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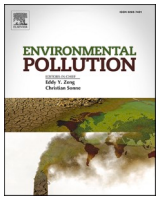

Evidence of environmental threat caused by sea-dumped chemical warfare agents: Exposure status of hagfish in the skagerrak strait☆

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ABSTRACT

Ammunitions containing toxic chemical warfare agents (CWAs) that were seized from Germany at the end of World War II were disposed mainly by sea-dumping in the Skagerrak Strait and the Baltic Sea. In an area located 25 nautical miles south-east of Arendal, Norway, several ships carrying cargo of chemical munitions were scuttled. Previous investigations have revealed that CWAs are leaking from containers and munitions into surrounding sediments in the area, raising concerns of bioaccumulation of these chemicals in marine biota.

In this study, Hagfish (*Myxine glutinosa*) was used as a model animal to investigate uptake of phenylarsenic CWAs by marine biota caught from the dumping area outside Arendal. Two laboratories analysed hagfish samples for primary degradation products of the phenylarsenic chemicals Clark I/II, namely diphenylarsinic acid and triphenylarsine oxide which have been previously found in sediment samples from the same area. The investigation showed that studied chemicals, originating from leaking munitions, are bioaccumulating in hagfish. These results support earlier findings of bioaccumulation of CWA-related phenylarsenic chemicals in different marine biota species living in the vicinity of dumping areas with increasing concern on environmental impacts caused by marine munitions. In addition, a novel biotransformation product of Clark I/II, methyldiphenylarsine oxide (MDPAO) was detected in studied fish samples for the first time. Based on findings reported in this study, biotransformation products of phenylarsenic CWAs should be considered as target chemicals in future evaluations of CWA exposure in marine biota. As the information on bioaccumulation and biological effects of CWAs in marine species are narrow, results gained in this study are essential for risk assessment related to marine munitions, as well as for future monitoring campaigns.

1. Introduction

Sea-dumped chemical warfare agents (CWAs) have raised international attention in recent years as the knowledge on the status of munitions, as well as the bioaccumulation and biological effects of leaking compounds have increased. Sea dumping was considered a cheap solution to get rid of chemical weapons, based on the assumption that they do not pose any environmental threats. It has been estimated that one million tons of chemical weapons (CWs) have been disposed in seas and oceans worldwide(Bełdowski et al., 2017; Glasby, 1997). The Skagerrak Strait, located between Denmark, the southeast coast of Norway and the west coast of Sweden, was one of those areas where CWs were disposed by sea-dumping (HELCOM, 1993; Knobloch et al., 2013). According to several sources, the total amount of CWs seized from Germany and dumped in the Skagerrak area by the Allies was between 130,000 and 160,000 tonnes (gross weight) (Stock, 1996; Tørnes et al., 2020; Knobloch et al., 2013; Arison, 2013). A large portion of these chemicals were loaded on condemned ships and sunk in an area 25 nautical miles south-east of Arendal in Norwegian waters and near Måseskär off the Swedish west coast (Tørnes et al., 2006, Tørnes et al., 2020; Knobloch

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et al., 2013). In 1989, the Norwegian Defence Research Establishment (FFI) carried out a survey of an area 16 × 8 km in size, 25 nautical miles south-east of Arendal area (Fig. 1.) During that investigation, 15 possible CW-containing shipwrecks were identified by use of a side-scan sonar (Fonnum, 1997). The number of possible CW-containing shipwrecks in this area has later been updated to more than 36 using modern underwater surveillance techniques (Hansen et al., 2019). The exact number of CW-containing wrecks cannot be stated without visual inspection. In an investigation carried out by FFI in 2002, sediment samples were collected in the vicinity of several wrecks. Sediment analyses revealed the presence of relative high concentration (up to 1100 mg kg^{-1} in dried sediment) of phenylarsenic chemicals originating from leaking sea-dumped munitions (Tørnes et al., 2006). In the present study, phenylarsenic chemicals were investigated for possible uptake in marine biota, collected near one of the shipwrecks.

Although Skagerrak and the Baltic Sea are among the areas with largest quantities of sea dumped CWAs, this environmental threat is a worldwide issue concerning not only Europe, but also North American, Asian and Australian waters (Glasby, 1997; Missiaen and Henriet, 2002; Bełdowski et al., 2017).

The information on bioaccumulation, distribution, metabolism, and toxicity of CWAs in marine species are quite narrow. However, in recent years there have been some achievements regarding the knowledge on bioaccumulation and biological effects of phenylarsenic CWAs.

Phenylarsenic CWAs are lipophilic and thus also predicted to bioaccumulate (Missiaen et al., 2010). The chemicals are prone for hydrolysis and subsequent oxidation when subjected to water environment leading to the assumption that phenylarsenic CWAs might be detected in marine biota samples in their oxidized form. Natural degradation pathway of Clark I in water environment is presented in Fig. 2.

To date, only two publications have demonstrated the presence of degradation products of CWA-related phenylarsenic chemicals in marine biota (Niemikoski et al., 2017; Niemikoski et al., 2020a), and at very low concentration levels. A few studies are available addressing the negative biological effects on aquatic organism caused by sea-dumped chemicals (Höher et al., 2019; Brzeziński et al., 2020; Czub et al., 2020a; Czub et al., 2020b, Ahvo et al., 2020, Straumer et al., 2020). The metabolism of phenylarsenic CWAs have been studied using *in vitro* models relevant to Baltic Sea species (Niemikoski et al., 2020b; Niemikoski et al., 2021). Increased knowledge on the biotransformation of these chemicals has led to the presumption that reactive metabolites might have a significant role for increased toxic properties caused by phenylarsenic chemicals (Niemikoski et al., 2021).

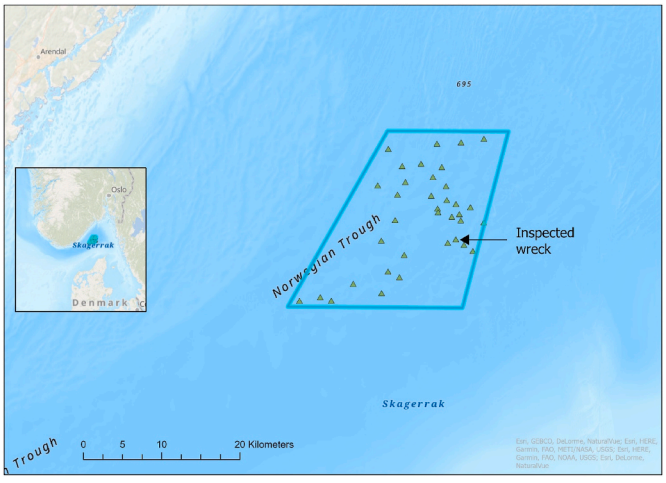

Fig. 1. The area where the scuttled wrecks (green triangles) are located. The inspected wreck is indicated with an arrow. Anchoring and fishing is prohibited in the marked area. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

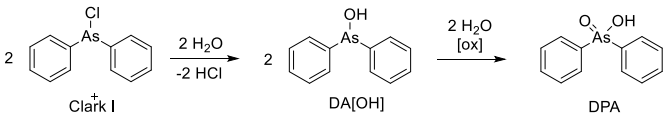

Fig. 2. Degradation pathway of Clark I in water environment.

The study by Niemikoski et al. (2020b) suggested that persistent biotransformation products of phenylarsenic CWAs, e.g. methylated forms, should be used as target chemicals when monitoring their exposure levels in marine biota. By recent years, the lack of information on biotransformation of CWA-related chemicals, and therefore the absence of their corresponding reference chemicals needed for development of sensitive analytical methods for detecting these chemicals, have been the main obstacle to assess the total levels of CWA contamination in marine biota.

In this study, hagfish (*Myxine glutinosa*) was used as a model species to investigate uptake of phenylarsenic CWAs by marine biota in the Skagerrak dumping area. Hagfishes are known to live in deep waters deeming them to be a possible species for modelling the *in situ* exposure of sea-dumped CWAs (Angulo and Del Moral-Flores, 2016). Two laboratories (A and B) analysed hagfish samples for primary degradation products of CWA-related phenylarsenic chemicals, diphenylarsinic acid (DPA) and triphenylarsine oxide (TPAO) using targeted analysis. These compounds have been previously reported in sediment samples from the same area. Additionally, suspect screening was applied to study whether the hagfish samples contain any biodegradation products of phenylarsenic CWAs.

2. Material and methods

2.1. Reference chemicals

2.1.1. Laboratory A

DPA was obtained from Envilytix GmbH (Wiesbaden, Germany), and TPAO was obtained from Acros Organics (Geel, Belgium). Methyldiphenylarsine oxide (MDPAO) was synthesized in-house using method published by Niemikoski et al. (2020c). Concentrations of reference chemical stock solutions were determined by nuclear magnetic resonance (NMR) spectroscopy. Stock solution for DPA and TPAO (10 mg/mL) were prepared in acetonitrile (ACN) (Honeywell Fluka, Seelze, Germany).

2.1.2. Laboratory B

Clark I (95%) was synthesized in house and triphenylarsine was obtained from Merck KGaA (Darmstadt, Germany). DPA and TPAO (10 μg/mL) were prepared from triphenylarsine and Clark I, respectively, by oxidizing the chemicals with 2% hydrogen peroxide (H_2O_2) in water + ACN (1 + 1). ACN and H_2O_2 were obtained from Merck KGaA (Darmstadt, Germany).

2.2. Hagfish sampling

The sampling of hagfish took place onboard the RV IMOR vessel in June 2017 at two positions close to one wreck in the Skagerrak area, outside Arendal (Fig. 1), Norway (position 1, 100 m from the wreck: 58°16.13′ N 9° 40.97′ E and position 2, 190 m from the wreck: 58°16.14′ N 9° 40.95’ E). The sampling depths were 573 m (position 1) and 578 m (position 2). Hagfish were trapped with a specific equipment consisting of a metal rig with 9 fitted plastic bottles inside. Remnants of cods and mackerels were used as bait, and the top of the bottles was cut off and placed back in reverse to prevent the hagfish from escaping. Trap bottles were kept at the sea bottom for 12 h.

A total number of twenty hagfish, randomly collected from sampling position 1, were prepared onboard for analysis at Laboratory A. The fish were sacrificed by decapitation, after which the muscle as cut fillet was

removed. Samples were kept at −20 °C prior to chemical analysis. Ten hagfish were selected for analysis at Laboratory B, five from each of the two positions. The fish were sacrificed by decapitation and stored at −20 °C until dissection at the laboratory.

2.3. Sample preparation

2.3.1. Laboratory A

The method for preparation of muscle samples was based on method published by Niemikoski et al. (2017). In general, 5 ± 0.5 g of hagfish cut fillet was extracted with ACN and 33 % H_2O_2 followed by centrifugation (4000 g, 4 min) and concentrated to a final volume of 0.25 mL prior to the chemical analysis.

2.3.2. Laboratory B

Muscle, backbone, liver and eggs from the hagfish were dissected and prepared separately. Dissected material from the five individuals from each sample position were pooled and homogenized using a hand blender. Homogenates were extracted according to the following procedure: 1.5–2 g (0.75 g for egg samples) was weighed in 15 mL polypropylene tubes and added 5 mL of ACN and 0.375 mL 30 % H_2O_2. Samples were shaken for 15 min at 1800 rpm on a Multi Reax test tube shaker (Heidolph Instruments GmbH & CO., Schwabach, Germany) followed by centrifugation (4600 g, 4 min) (ThermoFisher Scientific, Thermo Electron LED GmbH - Langenselbold, Germany). Sample clean-up was performed on Bond Elut C8 (Agilent Technologies, Santa Clara, CA, US) cartridges (200 mg). The cartridge was conditioned with 2 mL of ACN + water (1 + 1). Sample extract was mixed with 5 mL of water and eluted through the cartridge, where after it was washed with 2 mL ACN + water, 1 + 1. Sample and wash eluates were combined, vacuum centrifuged to dryness and redissolved in 0.5 mL of ACN - water (1:1). Additional online sample clean-up was performed by column switching in the LC-MS/MS analysis, described in the Supplementary information (Material and methods).

Homogenates were prepared in triplicate for cut fillet and backbone, and in one parallel for liver and eggs due to limited sample amount. Eggs were found only in one and two individuals, respectively, from the two sample locations.

2.4. Chemical analysis

Both laboratories analysed the samples by liquid chromatography–tandem mass spectrometry (LC-MS/MS) in the multiple reaction monitoring (MRM) mode. Instrument setups and method parameters are presented in the Supplementary data (Tables S1–S6). The limit of quantification (LOQ) values for studied chemicals in LC-M/MS analysis are presented in Table 1. The uncertainty in measurements was 30 %.

Chemical analyses were based on validated method by both laboratories. Spiked cod extracts were used for quality control sample to ensure the performance of the method. Also, both the solvent and matrix extract blanks were analysed to examine the possibility of cross-contamination generated from sample preparation and carry-over from LC-MS/MS analysis. No contamination or carryover was observed (Supplementary data Figures S-1 – S-3).

Additionally, laboratory A preformed confirmation analysis for detected chemicals by ultra-high performance liquid chromatography–high-resolution mass spectrometry (UHPLC-HRMS). UHPLC-HRMS analysis were applied for the samples in which the concentration level was below LOQ in the LC-MS/MS analysis. Same instrument was used for suspect screening in order to analyse biotransformation products. Detailed description of instrument parameters is presented in the Supplementary data (Tables S7 and S8).

Table 1
Limit of quantification for studied chemicals in fish samples.

	LOQ [μg kg^{-1}]	
	DPA	TPAO
Laboratory A	1	2
Laboratory B	2.5	0.5

3. Results and discussion

Hagfish collected from positions nearby a shipwreck in the Skagerrak CW dumping area, located 25 nautical miles from Arendal, Norway, were investigated for the presence of the CWA-related phenylarsenic chemicals, DPA and TPAO, as well as for possible biodegradation products. The structures of chemical compounds that have been found in hagfish samples are presented in Fig. 3.

This is the first study providing information on bioaccumulation of CWA-related chemicals in marine biota species living in this area. Analysis results are presented in Tables 2 and 3. In Laboratory A, CWA-related target chemicals were identified in 90 % of the individually analysed muscle samples (n = 20). Concentration levels above LOQ were found in three individuals, varying from 2 to 13 μg kg^{-1}. In laboratory B, TPAO was found at levels below LOQ in all investigated pooled tissue types. DPA was only detected in egg tissue, probably due to a poorer sensitivity of this compound for Laboratory B. In most cases the detected concentration levels were below LOQ values.

Identification criteria of the detected chemicals used by Laboratory A was based on the on the guidelines of the European Union and the guidelines of the Organisation for the Prohibition of Chemical Weapons (OPWC) for the analysis of biomedical samples (Organisation for the prohibition of chemical weapons (OPCW), 2018, The Commission of the European Communities, 2002). In the LC analysis, retention time of the identified chemical should not differ by more than ±0.2 min from the calibration standard. For MS/MS technique, the maximum permitted tolerance for the ratio of the areas of the confirming ion (q) and the quantitative ion (Q) is ±20% compared to the reference spectrum of the calibration standard when the relative peak intensity is more than 50% of the Q peak. Example of the relative ion intensities of the calibration standard (1 μg kg^{-1}) and the muscle sample from hagfish #16 are presented in the Supplementary data (Fig S-4 and Table S-9). In analysed samples where the detected concentration was below the LOQ value, the presence of the analyte in the samples was verified by HRMS (Laboratory A). The acceptable difference between measured and theoretical mass must be ≤ 2.5 ppm and the retention time of detected chemical shall not differ more than ±0.2 min from the reference chemical.

Identification criteria for Laboratory B were for the MS/MS confirmation ion to have a mass accuracy relative to theoretical value ± 0.010 Da, retention time match of ±0.1 min from calibration standard, and intensity >50 counts. Fig. 4 shows the extracted ion chromatograms (EICs) of specific MS/MS fragments of DPA and TPAO from analyses of a calibration standard and the muscle extract of sample B1.

The findings of TPAO distributed in fish muscle and liver tissues, as well as in backbone and eggs, together with DPA found in eggs, suggest that arsenic CWA-related chemicals are prone to bioaccumulation. This is in line with a previous study conducted with cod (*Gadus morhua*) in the Baltic Sea area, where CWA-related chemicals were detected in fish muscle and liver tissues, and some traces of DPA was found also in cod bile (Niemikoski et al., 2020a).

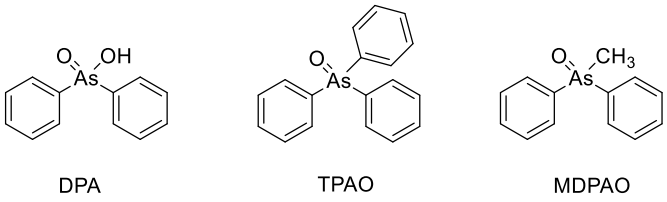

Fig. 3. Phenylarsenic chemicals detected in hagfish samples.

Table 2
Results from hagfish analysis by Laboratory A.

Sample ID	Number of individuals	Muscle	
		Chemical concentration [μg kg^{-1}]	
		DPA	TPAO
1	1	<LOQ*	6.1
2	1	<LOQ*	<LOQ*
3	1	N.D.	N.D.
4	1	<LOQ*	<LOQ*
5	1	ND	<LOQ*
6	1	<LOQ*	<LOQ*
7	1	ND	<LOQ*
8	1	<LOQ*	12.6
9	1	ND	<LOQ*
10	1	ND	<LOQ*
11	1	<LOQ*	<LOQ*
12	1	<LOQ*	<LOQ*
13	1	<LOQ*	<LOQ*
14	1	<LOQ*	<LOQ*
15	1	1.9	3.7
16	1	<LOQ*	<LOQ*
17	1	<LOQ*	<LOQ*
18	1	N.D.	<LOQ*
19	1	N.D.	N.D.
20	1	N.D.	<LOQ*

< LOQ = below the limit of quantification, * = positive finding, the confirmation done by HRMS, N.D. = no detection.

In addition, this is the first time when the presence of biotransformation product of CWA has been reported in biota samples. Methyldiphenylarsine oxide (MDPAO) was detected in some of the hagfish muscle samples by UHPLC-MS/HRMS (Laboratory A). The EICs for protonated molecule $[M+H]^+$ of MDPAO (*m/z* 261.02557) and the specific fragments for phenylarsenic chemicals (*m/z* 226.98336, 168.96286, and 154.07777) formed when higher-energy collision dissociation (HCD) 50 % was used are presented in Fig. 5, together with a mass spectrum of the compound.

This is the first time when biotransformation products of CWAs are found in marine species living in the vicinity of dumped chemical munitions. Previously MDPAO have been demonstrated to be one of the phase II metabolite of Clark I when S9 fraction isolated from cod liver has been applied in *in vitro* metabolism studies (Niemikoski et al., 2020b). The same study suggested that MDPAO is formed from Clark I and DPA by oxidative methylation via glutathione (GSH) conjugation catalysed by methyl-*S*-transferase isoenzyme. The other study published by Niemikoski et al. (2021) showed that conjugation with intracellular GSH enhanced the toxic properties of DPA stating that fish are facing increased toxicity due to biotransformation reactions. To date, the toxicological relevance of MDPAO for marine species is yet unknown.

Rantanen et al. (2024) demonstrated that methylated phenylarsenic chemicals, including MDPAO are formed as a result of microbiological activities in marine sediments. Methylated biotransformation products of phenylarsenic chemicals have been detected in several sediment samples collected from different dumping areas in the Baltic Sea (Niemikoski et al., 2020c). These previous findings together the results presented in this paper supports the presumption that the persistent biotransformation products of phenylarsenic chemicals, such as MDPAO, should be considered as target chemicals in future studies when determining the total concentrations of CWA-related phenylarsenic chemicals in marine biota. Even so, it is not possible to state from the present findings whether the presence of MDPAO in fish muscle is due to metabolism of Clark I or a result of contact with sediment containing MDPAO. Relatively high concentrations of phenylarsenic compounds have been found in sediment samples in the Skagerrak dumping area (Tørnes et al., 2006) but MDPAO have never been analysed, leaving the question open whether it is formed in sediment or in fish. This highlights the necessity of examining the presence of biotransformation products not only in marine biota, but also in sediment samples and bottom water phase in the future.

Along with this study, hagfish samples were also collected for biochemical and histological biomarker analysis. Negative biological effects were recorded; the selected biochemical responses in samples collected next to wrecks deviated from those taken from reference sites (Ahvo et al., 2020) and in histopathological studies showed the presence of pre-neoplastic or neoplastic lesions was predominant compared to hagfish from the reference site although the differences were not statistically significant(Straumer et al., 2020). The results of this study combined with previous investigations on biological assays, supports the presumption that these CWA-related chemicals have negative effects on marine organisms that live near chemical weapons dumping sites. These results are also in line with previous investigations conducted in the Bornholm and Gotland dumping areas when increased geno- and cytotoxicity levels were reported in different fish species collected close to known CWA dumpsites compared to samples collected from stations located further away (Baršienė et al., 2014; Pažusienė et al., 2021).

As the knowledge on biological effects as well as the toxic mechanism of sea-dumped phenylarsenic CWAs and their degradation

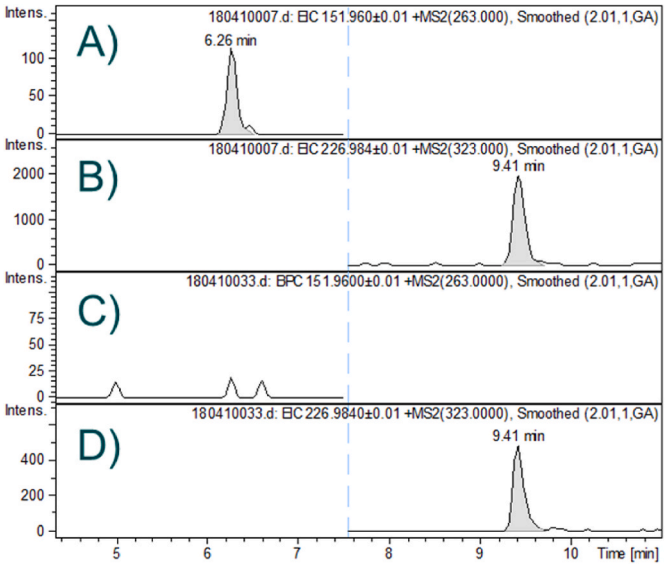

Fig. 4. LC-MS/MS extracted ion chromatograms of DPA (A) and TPAO (B) in the calibration standard (1.5 μg kg-1) and in hagfish muscle sample B1 for DPA (C) and for TPAO (D).

Table 3
Results from pooled hagfish analysis by Laboratory B. Sample ID B1 is from sample position 1, and B2 from sample position 2.

Sample ID	Number of individuals	Muscle		Backbone		Liver		Eggs	
		Chemical concentration [μg kg^{-1}]							
		DPA	TPAO	DPA	TPAO	DPA	TPAO	DPA	TPAO
B1	5	N.D.	0.56	N.D.	< LOQ*	N.D.	< LOQ*	< LOQ*	< LOQ*
B2	5	N.D.	< LOQ*	N.D.	< LOQ*	N.D.	< LOQ*	8.4	< LOQ*

< LOQ = below the limit of quantification, * = positive finding (see identification criteria), N.D. = no detection.

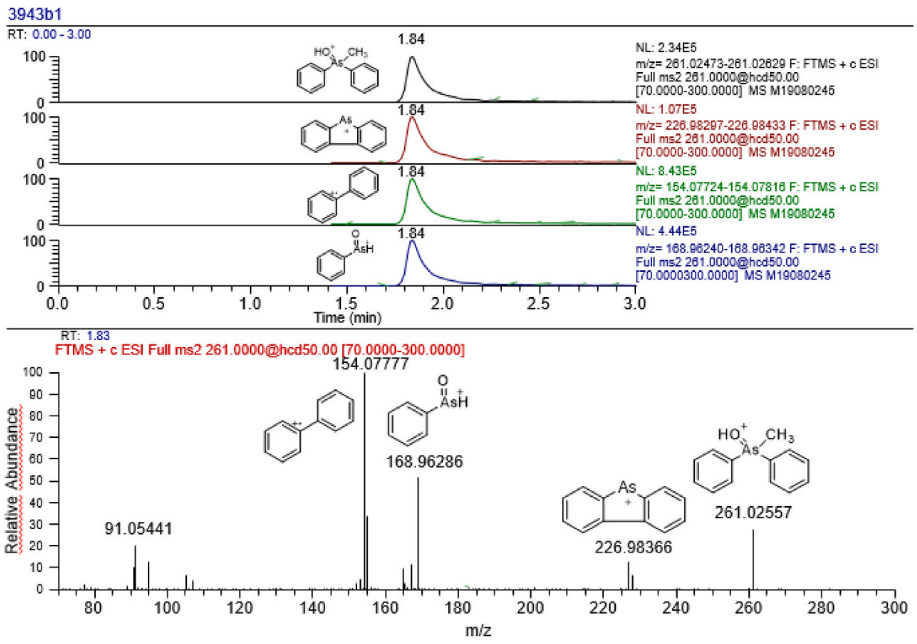

Fig. 5. EICs of specific fragments (A) generated from $[M+H]^+$ of MDPAO and MS/HRMS spectrum (B) for MDPAO analysed in a hagfish sample.

products on marine species are still very limited, more inputs for scientific research and elaboration are required for risk assessment protocols. The results obtained in this study contributes to the risk assessment related to sea-dumped CWs providing information on bioaccumulation and metabolism on phenylarsenic CWAs. One of the challenges of studying CWAs in marine samples is difficulty to obtain samples from CW dumping areas as well as very limited number of laboratories having capabilities for analysing CWA-related chemicals from marine samples. Besides, resources for this type of research are limited as the research of harmful substances in the Baltic Sea and North Sea areas is focused on widely used chemicals, such as POPs and other emerging compounds.

To our knowledge, only a few biota sampling campaigns have been carried out in the Skagerrak and Baltic Sea areas. In these campaigns, the focus has been on different fish species, such as cod, flounder and hagfish and no other species have been analysed. Because of limited amount and variety of marine biota samples, it is impossible to predict the entry point of CWA-related chemicals to the food web. The compounds could be transferred in fish through direct exposure, or by food or from the sediment/bottom water. By studying lower trophic species in the future, the biomagnification and bioavailability of compounds might be determined. The knowledge available so far does not answer these questions, which are important when assessing the risk to the entire marine ecosystem.

4. Conclusion

This is the first study providing information on bioaccumulation of phenylarsenic CWAs in marine species caught from the Skagerrak area near Arendal. Moreover, a methylated analogue of DPA, MDPAO, was detected in fish muscle tissue for the first time, suggesting that it has bioaccumulative properties. In future investigations, in addition to primary degradation products of phenylarsenic CWAs, biotransformation products should be considered when evaluating the bioconcentrations of phenylarsenic chemicals originated from leaking munitions in marine biota. To date, there are no ecotoxicological data for biotransformation products of CWAs and their behaviour in marine ecosystem in unknown. Results presented in this paper are important for risk assessment related to sea-dumped CWs providing new information on transformation and biodistribution of CWA-related chemicals.

Results presented in this paper supports earlier findings and brings more evidence to the fact that dumped CWs pose a risk for marine biota living in the vicinity of dumping sites. The growing pressure for offshore constructions, like wind power stations and underwater cables, as well as the concern the possible adverse impacts on the marine environment have raised the discussion about prospective monitoring campaigns in areas where CWs have been submerged. More information on the current situation of sea-dumped CWs in marine environment is required to implement real-time risk assessment.

CRediT authorship contribution statement

Hanna Niemikoski: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bent Tore Røen:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Marita Ljønes:** Methodology, Data curation. **John Aasulf Tørnes:** Writing – review & editing, Visualization, Investigation, Data curation. **Paula Vanninen:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.125391.

Data availability

The data that has been used is confidential.

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