The use of Atlantic hagfish (*Myxine glutinosa*) as a bioindicator species for studies on effects of dumped chemical warfare agents in the Skagerrak. 2. Biochemical biomarkers

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Abstract

The sea bottom of the Skagerrak Strait (North Sea) contains munitions loaded with chemical warfare agents (CWA), mostly stored in shipwrecks scuttled intentionally after the end of the World War II. The munition shells inside the wrecks are in different states of deterioration and corrosion and their environmental risk potential is unknown. The Atlantic hagfish (*Myxine glutinosa*), a sediment-dwelling chordate, was used as a model organism to study the potential impact of dumped CWA on the local ecosystem by using biochemical biomarkers. The hagfish were collected in 2017 and 2018 at three sampling sites: in the immediate vicinity of a wreck with CWA in the Skagerrak, a few kilometres from the wreck, and a reference site 21 km from the wreck, considered to be free of CWA. Significant differences were observed between the wreck site and the reference sites in the activities of glutathione reductase, superoxide dismutase and glutathione *S*-transferase, while the activity levels of catalase and acetylcholinesterase were identical at all sites. The recorded differences demonstrated negative biological effects in the hagfish sampled close to the dumped chemical munitions. Due to the limited knowledge of hagfish biology and of the extent of CWA contamination in Skagerrak, the results presented here warrant more research to further elucidate the potential environmental risks of the scuttled wrecks. The usefulness of the species as a bioindicator organism is further discussed.

Keywords: chemical munitions; hagfish; oxidative stress; biochemical biomarkers

Introduction

After World War II, chemical and conventional weapons were largely disposed by sea dumping. In the Skagerrak Strait (North Sea), 36 ships loaded with an estimated 168,000 tons of chemical warfare ammunitions inside ships were scuttled in Norwegian and Swedish waters (HELCOM CHEMU 2/2/5, 1993; Tørnes et al., 2002, 2006). In the adjacent Baltic Sea, 40,000 tons of chemical warfare materials were dumped, mostly in the Bornholm Basin and Gotland Deep (Bełdowski et al., 2014; Knobloch et al., 2013; Missiaen et al., 2010, Hansen et al., 2019). Currently, the ammunitions and shipwrecks are in different states of deterioration and many of them are already releasing the toxic chemicals into the water column and sediment, and the situation is certain to deteriorate in the near future as corrosion proceeds. Sulphur mustard and arsenic-containing CWA compounds have been detected in sediment samples in the Baltic Sea and Skagerrak dumpsites (Tørnes et al., 2006; Paka & Spiridonov, 2001; Hansen et al., 2009). Arsenic is known to accumulate in aquatic organisms and is highly toxic (Mason et al., 2000; Sharma & Sohn, 2009). Arsenic CWA-related compounds have been detected in lobster (*Nephrops norvegicus*) and flatfish (witch flounder *Glyptocephalus cynoglossus*) collected near a CWA dumpsite in Måseskär, west coast of Sweden (Niemikoski et al., 2017).

Previously, in the Skagerrak dumpsite Tørnes et al. (2002, 2006) investigated four wrecks in detail, of which "Wreck 13" was selected for this study. In the 2002 examination by using a remotely operated vehicle (ROV) (Tørnes et al. 2002), bombs, artillery shells and unknown containers were observed in "Wreck 13", some corroded and some intact. "Wreck 13" has not been identified and the amount of CWA onboard is not known, but the nearby wreck of D/S *Sesostris* was scuttled with 4500 tonnes of CWA. Thus, "Wreck 13" can be assumed to contain similar amounts of CWA. It lies in the depth of 677 m, with average bottom

temperature of 5ºC and salinity of 35. The bottom current in Skagerrak is estimated at 0-19 cm s^{-1}(NGU, 1997). Tørnes et al. (2002) measured a bottom current of 2.0 cm s^{-1}close to "Wreck 13". Sedimentation rate in the area is slow with only 1.5-4.5 mm $year^{-1}$ (NGU, 1997).

Tørnes et al. (2002) conducted sediment sampling in the area and chemical analysis indicated sulfur mustard degradation products as well as the arsenic-based riot-control agent Clark I and its degradation products in the samples from "Wreck 13". The highest concentration of Clark I (40 mg kg^{-1}) was found in the 1-3 cm top sediment layer, indicating that the compound remains very stable in the deep sea environment and that the contamination has already continued for quite a while. Clark I has potential to bioaccumulate and moderate potential to bioconcentrate in the food web (Tørnes et al., 2002).

The Atlantic hagfish (*Myxine glutinosa*) is a demersal fish species found on muddy bottoms at depths up to 1,000 m. It is an eel-like scavenger, completely blind, with a specialised olfactory sense able to detect chemical cues of fallen carrion or fish by-catch over long distances (Tamburri & Barry, 1999). The hagfish is famous for its defensive mechanism of producing large quantities of slime from its abdominal mucus sacs when disturbed (Jørgensen et al., 1998). The unique hagfish slime reacts with water and expands to almost a threefold volume, helping the hagfish, e.g., to deter predators by blocking their gills (Zintzen et al., 2011, 2013). In addition to scavenging, hagfish are known to predate on shrimp and polychaetes (Glover & Bucking, 2015a). Hagfish themselves are a significant prey item for Skagerrak adult harbour porpoises (Börjesson et al., 2003), and *M. glutinosa* and its eggs have been identified in the stomachs of cod, spiny dogfish and halibut in the coast of USA (Martini et al., 1997), creating a possible link of contaminant transfer and bioconcentration to higher trophic levels.

The species *Myxine glutinosa* is locally very abundant in the Skagerrak (Casini et al., 2005) and is traditionally known for being highly site-specific as it requires specific bottom

substrata and high-salinity (Martini et al., 1998), making it a potentially useful biomonitoring species for CWA exposure and biological effects.

The hagfish burrows in sediment, spending most of the time there and emerging occasionally to feed (e.g., Lesser et al., 1997). Skin contact with sediment could be a potential intake route for hazardous substances; Glover et al. (2011, 2015b, 2016) have shown that inorganic and organic nutrients, iron, and nickel are taken up via the skin of the hagfish. During the past decades, the approach of determining biological effects of contaminants on biota by using various biomarkers has been widely elaborated and applied both in laboratory and field studies. In living organisms, toxic effects are largely mediated by an increased production of reactive oxygen species (ROS), leading to an imbalanced redox state where ROS are not adequately neutralized by the antioxidant defence system (ADS) and cause damage to macromolecules such as lipids, proteins and DNA (Baussant et al., 2009; Regoli and Giuliani, 2014). Various contaminants presenting different groups of chemicals are known to induce the ADS and exposure to them thus leads to oxidative damage in organisms through the production of ROS (Regoli and Giuliani, 2014). Catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) are used as the main enzymatic antioxidants that provide cellular defence against endogenous and exogenous ROS. The activity of glutathione *S*-transferase (GST) is related to the functioning of the ADS but it is also a key enzyme in the detoxification Phase II (conjugation), and is a widely used biomarker of exposure to various types of contaminants (e.g., Kopecka et al., 2006; Richardson et al. 2008; Turja et al. 2014b). Similarly, inhibition of acetylcholinesterase (AChE) enzyme activity is a commonly used biomarker of neurotoxic effects (e.g., Bocquené and Galgani, 2002; Maisano et al., 2017). To our knowledge, the only commonly used biomarker enzyme that has been measured and characterized in the hagfish before this study is acetylcholinesterase (AChE) (Sanders et al., 1996).

The aim of the present study was to compare data on these selected biochemical biomarkers in the hagfish between three different sampling sites, one in the immediate vicinity of a scuttled ship containing CWA ammunition and at two alleged reference sites. In addition to the biochemical biomarkers, parallel studies on liver histopathology were carried out by Straumer et al. (2020): due to similarities in the background and sampling set-up of both studies, references to that paper have been given here to avoid unnecessary repetition of information.

Material and Methods

Hagfish were collected in the Skagerrak from (1) the immediate vicinity of the scuttled ship "Wreck 13" contaminated by CWA, from here on referred to as "WRECK" in June 2017, (2) a site few kilometres from WRECK not suspected to be contaminated by CWAs in June 2017 ("REF 1"), and (3) a reference site 21 km from WRECK in January 2018 ("REF 2"). The locations of the sampling sites are shown in Fig. 1, and geographical coordinates of all sampling sites and the number of the hagfish collected are given in Table 1. The individuals were measured for length, sacrificed by decapitation, and dissected. Samples of the livers and muscle tissues were snap frozen in liquid nitrogen, and later stored in -80°C until analysis.

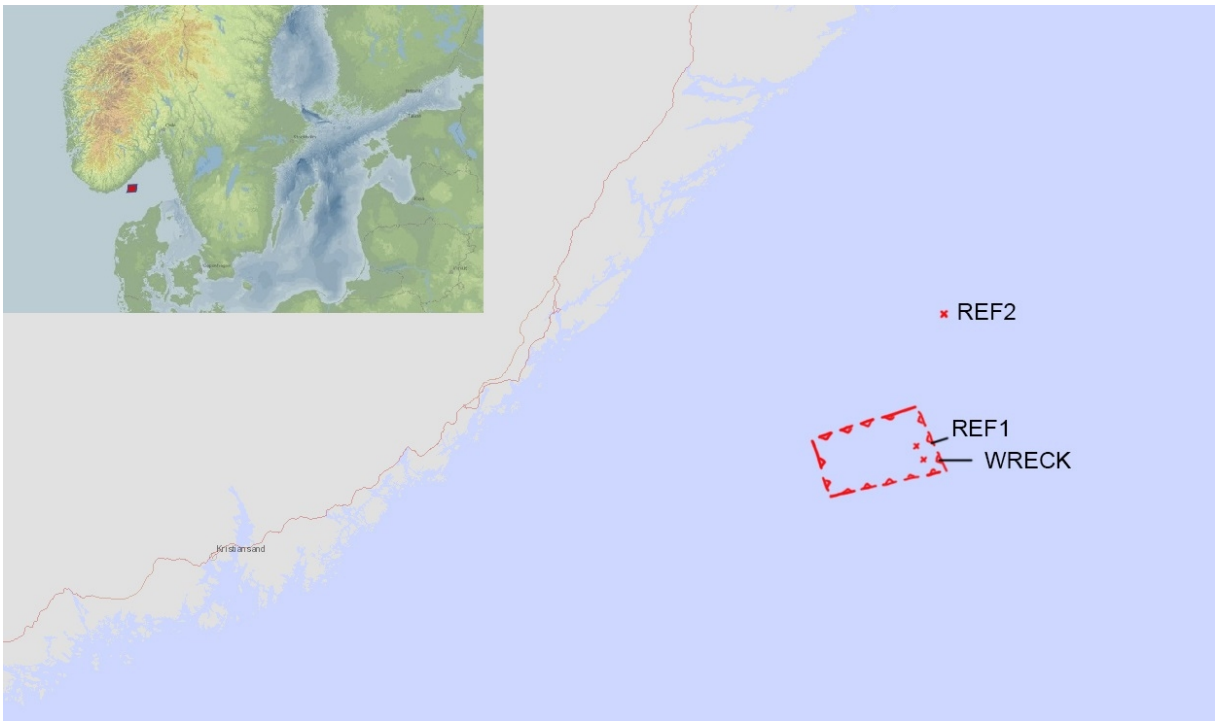

Figure 1. Smaller map: location of the study area in Skagerrak. Larger map: locations of the sampling sites: WRECK - samples collected next to the wreck, REF1 - samples 2 km from the wreck, and REF2 - samples 21 km away from wreck. Source: HELCOM.

Table 1. Sampling sites, their geographical coordinates and number of hagfish samples collected during each campaign.

Area	***Station*** **coordinates**	***Station*** **name**	***Campaign*** **RV IMOR June 2017**	**RV IMOR June 2017**	**H.U. Sverdrup II January 2018**
"Wreck 13", contaminated by CWA	58°15.9677 N - 9°41.0057' E	WRECK	N = 35		
2 km from "Wreck 13"	58°16.9913 N - 9°40.00' E	REF1		N = 9	
Skagerrak reference site	58°27.302 N - 9°44.052' E	REF2			N = 20

Biomarker analysis

Liver samples for the measurement of the ADS response and detoxification were individually homogenized in 100 mM potassium phosphate buffer (pH 7.4) and centrifuged at 10,000 *g* for 20 min at 4°C. The supernatants were stored at -80°C. Muscle samples for the neurotoxicity biomarker were individually homogenized in 20 mM Na-PO4 + 1M NaCl (pH 7.0) containing

1% Triton-X100 (buffer prepared according to Sanders et al. [1996]) and centrifuged at 10,000 *g* for 20 min at 4°C. The supernatants were stored at -80°C.

All enzyme activity rates were measured in 96-well plates (Greiner) using a microplate reader (Infinite 200, TECAN) and analysed with Magellan software (TECAN). The reaction rate was evaluated according to the best linearity range of the curve. The activities were adjusted to the protein concentrations of the samples determined on microplates using the Bradford (1976) method and a bovine serum albumin standard. The number of individuals analysed per site was 9-35 (Table 1).

Antioxidant enzymes

CAT activity was measured according to Claiborne (1985), modified by Vuori et al. (2015). Briefly, the activity is measured as the change in UV absorption in a mixture containing 4.3 µM H_2O_2 at a final concentration in phosphate buffer (100mM K-PO_4, pH 7.0). GR activity was measured according to Vuori et al. (2015) as the change in absorbance at 340 nm in a mixture containing 1 mM GSSG (oxidized glutathione), 0.75mM DTNB and 0.1 mM NADPH at a final concentration in EDTA-phosphate buffer (100 mM K-PO4 + 2 mM EDTA, pH 7.5). SOD was measured using a commercial kit (Sigma Aldrich 19160).

Detoxification

GST activity was measured according to Habig et al. (1974), modified for microplate, as the colour change at 340 nm in a mixture containing 2 mM GSH (reduced glutathione) and 1 mM CDNB (1-chloro-2,4 dinitrobenzene) at final concentration in Dulbecco's buffer.

Neurotoxicity

AChE activity in the muscle tissue was measured according to Bocquené and Galgani (1998), modified for microplate as the colour change at 412 nm in a mixture containing 0.5 mM DTNB (5,5´-dithiobis 2-nitrobenzoic acid) and 2.6 mM ACTC (acetylthiocholine iodide) at a final concentration in phosphate buffer (20 mM Na-PO_4, pH 7.0). Protein concentration measurements of the muscle homogenates were performed from freshly thawed aliquots of the samples since a longer incubation time was observed to make the measurement impossible, possibly due to slime gland residues in the muscles (Spitzer & Koch, 1998).

Statistical analysis

All biomarkers were analysed with ANOVA followed by Tukey's post-hoc HSD test using the SYSTAT 11™ software. Before the test all the data were checked for normality of distribution using the Shapiro-Wilk test and for homogeneity of variances using the Kolmogorov-Smirnoff test. In case of deviations in normality the data was $\log_{10}$ transformed and re-analysed.

Results

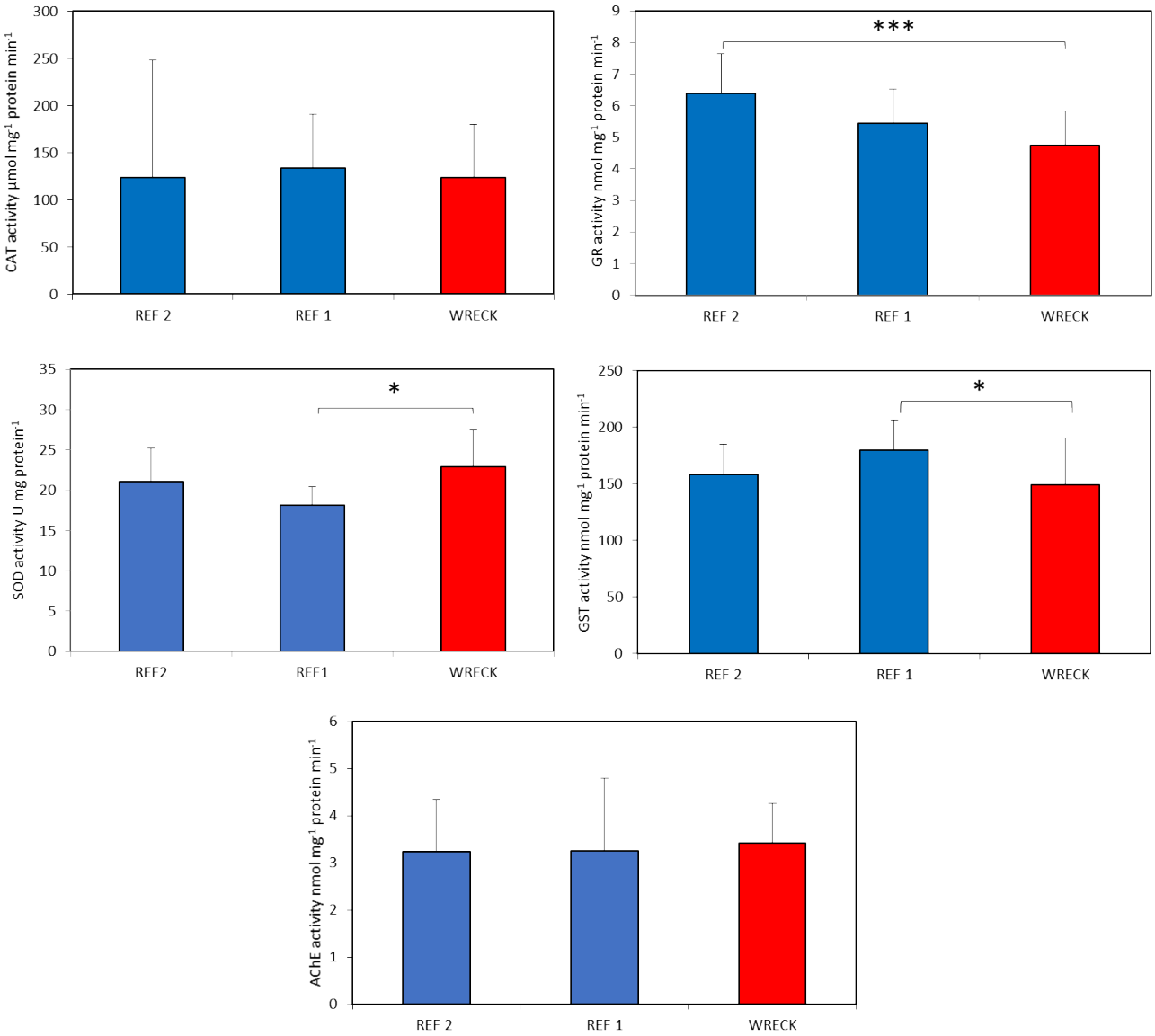

Figure 2. *Myxine glutinosa*. Mean levels (+ SD, vertical bar) in biomarker levels in the liver (CAT, GR, SOD and GST) and muscle (AChE) tissue in individuals collected from the reference site and the CWA wreck site. *** = statistical significance at $p < 0.001$, * = $p < 0.05$.

GR showed a statistically significant difference between REF 2 and the WRECK site with lowered activity rates at the latter (ANOVA $F_{2, 59} = 12.870$, $p < 0.001$) (Fig. 2). In addition, the difference between REF 1 and REF 2 was close to being significant ($p < 0.081$). GST showed a significant difference between REF 1 and the WRECK site (ANOVA $F_{2, 59} = 2.906$, $p < 0.05$). SOD activity was significantly elevated at the WRECK site compared to REF 1

(ANOVA $F_{2, 31} = 4.218$, $p < 0.05$). No differences in CAT or AChE could be detected between the sites.

Discussion

This study presents one of the very few datasets of biochemical biomarkers measured in hagfish, and the first one performed on individuals captured from a known CWA munition dumping area to examine potential biological effects caused by exposure to these chemicals. Since hagfish is a widespread species in the study region, the research carried out also serves as an effort to examine the usefulness of the species as a biomonitoring organism for the effects of environmental contaminants in general.

Significant differences between individuals collected from the known hotspot area compared to the alleged reference areas were found in GR, SOD and GST. GR activity was observed to be significantly lower in the individuals collected from the WRECK site while SOD showed higher activity, indicating differences in the functioning of the ADS. Elevated SOD activity, together with increased CAT and glutathione peroxidase activity, is considered as a first line antioxidant defence response. This is followed by the increase in ROS scavenging molecules such as GSH, including the control of its redox state by GR. Usually, the activity of GR is elevated under exposure to reasonably low levels of contaminants since more GSH is needed for the neutralization of ROS formed during xenobiotic metabolism, and also for the Phase II conjugation of intermediate molecules to more excretable forms (e.g., Regoli and Giuliani, 2014). However, the so-called bell-shape response especially in biochemical biomarkers (Forbes et al., 2006; Regoli and Giuliani, 2014) is often seen under exposure to high levels of contaminants, signifying the incapability of the organism to respond to the stress by

increasing the production of GR, and in case of failure to use other compensating protective measures the situation results in oxidative stress manifested as damage to various macromolecules (e.g., Livingstone, 2001).

Since no previous data on most of these biomarkers are available for comparison (data for AChE in Sanders et al. [1996] was reported using a different unit than in the present study), it is impossible to conclude whether the mean enzyme activities measured here represent the baseline rates, i.e., of non-exposed animals, or if the individuals from all study areas are exposed to contaminants, in this case including also CWA. In the latter case, all the biomarkers would show responses in all areas of collection and the lowered GR and GST could be explained by the bell-shape response showing that a stress threshold has been reached for these functions, and the functioning of the ADS is compromised. Also, an elevated level of SOD at the WRECK site suggests increased needs for the prevention of the excessive formation of ROS in hagfish collected from this site.

Finally, it should also be noted that regarding the differences observed in GR activity levels the REF 2 samples were taken in winter (January) contrary to the WRECK site samples obtained in summer (June). Environmental and endogenous biological factors and their interactions caused by seasonality are well-known to influence many biomarker responses in aquatic organisms, including fish (e.g., review by van der Oost et al., 2003). No research on seasonal variability in biomarkers has been conducted in the hagfish while only a handful of studies are available to confirm possible physiological changes that may occur in *M. glutinosa* during the annual cycle, related, e.g., to feeding, growth and reproduction. Foster & Moon (1986) detected seasonal changes in the glycogen content of *M. glutinosa* liver and muscle, possibly due to food limitation in the winter. Food limitation and changes in liver metabolism could also affect the oxidative stress biomarkers measured in the present study.

Physical conditions (e.g., temperature and light) at the depth range of the current study remain quite constant throughout the year; however, Powell et al. (2004) suggested that seasonal changes in the deposition of organic detritus may offer reproductive cues to the hagfish. Their study found seasonal differences in the concentration of oestradiol and progesterone in this species with the highest concentrations of both hormones occurring in January. The authors concluded that the hormonal peaks in January start the reproduction process and the production of vitellogenin, and further hypothesizing that the spawning occurs in April or May (Powell et al., 2005). Kavanaugh et al. (2005) also found elevated concentrations of gonadotropin-releasing hormone (GnRH) in both male and female hagfish in April and May, correlating with gonadal maturity. Female hagfish also exhibited increased GnRH in January and November, coinciding with the presence of the largest eggs. Female hagfish with large eggs have also been shown to exhibit significantly different hepatosomatic indices compared to other groups (Patzner & Adam, 1981). Reproductive physiology of the hagfish is still very poorly known but seasonal variability in biomarker responses related to reproduction cannot be ruled out especially as the cause of the observed difference in GR activity.

Granbom (1996) found no statistically significant differences in AChE activities between mussels and crabs caged next to chemical munitions and caged 1 km away in the Måseskär dumping area. Crabs from the reference area had a slightly lower AChE activity than the ones caged near the wreck, similar to this study. However, as discussed in the present article, CWA contamination in the Baltic Sea and Skagerrak could be more widespread than currently known, and therefore it cannot be ruled out that the reference area in the paper by Granbom (1996) could also be contaminated. In fact, he found the highest sulfur mustard concentration in a reference sample 1 km away from the wrecks. No arsenic based CWA were examined in that study.

In the Baltic Sea, no statistically significant differences in biomarkers were found in cod (*Gadus morhua*) collected from a known dumping area and a reference area during the CHEMSEA project. Similar to the results of the current study, some individual biochemical biomarkers showed more differences between sampling areas, but variation between sampling years and locations was also large (Bełdowski et al. 2014, 2016). However, a more recent study of cod from the Bornholm Basin showed significant differences in biomarkers between the dumping site and reference area, and also between individuals with CWA degradation products detected in the tissue (Niemikoski et al., in prep.). Even in the more well-known species the responses to CWA dumpsites are varied. The current study on the hagfish adds to this growing list of species studied for dumped munition biological effects and increases our knowledge of the effects of CWA on the whole ecosystem.

In comparison with teleost fish, the observed AChE and GST activity levels in the hagfish are lower (e.g., Kopecka et al., 2008; Schnell et al., 2008), while GR, CAT and SOD activities in the hagfish are similar to the activities measured in Atlantic and Baltic cod from assumedly clean sites (Hasselberg et al., 2004, Niemikoski et al., in prep.). Since all the biochemical biomarkers could be successfully quantified, the hagfish can thus be considered a suitable biomarker monitoring species for deep marine areas where the more commonly used model fish species are not available. However, the differences in hagfish biology need to be considered when discussing the results and more research is still needed to determine the baseline values of biomarkers.

Together with the paper by Straumer et al. (2020), the biological responses of the Atlantic hagfish to CWA contaminated wrecks were studied on two different biological organization levels. In Straumer et al. (2020), hagfish from a reference site in the North Sea ca. 400 km from the wreck area showed a lower prevalence of all the measured histopathological lesion categories compared to those collected from the Skagerrak study sites. These hagfish also had

the highest number of non-affected livers. However, no statistically significant differences in the lesion prevalence could be observed between the sites. In the same study, pre-neoplastic lesions or tumours could not be found in hagfish from the North Sea reference site, while also the lowest prevalence of non-specific lesions was observed at that site. Furthermore, pre-neoplastic lesions (foci of cellular alterations) were recorded only in the Skagerrak samples. The authors concluded that hagfish from the CWA dumping site were more frequently affected by pre-neoplastic or neoplastic lesions, especially compared to individuals from the North Sea reference site, thus not ruling out the possible effects of CWAs, especially arsenic-based ones, which have shown to induce liver and kidney damage in the rainbow trout (Kotsanis et al., 1999) and have a high toxic and carcinogenic impact in general (Mason et al., 2000; Cohen et al., 2008; Obijanu, 2009; Sharma & Sohn, 2009). Also, a range of other histopathological liver changes were recorded by Straumer et al. (2020) with a higher incidence in the Skagerrak populations, including macrophage aggregates and putative hydropic degeneration of hepatocytes. Other researchers have also provided evidence that dumped chemical munitions have a negative impact on the health of fish (Della Torre et al., 2010, 2013), but in some cases no differences between allegedly non-contaminated and CWA-affected areas have been found (Faber, 2014). The observed similarity in some of the biological effects between the WRECK and the reference sites could be due to a larger geographical spread of CWA contamination and ammunitions in the region than previously thought (Tørnes et al., 2006). In fact, hagfish analysed for CWA residues showed that almost all the WRECK samples and REF1 samples contained oxidation products of arsenic CWAs (Niemikoski et al., in prep.). The hagfish is not an entirely resident species (Walvig, 1967) and while it is most likely that the uptake of CWA contaminants occurs mainly through local water, sediments or food, it is possible that the hagfish may have consumed CWA contaminated food without residing in the CWA polluted area. The current studies thus do not

rule out the possibility of a widespread contamination by CWAs or other contaminants in the Skagerrak as a cause of the observed biological effects.

Conclusions

The results of the present study indicate differences in the selected biomarkers between hagfish collected next to a wreck containing a large amount of CWA munitions compared to the reference sites, indicating negative biological impacts caused by the CWAs. However, according to information on tissue and sediment concentrations of CWA residues from the area it is probable that the contamination is more widespread than previously thought, and, therefore, a true reference area is likely missing. Regarding the hagfish, due to its preference for deep waters and sediments it can be regarded as a suitable target species for further studies of submerged munitions in deep areas. However, more information is required to fully understand the links between CWA exposure and the observed biological effects.

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