar = 500 µm).

In Fig. 11 A and B a cholangiocellular adenoma of more than 500 µm in diameter is shown, characterized by dilated, irregular bile ducts, lined by moderately pleomorphic cells associated with moderate amount of fibrous stroma. Increased numbers of MAs are also visible.

## 4. Discussion

Due to the important role of the liver in general metabolism and contaminant detoxification, the study of liver histopathology has proven

to be a powerful biomarker in marine environmental monitoring with regard to biological effects of hazardous substances (Hinton et al., 2001; Feist et al., 2004; Della Torre et al., 2010). The special biology of the Atlantic hagfish, a scavenger, living on and partly in the sediment and, in case of the present study, in the immediate vicinity of the sunken ships and ammunition – makes this species an appropriate target species for examining possible effects of CWA. The hagfish's mode of life can be causative for intake and subsequent metabolism of chemical warfare agents and for a subsequent onset of pathological changes.

Liver histopathology of Atlantic hagfish has been studied before by Falkmer et al. (1977) and Falkmer (1998), partly describing lesions also found in the present study. However, the present study constitutes to our knowledge the first to investigate the occurrence of histopathological liver lesions in hagfish from a munitions dumping area contaminated by CWA in comparison with two unaffected reference sites.

Della Torre et al. (2010, 2013) provided evidence that chemical munitions dumped in the southern Adriatic Sea have a negative impact on the health of the fish species Blackbelly rosefish (*Helicolenus dactylopterus*) and conger (*Conger conger*). Also, in the present study, a higher rate of necrosis, fibrosis and macrophage aggregates (MA) (both: non-specific lesions) in liver could be shown in the dumping area in comparison to the reference sites. In contrast, the study by Faber (2014) did not reveal differences in liver histopathology between cod (*Gadus morhua*) from CWA dumpsites in the Baltic Sea (e.g. east of the island of Bornholm) and from munitions-free reference sites.

Although, in the present study, no statistically significant differences were found in prevalence of liver histopathology categories between the

three Skagerrak and North Sea sampling sites, the data indicate that fish from the CWA dumping site as well as from the reference site in Skagerrak were more frequently affected by pre-neoplastic lesions or neoplastic lesions, compared to the fish from the North Sea reference site. Additionally, a higher prevalence of non-specific lesions (category 1) in liver from CWA dumping site compared to both reference sites was remarkable. Thus, a causal link cannot be excluded, at least for the arsenic CWA. It is well known that arsenic compounds induce liver and kidney damage in rainbow trout (Kotsanis and Iliopoulou-Georgudaki, 1999) and have a high toxic and carcinogenic impact (Mason et al., 2000; Cohen et al., 2006; Obinaju, 2009; Sharma and Sohn, 2009).

Interestingly, there was no significant difference between the prevalence and the types of liver lesions recorded at the CWA dumping site and the reference site in the Skagerrak. Despite the distance of 21 km between sampling points at the Skagerrak reference site and the wreck site the histological results are similar. A reason for this could be a larger geographical spread of contamination with hazardous chemical warfare agents and ammunition in the region than previously assumed (Tørnes et al., 2006). In fact, examinations of hagfish samples for chemical ammunition residues revealed that almost all samples from the wreck site and in more than 2 km distance to that in the Skagerrak contained oxidation products of arsenic CWA (Niemikoski et al. in prep.). Furthermore, the extent of the hagfish movement radius and mobility has not yet been examined systematically. From a tagging experiment, there is some indication that hagfish undertake horizontal and vertical migrations and is not an entirely locally resident species (Walvig, 1967). In addition, the uptake mechanisms of toxic CWA are not yet sufficiently clarified. Chemical uptake may occur directly via the water or the sediment as well as indirectly through the diet. Therefore, it cannot be excluded that hagfish acquire contaminant burdens outside the normal resident area in the CWA dump sites.

These findings and hypotheses may explain the lack of differences in condition of hagfish livers from the Skagerrak wreck and reference site. As already mentioned, however, the small number of samples from the Skagerrak reference site ( $n = 14$ ) should also be taken into account and the studies of the three different areas should rather be regarded as a pilot study.

In the samples from the North Sea reference site, fewer lesions were identified in general and more liver samples were classified as healthy (NAD) than in those from both Skagerrak sites. Pre-neoplastic or neoplastic lesions were not found at all. This may be an indication that fish in this area were “true” reference fish, not affected by CWA-related contaminants. However, in this context, the small number of samples ( $n = 17$ ) available from that reference site is a limiting factor and has to be considered when interpreting the results.

Besides neoplastic and pre-neoplastic lesions, a range of other histopathological liver changes were recorded. Macrophage aggregates (MA) occurred at a high prevalence and at varying degrees of intensity. MAs contain pigments (melanin, lipofuscin and hemosiderin) and can accumulate in the liver over time and increase in range and volume. In addition, it is likely that the MAs are also activated by a stimulated immune system due to viral and bacterial infections or parasites. However, environmental stress also increases the susceptibility of fish to infectious disease with associated altered circulation of white blood cells and an increase in the abundance of macrophages (Agius and Roberts, 2003). As hagfish neither have a spleen nor a lymphatic system like other fish, it is possible that in the case of hagfish the liver accumulates more MAs due to increased activation of the immune system, a function that would be filled by the spleen in other species. Although fish length has been used as an indicator of age, it is regarded as an unreliable (Stentiford et al., 2010). However, it can be seen in this study that the longer and likely older fish have an increased prevalence of medium-grade and high-grade occurrence of MAs. The average length of the examined fish at both reference sites with a higher occurrence of MAs is 32 cm and at the dumping site 31 cm (mean length see Table 1). The fish with a tumour additionally showed a medium or high level of

MAs. However, this may rather be the result of a stressed immune system than of age. Unfortunately, there is a lack of data on age determination and age/length structure in hagfish (Powell et al., 2005). Therefore, a model incorporating the interactions in terms of age and sampling area could not be fitted. It has also been shown for other fish species that age also increases the probability of tumour growth, so that information on age of the fish (Lang et al., 2006; Vethaak and Jol, 1996; Stentiford et al., 2010) would be useful.

The extensive lipid-type, clear and spherical vacuolation (Fig. 7A) resembles the description of steatosis. Steatosis, characterized by vacuolation due to build-up of lipids in hepatocytes, by definition describes a specific pathological change that has been associated with, e.g., toxic contaminant effects (Braunbeck et al., 1990; Feist et al., 2004). However, a wide range of vacuolation is normal in most species of fish, very likely reflecting nutritional condition and maturity stage (Feist et al., 2004; Wolf et al., 2015; Wolf and Wheeler, 2018; Vethaak and Wester, 1996), rather than a pathological effect or a toxicity response (Moore et al., 1997). In few cases, also a vacuolation of nuclei was found. This could be a sign of an advanced age (Aravinthan et al., 2012). Seasonal liver changes in vacuolation are also possible, which can explain the differences between the three sampling sites, since the fish were caught at different times of the year (March and Reisman, 1995; Vethaak and Wester, 1996). In this study, the semi-quantitative assessment in three severity grades of vacuolation would indicate that the fish caught in June had a slightly stronger vacuolation on average than those caught in October and January. This would coincide with the statements made for flounder (March and Reisman, 1995; Vethaak and Wester, 1996). The sex can also influence the seasonal change in liver vacuolation, but is obviously also species-specific (Stentiford et al., 2003). However, no comparable literature data are available for hagfish.

In eight samples, from the CWA Skagerrak site, putative hydropic degeneration of hepatocytes was recorded (see Fig. 8 A and B), resembling lesions described as early toxicopathic non-neoplastic lesions by other authors (Myers et al., 1994; Stehr et al., 1998; Feist et al., 2004). The occurrence of such lesions would be another indicator of contaminant effects in the Skagerrak sites. However, so far data available are not sufficient to confirm the presence of this type of lesion in hagfish. Therefore, we decided to categorise the lesion as non-specific vacuolation and not as toxicopathic lesion.

## 5. Conclusions

The present study constitutes one of the few studies investigating possible links between exposure of wild fish to CWA from dumped WW II munitions with histopathological liver changes. The results indicate differences between the study sites, with the fish from the “true” reference site in the North Sea containing less liver pathology than the fish from the Skagerrak CWA and reference sites. Interestingly, the results of Della Torre et al. (2010, 2013) show partly the same histological changes in fish in heavily polluted CWA areas as found in hagfish examined in the present study. However, when interpreting the results of the present study, the low number of specimens examined has to be taken into account. Therefore, liver histopathology analyses and chemical residue testing in hagfish should be continued and should include more study sites, e.g. a “true” reference site in the Skagerrak.

Due to its preference for deep waters and sediments and due to the types of liver lesions found in the present study, the hagfish can be regarded as a suitable target species for further studies. However, comparatively little is known about biology and demography of the species and relevant information is required to fully understand the link between CWA exposure and biological effects. Data on other biological effects measured in the same hagfish that formed the basis of the present work will be provided in Part 2 of our contribution (Ahvo et al., 2020).



## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**K. Straumer:** Writing - original draft, Writing - review & editing, Resources, Formal analysis, Validation. **M. Kraugerud:** Writing - original draft, Writing - review & editing, Resources, Formal analysis, Validation. **S.W. Feist:** Writing - original draft, Writing - review & editing, Validation, Investigation. **A. Ahvo:** Writing - original draft, Resources. **K. Lehtonen:** Writing - original draft, Project administration, Validation, Conceptualization. **A. Lastumäki:** Resources. **M. Ljønes:** Resources. **J. Aa Tørnes:** Visualization, Writing - original draft, Project administration, Supervision, Resources, Validation. **T. Lang:** Investigation, Writing - original draft, Writing - review & editing, Project administration, Supervision, Resources, Validation, Conceptualization.

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