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Explosives leaking from dumped munition contaminate fish from German coastal waters: a reason for chronic effects?

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Abstract

Background Conventional munition dumped into the North Sea and the Baltic Sea close to the German coastline is corroding. A major concern is that biota, including fish, are negatively affected by toxic explosives leaking into marine environments. With the present study, we investigated fish living in close proximity to munition dumping sites for contamination and for signs of health impairments. The flat fish species common dab (*Limanda limanda*) was used as a model, since it lives in the vicinity of dumping sites and exhibits minor migratory activity. Since explosives are excreted via the bile, the bile fluids from dab were analysed. Further on we inspected the health status of the fish.

Results Dab caught in German coastal waters of the Baltic Sea and the North Sea were contaminated with explosives. Probably due to rapid metabolization, concentrations of the explosive 2,4,6-trinitrotoluene (TNT) were always below limit of detection, but its metabolites 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene were detected in bile fluid up to 26.36 ng/ml and 95.91 ng/ml, respectively. Only few fish from the Baltic Sea were positive for the explosive HMX, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine with a maximum concentration of 0.89 ng/ml. Highest concentrations of TNT metabolites in bile fluid were detected in dab collected near the dumping site "Kolberger Heide" in the bay of Kiel (Baltic Sea). However, also dab from the North Sea were significantly contaminated with TNT metabolites.

Conclusions The present study showed for the first time that fish living close to near shore munition dumping sites in the North Sea are contaminated with explosives. Various health indicators (body condition factors, externally visible fish diseases, parasites or liver anomalies) showed differences in health status between fish living in the North Sea and in the Baltic Sea, respectively. However, the health status of fish caught at the most contaminated site in the Baltic Sea was not worse compared to fish living in less contaminated areas. We conclude that fish living in the vicinity of dumping sites in the North Sea and the Baltic Sea can be significantly contaminated with explosives. However, obvious health impairments of the fish were not observed.

Keywords Dumped munition, Explosives, TNT, HMX, Baltic Sea, North Sea, Fish, Common dab (*Limanda limanda*), Monitoring

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Introduction

Marine dumped munition is a worrying legacy of the two world wars. Besides munition that reached the seafloor during military activity (unexploded ordnance, ammunition on sunken war ships, etc.), a huge amount of conventional munition was dumped intentionally in coastal areas of the North Sea and in the Baltic Sea during the de-militarization of Germany after World War II. It is estimated that about 1.6 Mio t of conventional munition is still present in German marine waters, 1.3 Mio t of which in the North Sea and 0.3 Mio t in the Baltic Sea [7]. Since about 75 years the metal shells of the dumped munition objects are corroding in the sea and chemicals, mostly explosive munition compounds, are leaking out and contaminate the marine environment [3, 35]. Among those, 2,4,6-trinitrotoluene (TNT) is one of the most abundant explosives in dumped munition and known to be genotoxic to fish [21]. During the two world wars TNT was also often mixed in formulations for military use with other explosives such as the nitramine octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX [7]).

Fish can take up TNT via food or form the surrounding water. In the liver of marine fish species, TNT is rapidly metabolized into 2-amino-4,6-dinitrotoluene (2-ADNT) and into 4-amino-2,6-dinitrotoluene (4-ADNT) [22]. Therefore, bioaccumulation of TNT in fish is minimal [5]. The two main metabolites of TNT exhibit acute toxicity and genotoxicity to fish, although the metabolites are less toxic than their precursor TNT [21, 22]. However, in marine organisms the concentrations of the two metabolites usually exceed those of TNT itself [2, 36]. Accordingly, concentration and toxicity of the two TNT metabolites are of relevance in the aquatic environment.

One of the most studied munitions dumping sites in Germany is the "Kolberger Heide" in the western Baltic Sea. Here solid explosive (gun wool) is lying open on the seafloor [14, 20]. Water samples collected directly at the surface of the solid explosive (< 1 cm) revealed TNT concentrations of up to 3100 µg/L, which declined rapidly with increasing distance to the munition surface, to 16 µg/L at a 1 cm distance and to 3.3 µg/L at a 50 cm distance [4]. However, explosive concentrations in sea water are in most cases much lower than these values. Koske et al. [23] detected TNT metabolites in bile fluids of the common dab (*Limanda limanda* L.), collected near the munition dumping site "Kolberger Heide". Additionally, Maser et al. [28] detected TNT metabolites in bile fluids of fish in the vicinity of a warship wreck loaded with munition and sunk in the North Sea. Therefore, fish bile is regarded a suitable matrix to assess TNT contamination of fish. Concentrations of explosives in bile reflect a recent exposure of the animal during the last hours or few days, because freshly formed bile fluid is released from the gall bladder to the intestine, each time the fish feeds. Explosives are then excreted with the faeces from the intestine. Detection of chemical residuals of explosives in bile confirms a recent exposure. However, especially the daily contact with explosives may endanger benthic organisms living in the vicinity of dumping sites, since it may have chronic impact on their health and biological fitness. In contrast, pelagic fish have usually limited contact to dumped munition, whereas bottom dwelling organisms like asteroidae (starfish) [14] or mussels [37] cannot escape the local contamination. Gledhill et al. [14] detected several chemical residuals of explosives in marine biota like asteroidea and tunicates, which were collected in the immediate vicinity of a munition dumping site.

For the present study, we have chosen the bottom dwelling, territorial flat fish common dab (*L. limanda*) as model species. Dab has often been used as a monitoring organism regarding chemical contamination in the North Sea and in the western Baltic Sea [12, 17–19, 34]. Furthermore, according to the OSPAR CEMP, dab is the first-choice flatfish species for chemical monitoring [31]. Dab bile fluid was successfully analysed for TNT metabolites [23], and dab were investigated extensively to monitor changes in fish health due to environmental variation and pollution [11, 24, 25, 38–40]. The studied array of dab diseases comprised viral (lymphocystis, hyperplasia), bacterial (skin/fin ulcerations) [38] and parasitic (X-cell gill disease, *Stephanostomum baccatum*, *Acanthochondria cornuta*, *Lepeophtheirus pectoralis*, *Cryptocotyle lingua*) infections, skeletal deformities, lipid metabolic disorder and hyperpigmentation (melanoma) [15, 30] as well as liver anomalies (LA), such as formation of nodules analysed as described by Bucke et al. [8].

If and how dab diseases interact with dumped munition was not investigated previously. However, the frequency of externally visible diseases of dab was positively correlated with mercury contamination of dab from the North Sea [24]. On the one hand, dab diseases monitored along a pollution gradient in the German Bight revealed that viral hyperplasia decreased with pollution, while lymphocystis showed the opposite trend [38]. For the present study, disease prevalence might change with explosive contamination if this has a negative effect on dab immunocompetence, which may give rise to more severe infectious diseases. On the other hand, explosives in the water may also directly affect parasites, e.g., on the skin or gills of dab and eventually reduce parasite burden. Given the proven genotoxicity of TNT and its metabolites in fish [21], formation of tissue neoplasms might be a possible consequence of chronic exposure with TNT. Indeed, it is suggested that dab collected near the "Kolberger Heide" munition dumpsite have elevated rates of liver tumours (Straumer and Lang, personal communication).

With the present study, we investigated if dab living near munition dumping sites along the German coastline are contaminated with explosives and exhibit changes in their health status (fish diseases, parasites or LA) at the same time. Since LA can lead to formation of neoplasms, nodules and even liver tumours, LA are therefore indicators of chronic exposure to the carcinogenic TNT and it is also carcinogenic metabolites. To the best of the authors' knowledge, the present study is the first to quantify explosives in fish from munition dump sites of German coastal waters of the North Sea.

During the last decade, the problem of marine dumped munition has received more and more attention (e.g., [10]), which led to the discussion about their remediation. In this context it is essential to know, whether and to which extend fish living close to different dumping sites are contaminated with explosives before remediation starts.

The goals of the present study are:

(1) Provide spatial overview on explosive concentrations in dab bile fluids to identify contamination hot spots in German coastal waters.
(2) Investigate the fish health status using a set of fish diseases, parasites and LA as indicators.
(3) Relate individual explosive contamination to fish health.
(4) Discuss whether the flatfish species dab is suitable for a possible future monitoring of explosives in fish, also related to planned remediation actions.

Methods

Study areas

Dab were collected during five cruises of the German research vessels (RV) Clupea from 2021 to 2023 and one cruise of the RV Solea in 2023 (Table S1). Since dab were collected with conventional fishing gear (bottom gillnets and beam trawls), that need seafloor contact for operation, fishing directly in/on munition dumping sites was not performed to avoid any risk from munition contact. However, sampling areas were selected near to known munition dumping sites. As source for the identification of dumping sites nautical charts were used, as well as maps provided on AmuCad.org [1]. Fish were collected with safety distance > 1 NM from the designated dumpsite areas. High-resolution seafloor imaging conducted by GEOMAR Kiel was used to place bottom gillnets in Kiel Bay (Kolberger Heide), Lübeck Bay and Schleimünde at munition contaminated sites with safety distance < 1 NM. The sampling of fish along the outer border of the dumping area includes the possibility to catch uncontaminated fish swimming into the area from the clean surrounding. This leads to a possible underestimation of the contamination of fish inside the area. Ten sampling areas in German North Sea waters are either located near the West Frisian Islands along the coastline of Lower Saxony and between the rivers Ems and Jade or near the North Frisian Islands including Heligoland along the coastline of Schleswig Holstein, northward, starting at the mouth of the river Weser. The locations of the sampling areas are shown in Fig. 1 and Table S2. Most sampling areas are located close to munition dumping sites or munition contaminated sites. For further information see Table S3.

Fish sampling, biological data recording

In total, 406 dab (*L. limanda*) were caught by bottom gillnets in the Baltic Sea and by bottom trawling with 3m and 7m beam trawls in the North Sea. Dab were sorted from the catches and transferred to tanks with running seawater to keep them in good condition before examination. Fish were anaesthetized with clove oil, weighed to the nearest g and length measured to the nearest cm. Externally visible fish diseases were recorded by visual inspection of each dab. The dab were then killed by decapitation and otoliths were removed for later age determination [27]. The body cavity was opened and bile fluid was collected with a syringe and stored at −20 °C. LA were recorded by visual inspection of the liver and liver nodules were counted if present. The biometric data were used to determine the condition factor (CF = weight [g] * 100/length [cm]^3) as an indicator of the general health status.

Chemicals and analytical materials

Acetonitrile (99.9%, HPLC quality), methanol (99.97%, UHPLC quality) and acetic acid (99%, p.a. quality) were obtained from Th. Geyer (Germany). Clove oil (≥ 80%, natural origin) was purchased from Carl Roth (Germany). Ammonium acetate was supplied by Honeywell Fluka (USA). HMX, TNT, 2-ADNT and 4-ADNT were obtained as certified reference standards from Accu-Standard (USA, purities > 99.0%). ß-Glucuronidase from *Helix pomatia* Type H-3 was purchased from Sigma-Aldrich (Germany). Chromabond Easy polystyrene-divinylbenzene-copolymer reversed-phase solid-phase extraction columns (1 ml/30 mg) were provided by Macherey and Nagel (Germany). Ultrapure water was prepared on-site with a Purelab Flex 3 system (Elga Veolia, High Wycombe, United Kingdom). The internal standard, isotopically labelled TNT ($^{13}C_7$ 99%; $^{15}N_3$, 98%) was purchased from Cambridge Isotope Laboratories, Inc. (USA, purities > 98%).

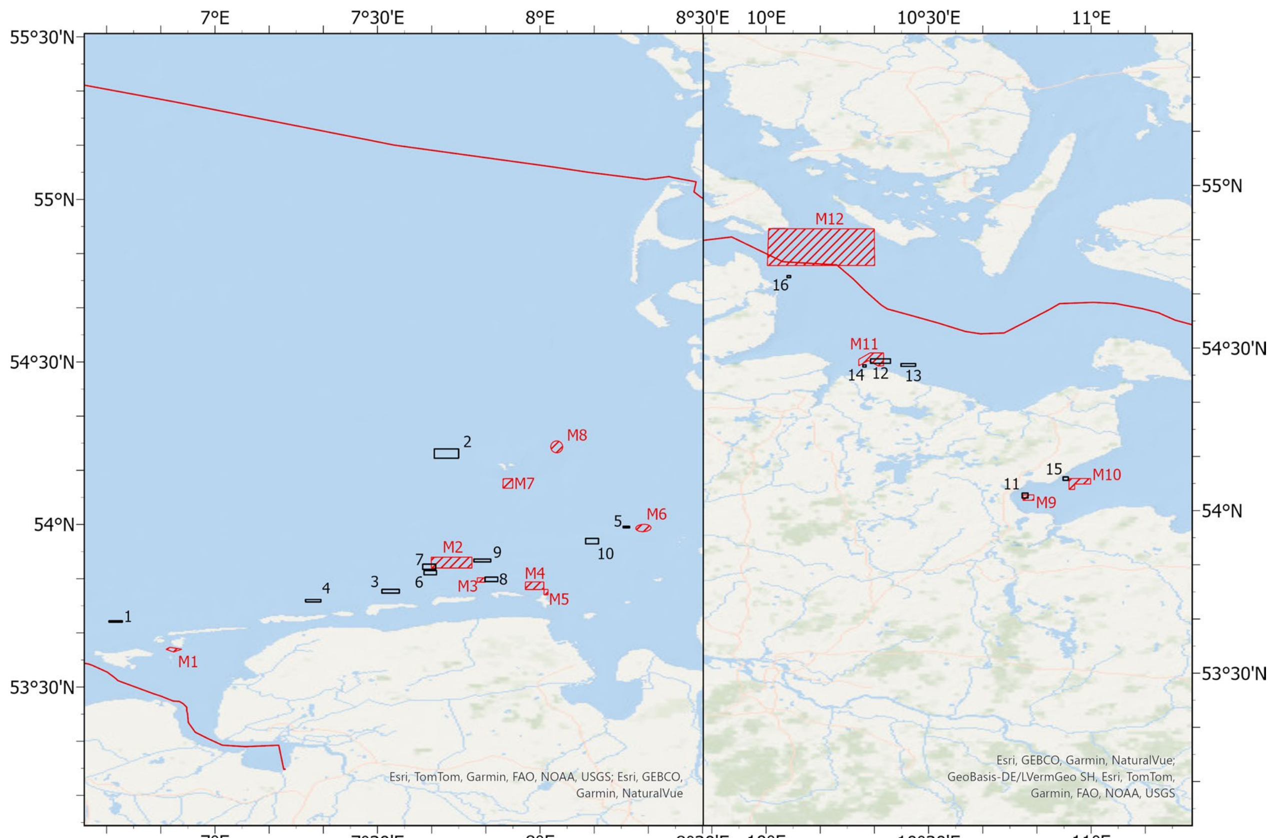

Fig. 1 Sampling areas of dab (black squares) and dumping sites of conventional munition (red hatched areas) close to the German coastline in North Sea (left) and Baltic sea (right). For area IDs compare Tables S2 and S3

Fish disease and parasite prevalence

Externally visible fish disease, parasites and LA were recorded for all 406 fish based on visual examination, following standard methodologies developed by the International Council for the Exploration of the Sea (ICES; [8, 11, 24, 25]). In addition, the body cavity was opened and the liver was inspected for visible anomalies. For each individual fish under investigation, the presence/absence of 15 health indicators were determined: lymphocystis (Ly), epidermal papilloma (Epap), skin ulceration (Ulc), fin rot/erosion (FloF), x-cell gill disease (KieHy), hyperpigmentation/dark melanoma (Mela), white hyperpigmentation (Pig), skeletal deformation (Skel), lipoma (Lip), *Stephanostomum baccatum* (Steph), *Acanthochondria cornuta* (Acanth), *Lepeophtheirus pectoralis* (Lepe), *Cryptocotyle lingua* (Cryp), myxosporidia (Myxo) and LA. Diseases are described in detail in Bucke et al. [8] and Lang and Wosniok [26]. A disease/parasite prevalence is the proportion of fish from a sampling area that exhibited this characteristic.

Bile sample preparation and HPLC–MS/MS analysis

The bile sample preparation and explosive quantification was adapted from Koske et al. [23], Bünning et al. [9], and Maser et al. [28]. For the extraction of explosive compounds 25 µl of each bile were transferred into reaction tubes filled with *ß*-glucuronidase solution containing 900 units as well as with IS solution of 5 ng $^{13}C^{15}N$-TNT to a final volume of 120 µl. Samples were then incubated at 37 °C in a heated shaker (Thermomix Compact, Eppendorf, Germany) for 22 h. After cooling to room temperature, each sample was quantitatively transferred onto a Chromabond Easy solid-phase extraction column (preconditioned with 600 µl methanol and 600 µl acetonitrile). The column was rinsed three times with 500 µl of ultrapure water followed by elution with 100 µl acetonitrile for four times.

Samples were reduced to dryness under a steam of nitrogen and diluted again in 75 µl acetonitrile and 175 µl ultrapure water before being transferred into a 200-µl insert within a 1.5-ml amber glass vial. Samples were immediately subjected to HPLC–MS/MS analysis. A volume of 25 µl of the prepared sample solution was injected in an Agilent 1290 Infinity High-Performance-Liquid-Chromatograph coupled to an AB Sciex QTrap 5500 Triple Quadrupole/Ion-Trap Mass Spectrometer (HPLC–MS/MS). Separation was achieved using an Acclaim Explosives E2 column (2.2 µm, 2.1 mm × 150 mm, Thermo Fisher Scientific) kept at 22 °C. Eluents were (1) ultrapure water with 10 mM acetic acid containing 10 mM ammonium acetate and (2) methanol. The gradient started with 50% methanol and increased to 60% within 5 min. Within the next 10 min methanol increased first to 80% and in another 10 min to 95%. The gradient changed to 100% methanol within 1 min before holding this status for further 9 min and the subsequent return to starting conditions. Flow was held constant at 0.22 ml/min. Ionization was conducted in negative ionization mode. Explosives and selected metabolites were detected via a multiple reaction monitoring mode based on their characteristic MS/MS transitions, previously optimized with commercially available standards. Peak detection was performed with the MultiQuant™ Software Version 3.0.2 (Sciex).

Quality assurance and treatment of censored data

For quantification the internal standard $^{13}C^{15}N$-TNT was used in combination with 10-point external calibration curves for each analyte covering the expected range of concentrations. Limits of detection (LOD) and limits of quantification (LOQ) were calculated according to DIN [13] for all explosive compounds adapted to the matrix fish bile. Matrix-specific LOD and LOQ were determined using spiked matrix samples. Uncontaminated dab bile samples were prepared as described above and spiked with all four explosive chemicals under investigation as well as with the internal standard at concentrations of 0.05–50 ng/ml. Explosives were determined as described before. Results are listed in Table 1. Censored data are values below LOD or LOQ. Concentrations below LOD/LOQ were regarded as estimates and used to calculate mean values before being substituted by LOD/LOQ in Table 3.

Statistics

Statistical analyses were carried out using Statistica Version 12.5 (Statsoft Europe, Hamburg, Germany). To test for possible differences in TNT metabolite contamination due to origin, health status and age, a two-factorial ANOVA (no interaction between factors) in combination with a Fisher LSD post hoc test was used, both with 95% significance. The principal component analysis (PCA) was performed using varimax rotation with explosive results and matching health status indicators. PCA was chosen to reduce dimensions and to reveal possible relations in the data set. For identification of sampling areas which were significantly positive for an explosive a *t*-test against the threshold value LOD (one-tailed, 95% significance) was carried out. Only sample sets showing significant positive difference to LOD in this test were regarded as contaminated.

Table 1 Detection limits (LOD) and quantification limits (LOQ) obtained for different munition compounds according to DIN [13] using matrix specific standards for 2,4,6-trinitrotoluene (TNT), 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)

Substance	LOD [ng/ml]	LOQ [ng/ml]
TNT	3.255	9.765
2-ADNT	0.049	0.147
4-ADNT	0.071	0.213
HMX	0.050	0.150

Values are given in ng/ml bile fluid

Results

Biological data and health indicators of fish

In total 406 dab were investigated for body condition parameters and 15 health indicators such as the prevalence of externally visible diseases and parasites as well as LA (dataset A). The results are displayed in Table 2 and Fig. 2. Overall, fish were 15.5 to 27.8 cm long and 1.7 to 4.8 years old, regarding mean values of sampling areas. The mean fish length in this study was 23.4 cm long and the mean age was 3.0 years. Body condition factor (CF) varied between the sampling areas from 0.84 to 1.13 with a mean CF of 0.97. Prevalence of diseases per sampling site ranged from 0% for e.g., white hyperpigmentation (Pig) at Borkum (Bork) to 76.5% for dark melanoma (Mela) at Scharhörn Reef (SchaRiff). Pig and *Cryptocotyle lingua* (Cryp) were exclusively found in fish from the Baltic Sea. On the other hand, lymphocystis (Ly), epidermal papilloma (Epap), fin rot (FloF), skeletal deformation (Skel), *Stephanostomum baccatum* (Steph), *Acanthochondria cornuta* (Acanth), *Lepeophtheirus pectoralis* (Lepe) and myxosporidia (Myxo) were detected in fish from the North Sea only. Fish in the Baltic Sea had a significantly higher age on average than fish caught in the North Sea (*t*-test; $p > 95\%$). However, the mean number of LA or any other fish disease did not differ significantly between North and Baltic Sea.

Fish health at the sites with highest contamination of explosives, Kolberger Heide West (KHW), did not differ significantly from other sites in the Baltic Sea. Also, the site in the North Sea, Wangerooge North (WangN),

Table 2 Biological data of dab (*Limanda limanda; dataset A*): number (*n*), total legth [cm], weight [g], age [y], condition factor (CF); 15 health indicators lymphocystis (Ly), epidermal papilloma (Epap), skin ulceration (Ulc), fin rot (FloF); x-cell gill disease (KieHy), dark melanoma (Mel), white hyperpigmentation (Pig), skeletal deformation (Skel), lipoma (Lip), *Stephanostomum baccatum* (Steph), *Acanthochondria cornuta* (Acanth), *Lepeophtheirus pectoralis* (Lepe), Cryptocotyle lingua (Cryp), myxosporidia (Myxo) and liver anomalies (LA)

Sampling area	ID	*n*	Length [cm]	Weight [g]	Age [y]	CF	Ly [%]	Epap [%]	Ulc [%]	FloF [%]	KieHy [%]	Mela [%]	Pig [%]	Skel [%]	Lip [%]	Steph [%]	Acanth [%]	Lepe [%]	Cryp [%]	Myxo [%]	LA [%]
North Sea																					
Bork	1	29	22.9	124.2	2.7	1.03	0.0	0.0	3.5	3.5	0.0	48.3	0.0	0.0	0.0	0.0	17.2	31.0	0.0	0.0	17.2
Hel-goWNW	2	32	19.5	74.9	1.7	0.99	6.3	0.0	0.0	0.0	0.0	59.4	0.0	0.0	0.0	0.0	0.0	15.6	0.0	0.0	3.1
Lang	3	30	23.1	115.0	3.2	0.92	0.0	3.3	0.0	0.0	0.0	63.3	0.0	0.0	0.0	3.3	26.7	40.0	0.0	0.0	33.3
NorN	4	30	22.9	110.9	2.9	0.91	6.7	0.0	13.3	10.0	0.0	53.3	0.0	3.3	0.0	3.3	20.0	30.0	0.0	0.0	23.3
SchaRiff	5	17	20.4	75.7	1.8	0.84	0.0	0.0	5.9	23.6	0.0	76.5	0.0	0.0	0.0	17.7	17.6	41.2	0.0	11.8	5.9
SpieN	6	33	22.2	108.0	2.4	0,94	9.1	9.0	3.0	0.0	0.0	51.5	0.0	0.0	0.0	9.1	9.1	9.1	0.0	0.0	18.2
SpiFaN	7	30	21.8	103.0	2.4	0.99	0.0	0.0	3.3	0.0	0.0	50.0	0.0	0.0	0.0	10.0	16.7	30.0	0.0	0.0	13.3
WangN	8	30	22.5	117.6	2.6	1.01	6.7	6.7	0.0	23.3	0.0	73.3	0.0	0.0	0.0	3.3	6.7	10.0	0.0	3.3	0.0
WaNMO	9	20	21.9	103.0	2.4	0.96	2.4	5.0	0.0	5.0	0.0	55.0	0.0	5.0	0.0	15.0	5.0	40.0	0.0	0.0	10.0
WesMuen	10	30	22.7	115.2	2.8	0.97	10.0	3.3	3.3	0.0	0.0	53.3	0.0	0.0	0.0	3.3	20.0	13.3	0.0	0.0	16.7
Baltic Sea																					
HaffkN	11	7	24.4	154.0	3.9	1.05	0.0	0.0	0.0	0.0	0.0	14.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
KH	12	36	27.8	200.6	4.8	0.94	0.0	0.0	2.8	0.0	0.0	16.7	5.6	0.0	0.0	0.0	0.0	0.0	8.3	0.0	13.9
KHO	13	44	27.4	198.1	4.8	0.94	0.0	0.0	2.3	0.0	0.0	4.5	18.2	0.0	0.0	0.0	0.0	0.0	4.5	0.0	18.2
KHW	14	12	27.3	217.5	4.7	1.05	0.0	0.0	0.0	0.0	0.0	0.0	8.3	0.0	0.0	0.0	0.0	0.0	8.3	0.0	8.0
PelzerhO	15	16	24.4	157.1	3.2	1.06	0.0	0.0	0.0	0.0	0.0	0.0	12.5	0.0	0.0	0.0	0.0	0.0	18.8	0.0	6.3
SchleiNO	16	10	26.0	199.0	4.6	1.10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0	0.0	10.0
All		406																			

Diseases and parasites are expressed as prevalence (percentage of fish exhibiting the alteration) for different sampling areas

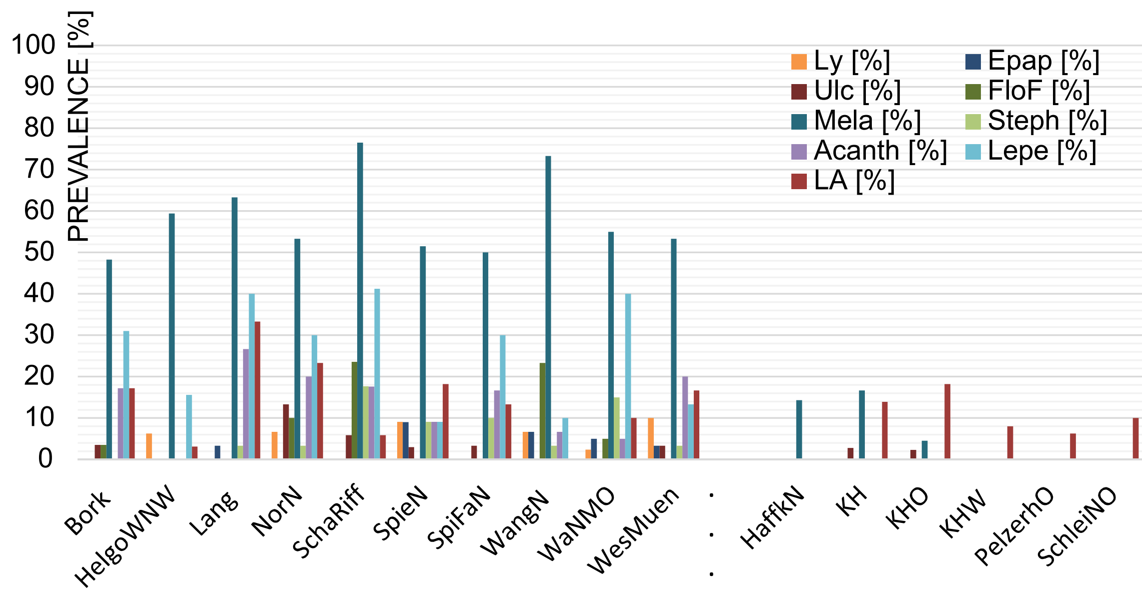

Fig. 2 Prevalence of selected fish diseases and parasites in different sampling areas: left of dotted line: North Sea areas, right of dotted line: Baltic Sea areas. Lymphocystis (Ly), epidermal papilloma (Epap), skin ulceration (Ulc), dark melanoma (Mela), *Stephanostomum baccatum* (Steph), *Acanthochondria cornuta* (Acanth), *Lepeophtheirus pectoralis* (Lepe) and liver anomalies (LA)

Table 3 Explosives in bile of dab (*L. limanda*), number (n), 2,4,6-trinitrotoluene (TNT), 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)

Sampling area	ID	*n*	TNT [ng/ml]	2-ADNT [ng/ml]	4-ADNT [ng/ml	HMX [ng/ml]
North Sea						
Bork	1	6	< LOD	< LOD	< LOD (< LOD– < LOQ)	< LOD
HelgoWNW	2	15	< LOD	< LOD	< LOD	< LOD
Lang	3	11	< LOD	< LOD (< LOD– < LOQ)	< LOQ (< LOD–0.559)	< LOD
NorN	4	14	< LOD	< LOD (< LOD–0.157)	0.071 (< LOD–0.230)	< LOD
SchaRiff	5	6	< LOD	< LOD	0.156 (< LOD–0.450)	< LOD
SpieN	6	10	< LOD	< LOD	< LOD	< LOD
SpiFaN	7	9	< LOD	< LOD	< LOD (< LOD–0.373)	< LOD
WangN	8	16	< LOD	<u>0.154</u> (< LOD–0.456)	< LOQ (< LOD–0.927)	< LOD
WaNMO	9	4	< LOD	< LOQ (< LOD–0.262)	0.319 (< LOD–1.211)	< LOD
WesMuen	10	13	< LOD	< LOD (< LOD–0.245)	< LOQ (< LOD–0.651)	< LOD
Baltic Sea						
HaffkN	11	3	< LOD	< LOD (< LOD–< LOQ)	< LOQ (< LOD–0.295)	< LOD
KH	12	31	< LOD	1.745 (< LOD–26.356)	<u>6.841</u> (< LOD–95.908)	< LOD (< LOD–0.205)
KHO	13	25	< LOD	<u>4.426</u> (0.219–16.987)	<u>18.483</u> (0.735–78.238)	0.034 (< LOD–0.620)
KHW	14	11	< LOD	<u>7.803</u> (3.289–13.747)	<u>28.523</u> (9.126–50.998)	< LOD
PelzerhO	15	14	< LOD	< LOD (< LOD–< LOQ)	< LOD (< LOD– < LOQ)	< LOD
SchleiNO	16	6	< LOD	<u>1.067</u> (0.251–1.742)	<u>4.762</u> (1.088–11.671)	0.148 (< LOD–0.885)
All		194	< LOD	1.333 (< LOD–26.356)	5.244 (< LOD–95.908)	< LOQ (< LOD–0.885)

Given are mean (minimum–maximum) concentrations for different sampling areas. LOD: limit of detection. LOQ: limit of quantification. Underlined mean values indicate a significant positive difference to LOD (*t*-test, $p = 0.95$)

with the highest contamination did not exhibit a different pattern of diseases or parasites compared to other sampling areas in the North Sea. Correlation between age and disease prevalence and sampling area was tested for dab from the Baltic Sea only because these fish were older. A significant correlation between age and LA was found in Kolberger Heide East (KHO) and Pelzerhaken East (PelzerhO). A significant correlation between age and *Cryptocotyle lingua* (Cryp) was observed in PelzerhO. A significant correlation between age and LA was detected in KHO and PelzerhO, whereas a significant correlation between age and white hyperpigmentation (Pig) was present in KHO, PelzerhO and Schleimünde North-East (SchleiNO) (results not shown). A clear correlation between LA and explosive contamination was not observed.

We conclude that the present data give no clear indication that dab from sites contaminated with explosive suffer more from the prevalence of externally visible diseases and parasites than dab from uncontaminated sites. However, the prevalence of LA, which might be precursors of liver tumours, seem to occur more often at sites contaminated with carcinogenic explosives like TNT and its metabolites (Tables 2 and 3). On the contrary, prevalence of LA, did not always match with elevated explosive concentration in the bile. This might be explained by the fact that explosives in fish bile mirror the acute contamination only, while liver tumours may develop on a different time scale, namely during years of chronic exposure.

Explosives in dab bile

From the total 406 dab, in a subset of samples ($n = 194$) concentrations of explosives were analysed in the fish bile fluid (dataset B). This was necessary, because not all fish had a bile volume of at least 25 µl—the methods limit for chemical analyses. However, 194 dab were distributed across all sampling sites, and 3 to 31 individual bile samples were analysed per sampling site (detailed n are given in Table 3). Each individual fish bile was analysed for the explosives TNT, 2-ADNT, 4-ADNT and HMX. Signals of 2-ADNT and/or 4-ADNT were present in most of the samples, however concentrations were often below LOD. In total 34% samples from the North Sea were > LOD for 2-ADNT and/or 4-ADNT. In the Baltic Sea 88% samples under investigations were positive for at least one explosive. In fish bile mean contamination in the sampling areas with 2-ADNT (4-ADNT) ranged for from < LOD to 0.154 (< LOD to 0.319) ng/ml in the North Sea and

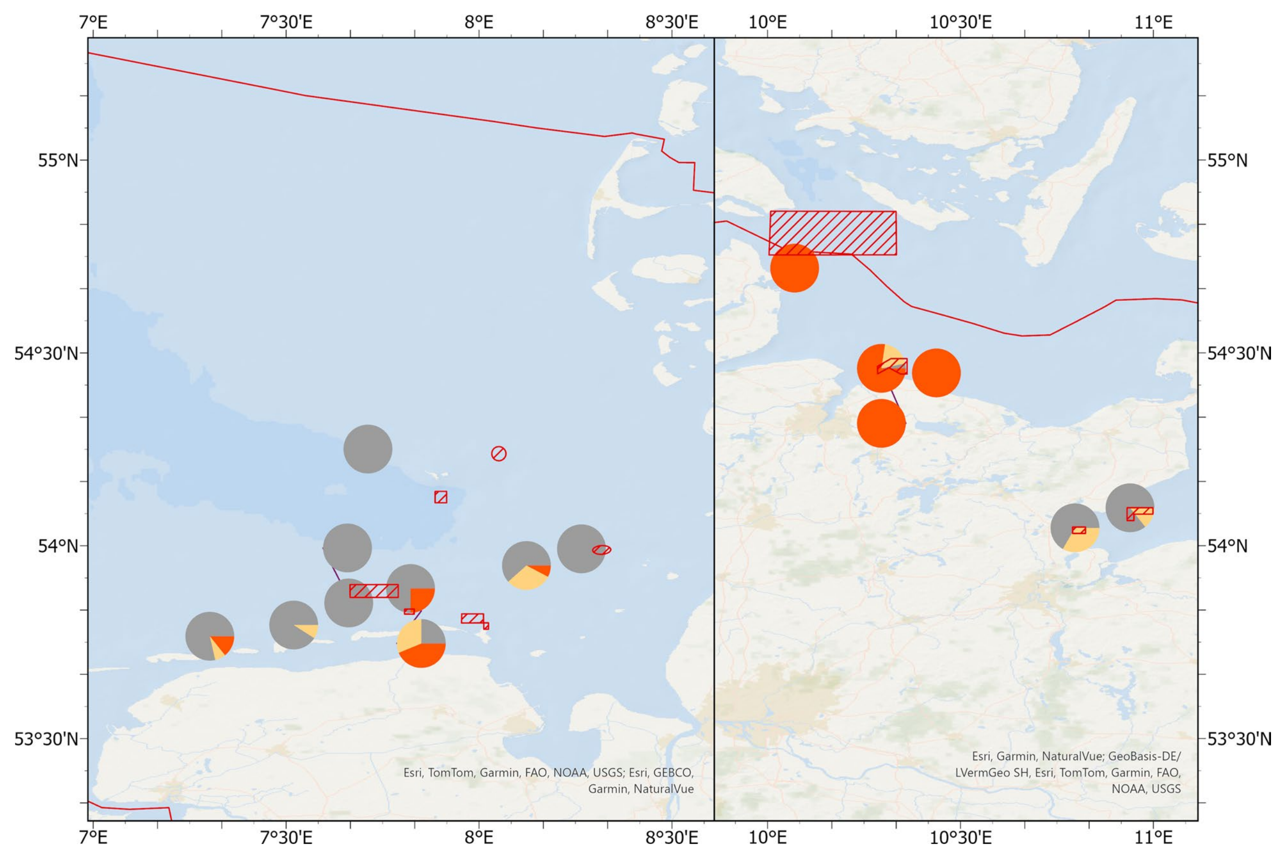

Fig. 3 Spatial distribution of 2-amino-4,6-dinitrotoluene (2-ADNT) in bile of dab (*Limanda limanda*). Given are percentages of individuals per sampling area below LOD (grey), between LOD and LOQ (light orange), and above LOQ (dark orange). Red hatched areas are dumping sites of conventional munition, red line is the border of the EEZ of Germany

from < LOD to 7.83 (< LOD to 28.523) ng/ml in the Baltic Sea. Results are displayed in Fig. 3 and Table 3.

Highest individual concentration of 2-ADNT in the North Sea was detected at Wangerooge (WangN) with 0.456 ng/ml as well as in the Baltic Sea with 26.356 ng/ml in fish from Kolberger Heide (KH). Highest individual concentration of 4-ADNT in the North Sea was detected at site Wangerooge North Minsener Oog (WaNMO) with 1.211 ng/ml as well as in the Baltic Sea with 95.908 ng/ml again in KH. HMX was detected in eight fish samples from the Baltic Sea only. The maximum HMX concentration of 0.886 ng/ml was found in a dab caught at the site Schleimünde North-East (SchleiNO). TNT was not detected in any fish bile—even not in concentrations below LOD, presumably due to its rapid metabolization to 2-ADNT and 4-ADNT in the liver. Concentrations of chemical residues of explosives are displayed in detail in Table 3.

Explosive concentrations differed significantly between sampling areas and was not related to sex or age of the dab. The results indicate, that dab from the North Sea living in the vicinity of the island Wangerooge (WangN) were significantly contaminated with explosives. In the other North Sea regions under investigation, only few individuals were positive for explosives. In the Baltic Sea, dab caught in the area Kolberger Heide (KH, KHO, KHW), as well as from the mouth of the river Schlei (SchleiNO) were significantly contaminated with at least one explosive. In contrast the Baltic Sea areas HafkN and PerzerhO did not reveal dab contaminated with explosives significantly above LODs (Table 3, Fig. 3).

Discussion

With the present study we have observed that explosives, leaking from dumped munition at sites in German coastal waters of the Baltic and the North Sea, are detectable in fish bile. This is in line with previous results from the dumping site "Kolberger Heide" in the Baltic Sea [23] and in the vicinity of munition-loaded wrecks in the North Sea [28]. To the authors knowledge we here show for the first time, that fish caught next to the island Wangerooge (WangN), near the German coastline of lower Saxony, were contaminated with explosives, which presumably originates from munition dump sites located nearby. This is the first documentation that dumped munition in the North Sea contaminate fish in their near surrounding.

Our results from the Baltic Sea are in accordance with Beck et al. [2] and Koske et al. [23], who found that dabs caught in the Baltic Sea near the dumping area "Kolberger Heide" (KH, KHO, KHW) were contaminated in mean with 1.60 ng/ml 2-ADNT and 17.06 ng/ml 4-ADNT. We also confirm the finding of Beck et al. [2] and Koske et al. [23], who detected HMX in some fish from the Kolberger Heide. The mean concentrations observed in fish caught at "Kolberger Heide" in the present study, exceeded measured values at all other sites in the Baltic Sea and the North Sea. This was presumably due to the fact that in the dumpsite "Kolberger Heide" solid explosive (gun wool) is lying open (without munition shell) on the seafloor [14, 20], from which significant amounts of explosives leak out [4]. In contrast, dumped munition in the investigated part of the North Sea typically has intact shells and is buried under sand [33]. The stronger water current in the North Sea dilutes leaking explosives more quickly than in the Baltic Sea.

Explosives and their metabolites are toxic, mutagenic and carcinogenic [6, 21]. Therefore, they may be harmful to marine ecosystems. We combined the chemical results with fish health indicators determined at the same individuals. These 15 health indicators comprised 9 fish diseases, 5 parasites as well as LA. Health indicators showed differences between dab caught in the North Sea and dab caught in the Baltic Sea, but significant correlations of health indicators to contamination with explosives were not observed. However, LA, which may be precursors of tumours showed a trend to be more abundant at sites at which dab were contaminated with explosives.

A principal component analysis shows that concentrations of explosives in fish bile and fish health indicators did not follow the same main factor. However, 2-ADNT and 4-ADNT were closely correlated, suggesting that both metabolites have the same source. HMX is located in a different quadrant of Fig. 4 than 2-ADNT and 4-ADNT, since HMX was not always detected alongside TNT metabolites. Fish diseases and parasites are widely distributed between the two main factors. Some fish diseases show only limited relations to each other. However, Pig and Cryp, only observed in fish from the Baltic Sea were clustered. Also, Ly, Epap, FloF, Skel, Steph, Acanth, Lepe and Myxo group in Fig. 4, probably because they were present in dab from the North Sea only. Also, the sum of variation in Fig. 4 explained by the first two factors is quite low with about 30%. Other factors than explosive contamination appear to be more important for the individual fish health.

Our results show that toxic explosives like TNT metabolites can be monitored successfully by analysing bile fluid of the flatfish species dab. Explosives exhibited a patchy distribution and peaked in the vicinity of sources like dumping sites. Therefore, the monitoring of explosive hotspots should be considered in the Baltic Sea as well as in the North Sea. Even though, if explosives like HMX, TNT and its metabolites are actually not in the list of monitored chemicals in European marine waters [16, 32], the authors of the present study expect that awareness for this topic will grow in the

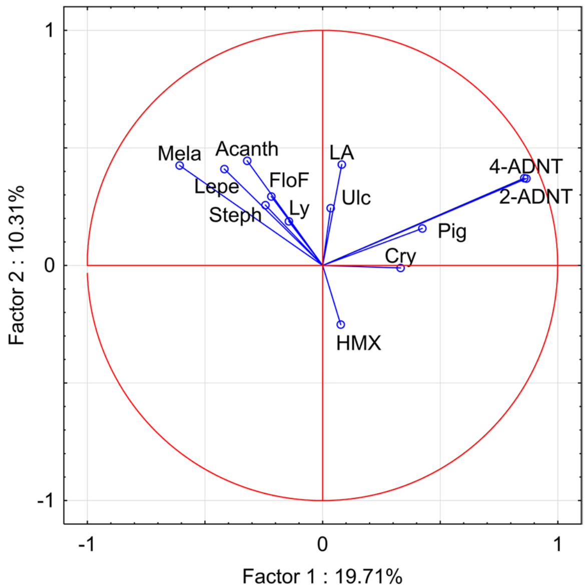

Fig. 4 Principal component analyses of fish health parameters (lymphocystis (Ly), skin ulceration (Ulc), fin rot (FloF), dark melanoma (Mela), white hypopigmentation (Pig), *Stephanostomum baccatum* (Steph), *Acanthochondria cornuta* (Acanth), *Lepeophtheirus pectoralis* (Lepe), liver anomalies (LA) and explosive concentrations (2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)) in bile. Diseases/parasites with zero-variation are not displayed

future because dumped munition in the seas are receiving more and more attention from multiple angles. The fact that marine dumped munition is corroding and releases toxic substances to the environment, spurs the motivation for remediation campaigns.

In fact, the German government is currently financing a 100 Mio € programme to stimulate the development of a large-scale clearance and remediation of marine dumped munition [29]. In 2024, remediation trials started in Lübeck Bay, where dumping of about 30,000 t of conventional munition after WW II is estimated. We recommend to include fish in a possible monitoring following a remediation action. We also recommend to analyse explosives in fish bile because it can mirror changes in contamination within few days before and after the remediation action. We further recommend the dab as suitable fish species for short-term as well as for long-term monitoring purposes and the assessment of its health status alongside with chemical contamination. Even if at present, contamination of fish with munition compounds is still low and health effects were not detectable, this may change with increasing corrosion of munition shells or may be different at yet undiscovered hot spots of contamination.

Conclusions

We conclude that fish living close to dumping sites in the Baltic as well as in the North Sea can be contaminated with explosives. Highest contamination was observed in the Baltic Sea at the hot spot "Kolberger Heide". In the North Sea a contamination hot spot could also be detected next to the island of Wangerooge in the vicinity of munition dumping sites. Based on fish health indicators, we have no clear indication that fish might suffer from contamination with explosives. The presented analytical method for explosives in dab bile is suitable for monitoring of environmental contamination at hot spots or for supplementation of remediation measures.

Abbreviations

2-ADNT	2-Amino-4,6-dinitrotoluene
4-ADNT	4-Amino-2,6-dinitrotoluene
Acanth	*Acanthochondria cornuta*
Bork	Borkum
CF	Condition factor
Cryp	*Cryptocotyle lingua*
Epap	Epidermal papilloma
FloF	Fin rot/erosion
HaffN	Haffkrug North
HelgoWNW	Heligoland West-North-West
HMX	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
ICES	International Council for the Exploration of the Sea
KH	Kolberger Heide
KHO	Kolberger Heide East
KHW	Kolberger Heide West
KieHy	X-cell gill disease
LA	Liver anomalies
Lang	Langeoog
Lepe	*Lepeophtheirus pectoralis*
Lip	Lipoma
LOD	Limit of detection
LOQ	Limit of quantification
Ly	Lymphocystis
Mela	Hyperpigmentation/dark melanoma
Myxo	Myxosporidia
NorN	Norderney North
PCA	Principal component analysis
PelzerhO	Pelzerhaken East
Pig	White hypopigmentation
RV	Research vessels
SchaRiff	Scharhörn reef
SchleiNO	Schleimünde North-East
Skel	Skeletal deformation
SpieN	Spiekeroog North
SpiFaN	Spiekeroog fairway North
Steph	*Stephanostomum baccatum*
TNT	2,4,6-Trinitrotoluene
Ulc	Skin ulceration
WangN	Wangerooge North
WaNMO	Wangerooge North, Minsener Oog
WesMuen	Weser mouth

Supplementary Information

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Supplementary Material 1.

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Author contributions
Conceptualization UK, JS; methodology NS, VT; validation NS; formal analysis UK; data curation MR, MG; investigation NS, VT, MG, MR; resources JS; writing—original draft preparation UK; writing—review and editing JS, MOA; visualization MOA; supervision, JS UK; funding acquisition JS UK; All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
The datasets generated and analysed during the current study are available in the OpenAgrar repository, https://doi.org/10.3220/DATA20240430113916-0.

Declarations

Ethics approval and consent to participate
All procedures were conducted in accordance with European directive 2010/63/EU on the protection of animals used for scientific purposes.

Consent for publication
Not applicable.

Competing interests
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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