



REVIEW

Environmental and human toxicology studies on explosive chemicals leaking from submerged munitions

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Funding information

German Federal Ministry of Education and Research, Grant/Award Number: "UDEM" 03F0747B; Schleswig-Holstein Ministry of Energy, Agriculture, the Environment, Nature and Digitisation (MELUND); State Government Schleswig-Holstein; German Environment Agency (UBA), Grant/Award Number: FKZ 3719 25 2990

Abstract

This review provides an overview of 15 years of munitions research by the Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein, Kiel, Germany. As early as 2009, it was possible to detect the TNT metabolite 4-ADNT in a quantity of 250 ng/g (wet weight) in blue mussels collected directly on munition items in the Kolberger Heide dumping area (Bight of Kiel, Baltic Sea, Germany). Based on these results, biomonitoring with blue mussels was established, in which uncontaminated mussels were exposed at certain distances from munition items and then retrieved after a few weeks in order to then be analyzed in the laboratory for energetic compounds (EC) including TNT and metabolites thereof. In following studies – in addition to mussels – also fish, sediments, water samples and passive samplers from specific locations were examined for the presence of EC. The study areas included dumping site regions from both the Baltic Sea and the North Sea, and were then extended to appropriate shipwrecks in the North Sea. The field studies were completed with laboratory investigations, which dealt with the development of a molecular biomarker as a kind of “early warning system” and with aspects of microbial degradation of EC. Finally, risk assessments for the marine environment and the human seafood consumer were carried out. The sections below are based on results from more than 10 scientific research projects and more than 20 publications in primary international research journals.

KEYWORDS

energetic chemicals, human seafood safety, marine environmental contamination, submerged munitions, TNT toxicity and carcinogenicity, toxic World War relics

1 | INTRODUCTION

Since World War I considerable amounts of warfare materials have been dumped at sea worldwide [1]. The hazard of sea-dumped munitions can be divided into three main categories: (1) injury of the human body due to spontaneous explosion by activation of

the ignition mechanisms, including the devastation and contamination of the surroundings with unexploded materials (2) threats to human health by direct physical contact with toxic chemicals (3) chronic contamination of the marine ecosystem and marine organisms by toxic munition constituents continuously leaching from corroding munition items,

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followed by the entry into the marine and human food chains [1].

Munition constituents have been found in soil, sediments as well as in surface- and groundwater at sites with military activities [2–4], and also in the marine ecosystem [5, 6]. Sea dumped munitions are able to contaminate the marine environment, because of the release of the toxic chemicals through corrosion and breaching or during blow-in-place detonations [7–9]. The current state of corrosion of dumped munitions is not exactly known in many cases and varies from ‘very little’ to ‘completely degraded away’ [1]. Especially in low-order detonations larger pieces of unexploded munition constituents are distributed in the environment. As a consequence, differently sized particles can be found in sediments and soil, and may dissolve slowly over time [10]. It is possible that these substances persist for several decades in the environment [5].

The secondary explosive 2,4,6-trinitrotoluene (TNT), a nitroaromatic energetic compound, belongs to the major ingredients in almost every munition formulation [2]. TNT has a low melting point and is thermally and chemically stable (Table 1). The solubility in water is 130 mg/L at 20°C [10] but decreases significantly at lower water temperature (approx. 50 mg/L at 6°C [11]). 2-Amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) are metabolites of TNT which occur during biotic transformation in the environment through the reduction of the respective nitro group to an amino group [10]. Laboratory exposure experiments have shown that TNT and its metabolites are able to put threats to marine organisms [12, 13]. Nipper et al. [13] investigated the toxicological effects of TNT and other energetic compounds on the marine species sea urchin (*Arbacia punctulata*), opossum shrimp (*Mysidopsis bahia*), redfish (*Sciaenops ocellatus*), macroalgae (*Ulva fasciata*) and polychaetes (*Dinophylus gyrotilatus*) with different toxicological endpoints. The toxicity of all compounds could be proven.

For humans, health risks have also been determined. In TNT-exposed humans, notable toxic manifestations have included aplastic anaemia, toxic hepatitis, cataracts, hepatomegaly and liver cancer [14, 15].

In the last decades, a controversial discussion was going on among decision makers whether munition dumped-sites should remain undisturbed because of no direct risk to humans. However, there was almost consensus that monitoring of toxic explosives released from corroded munitions in these areas should be continued [1].

Though it is possible to detect levels of pollutions and toxic chemicals in water, soil, and sediment samples by chemical analysis and measurement of their concentrations directly, information about the impact on biological systems and their organisms is limited [16]. Also, the determination of potential metabolic products, which can be toxic as well, is only possible in appropriate organisms. These organisms may then serve as bio-indicators or biomonitoring systems.

2 | THE BEGINNING: DETECTION OF ENERGETIC COMPOUNDS (EC) IN BALTIC SEA WATER USING BLUE MUSSELS AS BIOINDICATORS (ON BEHALF OF THE STATE OFFICE FOR AGRICULTURE, ENVIRONMENT AND RURAL AREAS OF SCHLESWIG-HOLSTEIN (LLUR))

After initial suspicion of the release of EC from dumped war munitions, the Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School Schleswig-Holstein (UKSH) was commissioned by the LLUR on October 6, 2009, to develop a reliable and reproducible analytical method for the detection of EC. The study was called “Development of a method for the detection of energetic compounds (EC) in blue mussels”. Between November 16, 2009, and April 23, 2010, mussel samples were collected by the Schleswig-Holstein State Ordnance Disposal Service and various EC (TNT and metabolites thereof) were examined. Physicochemical data of selected EC are listed in Table 1.

2.1 | Suitability of the blue mussel (*Mytilus edulis*) as a bioindicator

Based on physicochemical principles and accumulation processes lipophilic, persistent compounds with a log octanol/water coefficient > 3 accumulate in the blue mussels by several powers of ten [18]. Mean log octanol/water coefficients of approx. 1.60 (TNT), 1.84 (estimated for 4-amino-2,6-dinitrotoluene (4-ADNT) and 2-amino-4,6-dinitrotoluene (2-ADNT) and 1.64 (trinitrobenzene (TNB)) (see Table 1), suggest that an accumulation of EC in mussels has not to be expected due to low lipophilic properties. In line with this is the rapid metabolism of TNT in mussels, which leads to the formation of aminodinitrotoluenes [12, 19]. However, the weakly polar character may cause a membrane binding of these compounds and thus an accumulation of EC can be expected

TABLE 1 Physicochemical data of selected EC (adapted from [17])

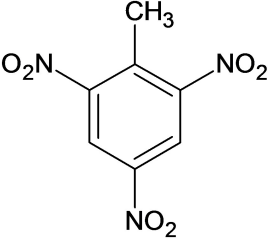
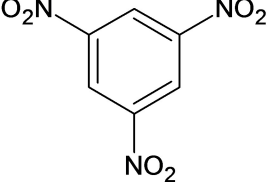
Structural formula	Physical data [17]
	CAS Number: 000118-96-7 Chem Name: 2,4,6-TRINITROTOLUENE Mol Formula: C ₇ H ₅ N ₃ O ₆ Mol Weight: 227.13 Melting Pt: 80.1 deg C Boiling Pt: Water Solubility: Value: 115 mg/L Temp: 23 deg C Type: EXP Ref: PHELAN,JM & BARNETT,JL (2001) Log P (octanol-water): Value: 1.60 Type: EXP Ref: HANSCH,C ET AL. (1995) Vapor Pressure: Value: 8.02E-006 mm Hg Temp: 25 deg C Type: EXP Ref: SPANGGORD,RJ ET AL. (1980) pKa Dissociation Constant: Value: Temp: Type: Ref: Henry's Law Constant: Value: 2.08E-008 atm-m ³ /mole Temp: 25 deg C Type: EST Ref: VP/WSOL
	CAS Number: 000099-35-4 Chem Name: 1,3,5-TRINITROBENZENE Mol Formula: C ₆ H ₃ N ₃ O ₆ Mol Weight: 213.11 Melting Pt: 121.5 deg C Boiling Pt: 315 deg C Water Solubility: Value: 278 mg/L Temp: 15 deg C Type: EXP Ref: YALKOWSKY,SH & HE,Y (2003) Log P (octanol-water): Value: 1.18 Type: EXP Ref: HANSCH,C ET AL. (1995) Vapor Pressure: Value: 6.44E-006 mm Hg Temp: 25 deg C Type: EXT

TABLE 1 (Continued)

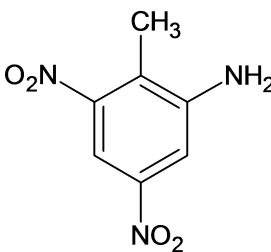
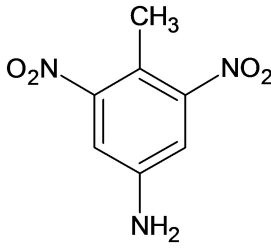
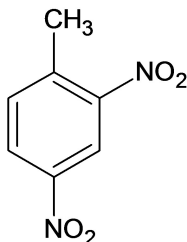
Structural formula	Physical data [17]
	Ref: OHE,S (1976) pKa Dissociation Constant: Value: Temp: Type: Ref: Henry's Law Constant: Value: 6.5E-009 atm-m ³ /mole Temp: 25 deg C Type: EST Ref: VP/WSOL CAS Number: 035572-78-2 Chem Name: 2-Amino-4,6-dinitrotoluene Mol Formula: C ₇ H ₇ N ₃ O ₄ Mol Weight: 197.15 Melting Pt: 174.5 deg C Boiling Pt: Water Solubility: Value: 1220 mg/L Temp: 25 deg C Type: EST Ref: MEYLAN,WM ET AL. (1996) Log P (octanol-water): Value: 1.84 Type: EST Ref: MEYLAN,WM & HOWARD,PH (1995) Vapor Pressure: Value: 1.07E-005 mm Hg Temp: 25 deg C Type: EST Ref: NEELY,WB & BLAU,GE (1985) pKa Dissociation Constant: Value: Temp: Type: Ref: Henry's Law Constant: Value: 3.27E-011 atm-m ³ /mole Temp: 25 deg C Type: EST Ref: MEYLAN,WM & HOWARD,PH (1991) CAS Number: 019406-51-0 Chem Name: 4-Amino-2,6-dinitrotoluene Mol Formula: C ₇ H ₇ N ₃ O ₄ Mol Weight: 197.15 Melting Pt: 171 deg C Boiling Pt: Water Solubility: Value: 1220 mg/L Temp: 25 deg C Type: EST
	

TABLE 1 (Continued)

Structural formula	Physical data [17]
	Ref: MEYLAN,WM ET AL. (1996) Log P (octanol-water): Value: 1.84 Type: EST Ref: MEYLAN,WM & HO-WARD,PH (1995) Vapor Pressure: Value: 1.07E-005 mm Hg Temp: 25 deg C Type: EST Ref: NEELY,WB & BLAU,GE (1985) pKa Dissociation Constant: Value: Temp: Type: Ref: Henry's Law Constant: Value: 3.27E-011 atm-m3/mole Temp: 25 deg C Type: EST Ref: MEYLAN,WM & HO-WARD,PH (1991) CAS Number: 000121-14-2 Chem Name: 2,4-DINI-TROTOLUENE Mol Formula: C7H6 N2O4 Mol Weight: 182.14 Melting Pt: 71 deg C Boiling Pt: 300 deg C Water Solubility: Value: 200 mg/L Temp: 25 deg C Type: EXP Ref: PHELAN,JM & BAR-NETT,JL (2001) Log P (octanol-water): Value: 1.98 Type: EXP Ref: HANSCH,C ET AL. (1995) Vapor Pressure: Value: 0.000147 mm Hg Temp: 22 deg C Type: EXP Ref: PELLA,PA (1977)
	CAS Number: 000606-20-2 Chem Name: 2,6-DINI-TROTOLUENE Mol Formula: C7H6 N2O4 Mol Weight: 182.14 Melting Pt: 66 deg C Boiling Pt: 300 deg C Water Solubility: Value: 182 mg/L

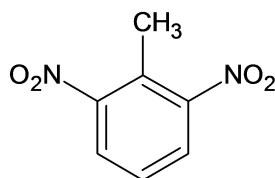


TABLE 1 (Continued)

Structural formula	Physical data [17]
	Temp: 20 deg C Type: EST Ref: SPANGGORD,RJ ET AL. (1980) Log P (octanol-water): Value: 2.10 Type: EXP Ref: NAKAGAWA,Y ET AL. (1992) Vapor Pressure: Value: 0.000567 mm Hg Temp: 25 deg C Type: EXP Ref: PELLA,PA (1977) pKa Dissociation Constant: Value: 1.8 Temp: Type: EXP Ref: SPANGGORD,RJ ET AL. (1980) Henry's Law Constant: Value: 7.47E-007 atm-m3/mole Temp: 25 deg C Type: EST Ref: VP/WSOL

which finally leads to a measurable accumulation of EC in the mussel tissue.

2.2 | Material and analytical methods

Two blue mussel pool samples of 20 mussels each (sample 1 and sample 2) from the munitions dumping area Kolberger Heide (Bay of Kiel, Baltic Sea, Germany) and, as a reference, commercially available North Sea blue mussels from Danish waters were investigated. The soft tissue was removed from the mussels and homogenized using an Ultra-Turrax. For detecting EC (1,3,5-trinitrotoluene, 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 2,4-dinitrotoluene, 2,6-dinitrotoluene) EC were extracted from the matrix by freeze-drying and liquid extraction with organic solvents using or Soxhlet extraction. After the organic phase had been separated and dried over calcium chloride or sodium sulphate, the solvents were removed under reduced pressure at low temperatures in a stream of nitrogen. The EC were then analyzed by GC-MS technology. The method developed was reproducible and had satisfactory recovery rates averaging 70%. The detection limit for the individual STV was around 0.2 mg/kg sample at that time [17].

2.3 | Results and validation of the findings

Blue mussel pool samples from the North Sea reference area (Danish waters) did not provide any indication of EC presence. However, mussels from the two areas of the munitions dumping area Kolberger Heide were shown positive for the presence of EC. While mussels living directly on the munition items (sample 1) contained 4-ADNT concentrations of approx. 250 ng/g wet weight, mussel samples from a distance of approx. 4.5 m from the munition item showed signals of lower intensity with regard to the retention times of 4-ADNT and 2-ADNT compared to sample 1. Thus, a reliable quantification was not possible for the latter. Considering the detection limit with the above method of 200 ng/g per EC, the method developed using GC-MS technology could, at that time, be carried out routinely for a trace analysis laboratory when concentrations of EC were higher than 200 ng/g [17]. Two points should be emphasized here: First, the mussels investigated here were wild living species that had been sitting their whole life on the munition items and therefore showed this high EC concentration of 250 ng/g wet weight, while transplanted mussels (Chapter 3) were exposed at varying distances from the munition items with a limited exposure time of 3 months. Second, the limit of detection for EC was considerably improved in the following years (Chapter 7).

3 | BIOMONITORING STUDIES WITH TRANSPLANTED BLUE MUSSELS AT DUMPING SITES IN THE KOLBERGER HEIDE (BALTIC SEA) NEAR KIEL BIGHT

Routine biomonitoring studies with the blue mussel *Mytilus* spp. were developed in the framework of the BMBF (German Federal Ministry of Education and Research) founded project UDEMM (“Umweltmonitoring für die Delaboration von Munition im Meer”) which took place from 2016 to 2019 [20]. The general aim of the UDEMM joint project was to investigate the potential contamination with conventional munitions in the Baltic Sea and specifically in the Kolberger Heide munition dumping area. In this regard, a concept should be developed for the first time that enables scientifically sound monitoring. New analysis methods, state-of-the-art technologies and numerous field studies should be developed and used. Due to the complexity of the topic, UDEMM was divided into four scientific work packages, whereby the Institute for Institute of Toxicology and Pharmacology for Natural Scientists had the task of developing an

ecological-toxicological biomonitoring for pollutant emissions from munitions in the sea in the sub-project ÖkoToxEMM (Untersuchungen der Beeinträchtigung des Ökosystems durch sprengstofftypische Verbindungen).

Since several decades, blue mussels, especially *Mytilus* spp. and *Mytilus galloprovincialis*, have been successfully used as bioindicators [21]. The advantages are manifold: Bivalves are filter feeding organisms filtering many liters of water every day and as a result concentrate chemicals in their tissues, the ability to metabolize organic contaminants is low relative to other marine organisms, they are robust and survive under moderate levels of different pollutions, and due to their sedentary property they are not only distributed worldwide in relatively stable local populations but at the same time represent a specific area. Moreover, on the one hand repeated sampling of natural occurring specimens is possible, on the other hand mussels are ideally suitable to be transplanted to test areas for controlled biomonitoring. Beyond that as primary consumers many bivalves are important sea food species and can be used as indicators for the entry of toxic substances into the marine food chain [22].

The aim of the present study was the implementation of a field experiment to conclude if it is possible to use blue mussels (*M. edulis*) as a biomonitoring system for the detection of TNT and its metabolites 2-ADNT and 4-ADNT in a former dumping ground for discarded military munitions (Figure 1).

Bioaccumulation of TNT and its main metabolites 2-ADNT and 4-ADNT leaking from corroded munitions at

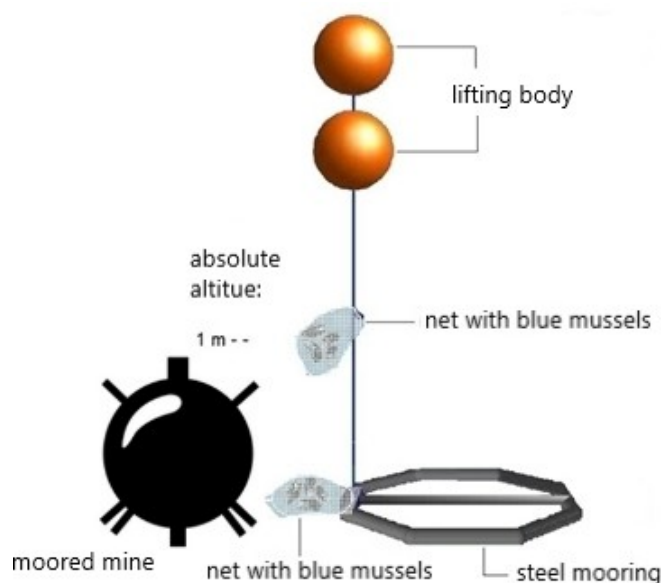


FIGURE 1 Construction scheme of the mooring positioned at Kolberger Heide.

the munition dumping site Kolberger Heide was evaluated in transplanted blue mussels (*Mytilus edulis*) near blast craters [23]. Over an exposure time of 93 days it was shown that the exposed blue mussels accumulate TNT, 2-ADNT, and 4-ADNT in their tissues. In all mussels deployed at the ground a body burden with 2-ADNT of 103.75 ± 12.77 ng/g wet weight and with 4-ADNT of 131.31 ± 9.53 ng/g wet weight was found. TNT itself has been found in six mussels with an average concentration of 31.04 ± 3.26 ng/g mussel wet weight. In the mussels positioned at one meter above the ground no TNT nor 2-ADNT could be detected, but 4-ADNT was found in all samples with an average concentration of 8.71 ± 2.88 ng/g mussel wet weight. This was the first study using blue mussels (*M. edulis*) as an active biomonitoring system for TNT and its metabolites 2-ADNT and 4-ADNT in a field experiment in a burdened area. Moreover, with this system, it was unequivocally proved that these toxic energetic compounds accumulate in the marine biota respective in the marine food chain, thereby posing a possible risk to the marine ecosphere and human health [23] (Figure 2).

In a second approach, the same experimental setup was used again in the Kolberger Heide at varying positions near a mound of around 70 moored mines [24]. In order to monitor any differences resulting from changing seasons, three exposure times were chosen. First exposure period: April–July 2016 (106 days); second exposure period: July–December 2016 (146 days); third exposure period: December 2016–March 2017 (92 days). Amounts of 4-ADNT in mussel tissue ranging from 2.40 ± 2.13 to 7.76 ± 1.97 ng/g (mussel wet weight) were found. Neither TNT nor 2-ADNT could be detected.

Considering seasonal differences, orientation and distances of the moorings to the mine mound no correlation between levels in mussel tissue was evident [24].

4 | “DON’T BLAST” – BLAST-IN-PLACE (BIP) OPERATIONS OF DUMPED WORLD WAR MUNITIONS IN THE OCEANS SIGNIFICANTLY INCREASE HAZARDS TO THE ENVIRONMENT AND THE HUMAN SEAFOOD CONSUMER

Relic underwater conventional and chemical munitions represent multiple hazards to human activities. As the precarious and first danger, uncontrolled self-detonations by ship traffic (civil and military), fisheries, water sports, cable constructions, wind farms and pipelines were primarily feared. Even more, after long periods of resting under water the sensitivity of the energetic materials increases due to deterioration of stabilizing components or recrystallization [25].

In order to prevent an endanger of today’s shipping traffic or the installation of pipelines and offshore plants by uncontrolled and unforeseen explosions, controlled blast-in-place (BiP) operations of these dangerous World War relicts are a common practice worldwide. For this purpose, contact donor charges are attached manually that initiate detonation of these munition items.

The German Baltic Sea is a marine area with large amounts of munitions deposited. For example, “Kolberger Heide” as a part of the Kiel Bight served as a

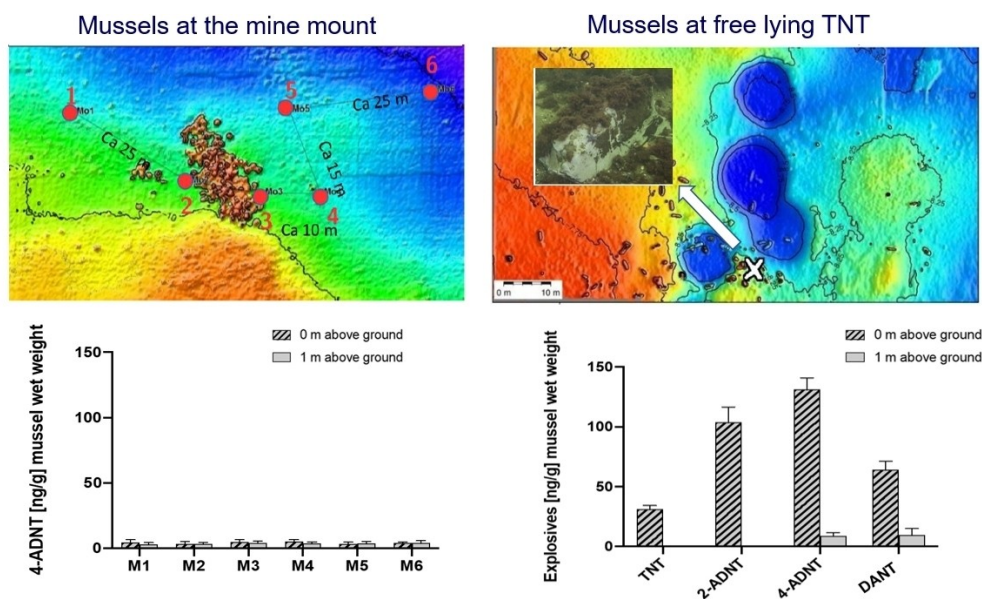


FIGURE 2 Energetic compounds accumulated in blue mussels.

dumping ground with at least 8 000 torpedo heads and 10 000 mines amongst other items such as ground mines and moored contact mines that were dumped after World War II [26–28]. Kolberger Heide also served for clearance of old munitions by BiPs, in case these munition items were considered as to form an acute threat. If located elsewhere in the Kiel Bight, munition items were also pulled to BiP places within Kolberger Heide for blasting. For instance, numerous 250–500 kg mines were intentionally detonated underwater via BiP [29, 30]. Unfortunately, these low-order detonation left substantial quantities of explosive material on the seafloor [31–33].

In the present field investigation blue mussels (*Mytilus* spp.) were placed as a biomonitoring system (Figure 2) in the area where BiP operations took place and where, by visual inspections by scientific divers, smaller and larger pieces of energetic materials were scattered on the seafloor [23, 34]. After recovery, the mussels were analyzed for TNT and its derivatives via gas chromatography and tandem mass spectroscopy.

Blue mussels exposed horizontally on the seafloor in 30 cm intervals in a region with larger and smaller pieces of scattered energetic material showed the highest concentrations of TNT metabolites in their tissues. The metabolite 4-ADNT appeared to be the most abundant energetic compound found in all samples, ranging between 70 to 145 ng/g mussel wet weight. The metabolite 2-ADNT varied between 22 and 55 ng/g mussel wet weight, while 2,4-DA-6-NT (2,4-diamino-6-nitrotoluene) ranged between 33 and 73 ng/g mussel wet weight. No TNT was found. Apparently, compared to corroding (but still encased) explosives [24], the free-lying energetic materials lying on the seabed, either in form of larger chunks or smaller pieces, led to an approximately 30–50-fold increased entry and concentration of energetic compounds within the exposed mussels [9]. Likewise, mussels exposed to explosives vertically in 30 cm intervals in the same region showed high levels of energetic chemicals in their tissue. While in an altitude of 10 cm from the ground level TNT, 2-ADNT and 4-ADNT were found at levels of around 40, 50, and 145 ng/g mussel wet weight, respectively, values decreased upwards the water column (at 40 cm: around 30 ng/g of 2-ADNT and 60 ng/g of 4-ADNT; at 70 cm: around 30 ng/g of 2-ADNT and 50 ng/g of 4-ADNT; at 100 cm: around 10 ng/g of 2-ADNT and 20 ng/g of 4-ADNT). No TNT was detected in blue mussels exposed in at 40 cm, 70 cm and 100 cm height.

The data obtained were then used to carry out a toxicological risk assessment. These data unequivocally demonstrate that low-order BiP operations of dumped

munitions in the sea lead to multiple increases in the concentration of TNT and its metabolites in the mussels when compared to similar studies at corroding but still intact mines. For this reason, BiP operations are explicitly criticized because of the resulting environmental hazards, which can ultimately even endanger human seafood consumers [9].

5 | CAN SEAFOOD FROM MARINE SITES OF DUMPED WORLD WAR RELICTS BE EATEN?

Toxicity studies suggest that chemical components of munitions are unlikely to cause acute toxicity to marine organisms. However, there is increasing evidence that they can have sublethal and chronic effects in aquatic biota, especially in organisms that live directly on the sea floor or in subsurface substrates. Moreover, munition-dumping sites could serve as nursery habitats for young biota species, demanding special emphasis on all kinds of developing juvenile marine animals.

Unfortunately, these chemicals may also enter the marine food chain and directly affect human health upon consuming contaminated seafood. While uptake and accumulation of toxic munition compounds in marine seafood species such as mussels and fish have already been shown, a reliable risk assessment for the human seafood consumer and the marine ecosystem is lacking and has not been performed until now. In this study, the first data and landmarks for a reliable risk assessment for humans who consume seafood contaminated with energetic compounds have been compiled, thereby following the general guidelines for a toxicological risk assessment of food as suggested by authorities.

Energetic chemicals such as TNT and its derivatives are not only known for their toxicity but also for their carcinogenicity [35, 36]. TNT has been tested for carcinogenicity in two-year bioassays in rats and mice. After administration of TNT via diet, carcinoma of the urinary bladder and hepatocellular neoplasms were observed in rats, while malignant lymphoma combined with lymphocytic and granulocytic leukemia in the spleen significantly increased in mice. US-EPA concluded that TNT is a possible human carcinogen (Class C) [35]. A study in humans found elevated levels of chromosomal aberrations in a subset of TNT-exposed workers who were also positive for *N*-acetyltransferase (*NAT1*) (rapid acetylator) and

exhibited the null glutathione-S-transferase (GST) T1 (*GSTT1*) or *GSTM1* genotype [14]. In Germany, TNT has been classified from the MAK Commission (The Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area) of the German Research Foundation (DFG) as belonging to Group 2 (“substances that are considered to be carcinogenic in humans”).

Generally, the risk assessment requires two different sets of data. Firstly, the hazard potential of a given chemical has to be determined by experimental investigations to define the toxicological endpoint in specific target tissues in animal and human studies (liver, kidney, blood, brain, eyes, skin). Secondly, the exposure has to be estimated, i.e., the nature and extent to which animals or human individuals are exposed to chemicals. From the combined assessment of the hazard potential and exposure, the actual risk is derived.

Maser and Strehse [9, 37] carried out a preliminary risk assessment for the consumption of Baltic sea blue mussels from the Kolberger Heide dumping area (Germany). Overall, this preliminary toxicological risk assessment of the mussels resulted in two different scenarios: 1) the regular consumption of mussels that were exposed in the immediate vicinity of corrosive mines (containing up to 5 ng explosives per g of tissue wet weight) [24] may not lead to an increase in the risk of cancer for humans. In contrast, 2) mussels that were exposed in the immediate vicinity of smaller and larger lumps of explosives such as hexanite contained significantly higher amounts of ECs (up to 350 ng/g of tissue wet weight) [9, 23] which would mean an increased risk of cancer with regular consumption of these mussels. It must be clearly emphasized here, however, that these preliminary calculations were carried out on the basis of a worst-case scenario where all energetic-related chemicals found in the mussels (2-ADNT, 4-ADNT, DANT) were considered to be as carcinogenic as TNT. Secondly, it was assumed that the average daily intake of fish and seafood of 39 g per person in Germany [38] consists only of these highly contaminated mussels. And thirdly, it was believed that affected people would consume these mussels on a daily basis throughout their lives (an average of 70 years) [9]. In other words, since the lifelong daily consumption of 39 g of mussels from the immediate vicinity of chunks of explosives lying on the sea bed is very unlikely, the consumption of mussels from the Baltic Sea can be described as safe from today's point of view [37].

6 | THE EXPLOSIVE TRINITROTOLUENE (TNT) INDUCES GENE EXPRESSION OF CARBONYL REDUCTASE IN THE BLUE MUSSEL (*MYTILUS* SPP.) AND MAY SERVE AS A NEW PROMISING BIOMARKER FOR SEA DUMPED WAR RELICTS

TNT is toxic to all organisms. In humans, TNT and metabolites are mutagenic and of relevant urothelial carcinogenicity as well as toxic to the liver, kidneys, eyes, skin, blood system and spleen [35]. Many studies have shown acute and chronic toxic effects on marine species like shrimp, fish, copepods, corals, amphipods and bivalves [12, 13, 39, 40]. Recently, Koske et al. [36] proofed the genotoxicity of TNT by using zebrafish embryos (*Danio rerio*). Schuster et al. [41] demonstrated negative multilevel biological effects in the Baltic blue mussel (*Mytilus* spp.). Interestingly, the mechanism of toxicity and carcinogenicity of TNT and its derivatives occurs through its capability of inducing oxidative stress and reactive oxygen species (ROS) in the target biota [35]. In HepG2 and HepG3 cells, ROS formation by TNT causes DNA damage and induces apoptosis [42]. Another study demonstrated that TNT induces oxidative stress in mouse, rat and human hepatoma cells [43].

Oxidative stress is able to perform peroxidation reactions of lipoproteins and lipids, thereby producing reactive carbonyl compounds which lead to modifications of DNA, lipids and proteins [44]. Detoxification by enzymatic carbonyl reduction of reactive carbonyl compounds to their corresponding alcohols therefore is a well working defence mechanism. This reaction is catalysed by enzymes from two different protein super-families, referred to as aldo-keto reductases (AKR) [45] and short-chain dehydrogenases/reductases (SDR) [46].

A subgroup of the SDRs, the carbonyl reductases, are of particular importance in the defense against oxidative stress. While neurodegenerative disorders in humans such as Parkinson's, Alzheimer's, Creutzfeldt-Jakob and Huntington's diseases are obviously aggravated by oxidative stress in the brain [47, 48], human CBR1 may contribute to neuroprotection due to its ability to metabolize reactive carbonyls derived from lipid peroxidation [49]. The role of CBR1 and CBR3 in the protection against ROS is underlined by their inducibility under conditions of oxidative stress [50, 51]. In addition, toxic chemicals with impact on the environment like paraquat and even treated sewage are able to

increase carbonyl reductase expression in *Oncorhynchus mykiss* and *Zoarcas viviparus* [52, 53]

In many biomonitoring studies, mussels are used as bioindicators for environmental pollution [54]. On the other hand, with regard to marine pollution by TNT and its metabolites, specific biomarkers are urgently sought to detect TNT contaminations in the environment and to perform a risk assessment both for the ecosphere and the human sea food consumer. Based on these facts that TNT exerts its toxicity through the development of oxidative stress and ROS, on the one side, and that the genes of carbonyl reductase are up-regulated in their expression by ROS, on the other, we examined whether the corresponding gene in the blue mussel is induced by TNT and whether carbonyl reductase is able to act as specific biomarker in this mussel species for TNT detection. To answer this question, identification and cloning of the carbonyl reductase gene in *Mytilus* spp. as well as laboratory exposure studies towards TNT and field experiments were performed [55].

We, finally, identified, the gene coding for carbonyl reductase from the blue mussel *Mytilus* spp. by a bio-informatics approach [55]. After cloning the gene, in both laboratory and field studies, it was shown that TNT induces a strong tissue and concentration-dependent induction of gene expression of carbonyl reductase in the blue mussel (Figure 3, Figure 4). Carbonyl reductase may thus serve as a biomarker for TNT exposure on a molecular level which is useful to detect TNT contaminations in the environment and to perform a risk assessment both for the ecosphere and the human seafood consumer [55].

7 | A TOOLBOX FOR THE DETERMINATION OF NITROAROMATIC EXPLOSIVES IN MARINE WATER, SEDIMENT, AND BIOTA SAMPLES ON FEMTOGRAM LEVELS BY GC-MS/MS

In order to assess contamination by submerged World War munitions as comprehensively as possible, with regard to present and future ecotoxicological consequences, the examination of large quantities of various abiotic and biotic samples for TNT, its metabolites, and other munitions components such as 1,3-dinitrobenzene and 2,4-dinitrotoluene is required. Gas and liquid chromatography techniques have been routinely used in the detection of nitro-organic explosives for over fifty years [56, 57]. Coupled to mass selective detectors, they do not only allow quantitative analysis, but also qualitative screening for unknown substances or unsuspected compounds in samples. Modern mass spectrometric techniques, such as triple quadrupole, time of flight, or Orbitrap mass spectrometry allow limits of detection, depending on the components, in the range of femto- to atto-gram per injection [58].

In the current study, sample preparation methods were developed and optimized for the detection of TNT, its metabolites 2- and 4-ADNT, and the explosive by-products 1,3-dinitrobenzene (1,3-DNB) and 2,4-dinitrotoluene (2,4-DNT). The goal was to develop a toolbox of methods which enable detection of these compounds in different abiotic and biotic samples at sub-nanogram levels. Depending on the expected concentrations, many samples can either be processed quickly, or purified and

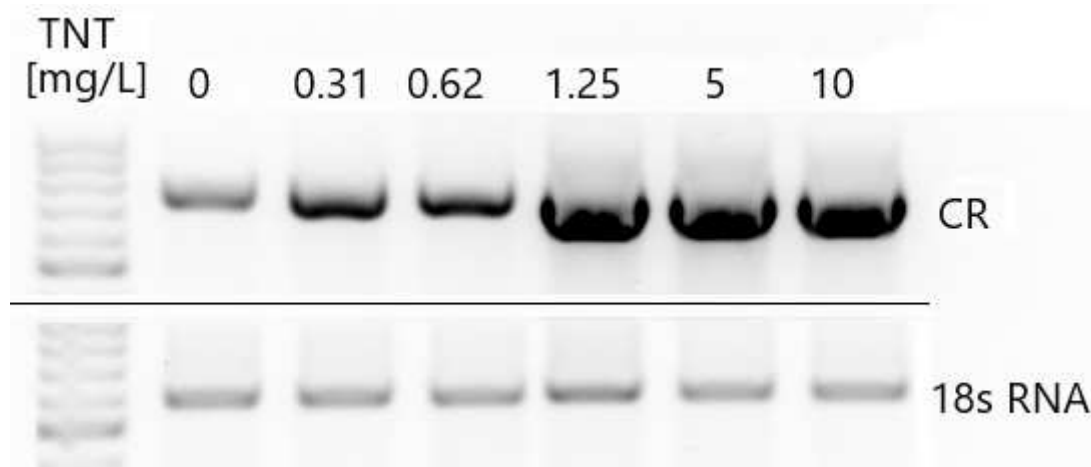


FIGURE 3 Induction of the carbonyl reductase gene in *Mytilus* spp. gills after a 96-hour treatment with TNT – representative agarose gel. Blue mussels were exposed to increasing concentrations of TNT (ranging from 0.31 to 10 mg/L) for 96 hours in laboratory aquarium studies.

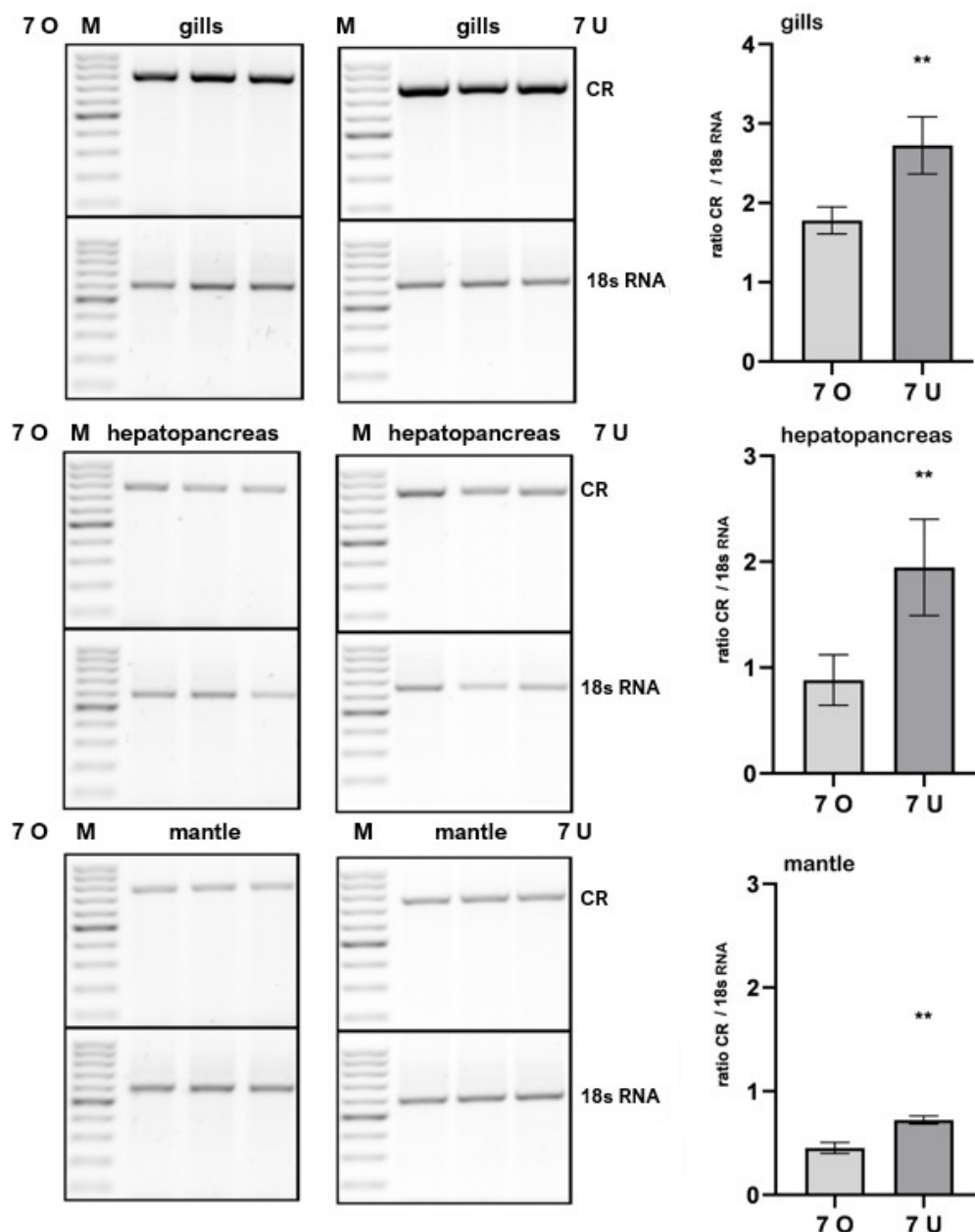


FIGURE 4 Induction of the carbonyl reductase in *Mytilus* spp. exposed in the munition dumping area Kolberger Heide. Mussels were exposed in a field study at the ground directly adjacent to a chunk of explosive material (7 U) or at a height of one meter (7 O). After recovery (58 days exposure) mussel tissues were analysed for carbonyl reductase expression. On the left-hand side: Agarose gels showing three replicates of PCR fragments per tissue. On the right-hand side: Densitometric analysis of the gels shown on the left-hand side (means with SD from $n=3$). Fold inductions were calculated from the carbonyl reductase expression relative to 18 s RNA as the housekeeping gene. Statistically significant changes are indicated by asterisks (** $p < 0.01$; unpaired t test with Welch's correction).

concentrated better through additional steps to achieve even lower detection limits [59].

As a result, reliable, fast, and sensitive determination of low energetic compounds concentrations in marine samples, such as water, sediment and marine biota are now possible [59] (Figure 5). The splitless injection method allows rapid measurement in only 6.25 min with limits of detection between 77 to 333 fg/ μ L. Combined with the applied extraction and sample preparation

methods, it can be adapted for multiple types of biotic and abiotic samples. For biota samples (e.g., *Mytilus* spp), in which the parent TNT concentration is lower compared to its metabolites, the large volume injection method allows detection limits that are even 10 times lower (of note: 8–47 fg/ μ L, corresponds 40 to 235 μ g on column) (Figure 5). Altogether, the methods described in the present investigation are among those with the lowest detection limits published to date and have

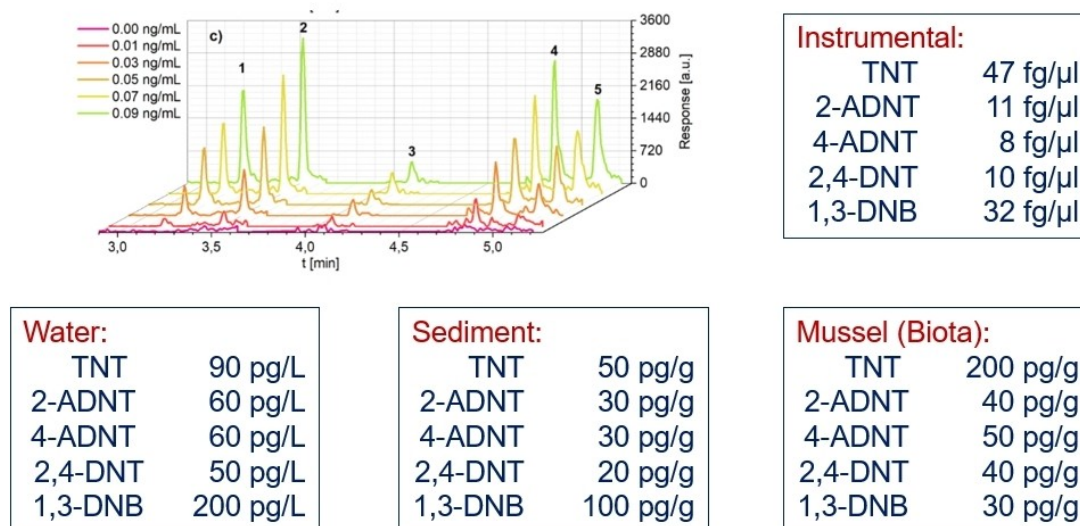


FIGURE 5 Chromatograms of the calibration standards used to determine the limits of detection (LoDs). Peaks are 1: 1,3-dinitrobenzene (1,3-DNB); 2: 2,4-dinitrotoluene (2,4-DNT); 3: TNT; 4: 4-amino-2,6-dinitrotoluene (4-ADNT); 5: 2-amino-2,4-dinitrotoluene (2-ADNT). The boxes give the LoDs for the respective matrices.

successfully been proven for their reliability on actual samples, i.e., samples obtained from different scientific research projects [60–63]. Furthermore, for the splitless method, using over 200 samples, the areas of an external 10 ng/mL standard remained constant for 1,3-DNB, 2,4-DNT, 2- and 4-ADNT, and only decreased by 50% for TNT. This allows automatic measurements over several days. The PTV method has been proven over a batch with 50 samples and has provided consistent results for 2- and 4-ADNT over the entire course of measurement.

8 | LONG-TERM TRENDS FOR BLUE MUSSELS FROM THE GERMAN ENVIRONMENTAL SPECIMEN BANK SHOW FIRST EVIDENCE OF MUNITION CONTAMINANTS UPTAKE

For more than 40 years, mussels such as blue mussels have been used as bioindicators in contaminant monitoring [21] because of their sedentary nature, worldwide distribution, easy accessibility, and resistance to certain pollutants. Natural substances and anthropogenic chemicals dissolved in seawater or bound to particles are taken up by mussels at a filtration rate of about 2–3 liters of seawater per hour and can be measured in the mussel soft body [21]. Recently, blue mussels have successfully been tested as indicators to monitor the leakage of explosive chemicals from munitions into the marine environment [54].

Since the 1980's, the German Environmental Specimen Bank (ESB) systematically collects, analyses and

archives blue mussels and 14 other species from the marine, freshwater and terrestrial compartment. The ESB is part of the German long-term monitoring infrastructure in support of environmental policy and regulation [64]. The samples can be used at any time to examine temporal trends of environmental stressors that were not known to be problematic at the time of sampling, could not be analytically detected, or were ignored. Due to the storage of the samples at temperatures below -130°C , the degradation and transformation processes within the tissues are stopped and the actual concentrations at the time of sampling can be analyzed retrospectively. Recent examples from retrospective analysis are metals [65], legacy and emerging chlorinated, brominated and fluorinated contaminants, and microplastics [66–68].

In the present study, we investigated the occurrence and trends of explosive chemicals and their metabolites in blue mussels from the annual collections of the German Environmental Specimen Bank sampled in the North Sea and Baltic Sea over the last 35 years [63]. This allowed not only to identify possible hazards to human health and ecosystems, but also to draw conclusions about the condition of the munitions lying in the sea bed of the sampling regions.

Blue mussels were sampled at three different locations – one at the coastline of the Baltic Sea and two in the North Sea region. The mussels analysed in this study have been collected since 1985 at the North Sea region Eckwarderhörne located in the Lower Saxony Wadden Sea, since 1986 close to the island of Sylt (Königshafen) located in the Schleswig-Holstein Wadden Sea (North

Sea), and since 1992 at Darßer Ort in the Bodden National Park of Western Pomerania (Baltic Sea); shown in Figure 6. According to the Ammunition Cadastre Sea (AmuCad <https://amucad.org/>), dumped munitions and UXOs can be assumed in all three sampling regions from both war and post-war activities, including designated dumping areas.

Samples were analyzed by GC-MS/MS for 1,3-dinitrobenzene (1,3-DNB), 2,4-dinitrotoluene (2,4-DNT), 2,4,6-trinitrotoluene (TNT), 2-amino-4,6-dinitrotoluene (2-ADNT), and 4-amino-2,6-dinitrotoluene (4-ADNT). The first signals indicating trace levels of 1,3-DNB were observed in samples from 1999 and 2000. EC signals were also found below the limit of detection (LoD) in subsequent years. From 2012 onwards, signals just above the LoD were detected. The highest signal intensities of 2-ADNT and 4-ADNT, just below the limit of quantification (LoQ) (0.14 ng/g dry weight and 0.17 ng/g dry weight, respectively), were measured in 2019 and 2020. This study clearly shows that corroding submerged munitions are gradually releasing EC into the waters and can be detected in randomly sampled blue mussels, even

though the concentrations measured are still in the non-quantifiable trace range [63].

As a conclusion, retrospective analysis of ESB mussel time series showed that environmental contamination from TNT based munitions is increasing along the sampling areas in the German North and Baltic Sea. The data necessitate further monitoring in the coastal regions that were battle grounds in both World Wars and dumping grounds for World War II munitions. Potential main sources are leaking energetic compounds after progressive corrosion of metal casings of dumped munitions over the last decades. More recent exposure may originate from targeted blasting of submerged munitions in the sea or on sandbanks, which should be avoided because they temporarily release considerable quantities of explosive compounds into the environment, and due to under water noise harm marine mammals. Relic munitions still lying in the sea should be recovered as soon as possible and safely destroyed on land or on a floating platform also because progressive corrosion of metal casings hinders their identification in the future. The retrieval of submerged munitions should therefore be

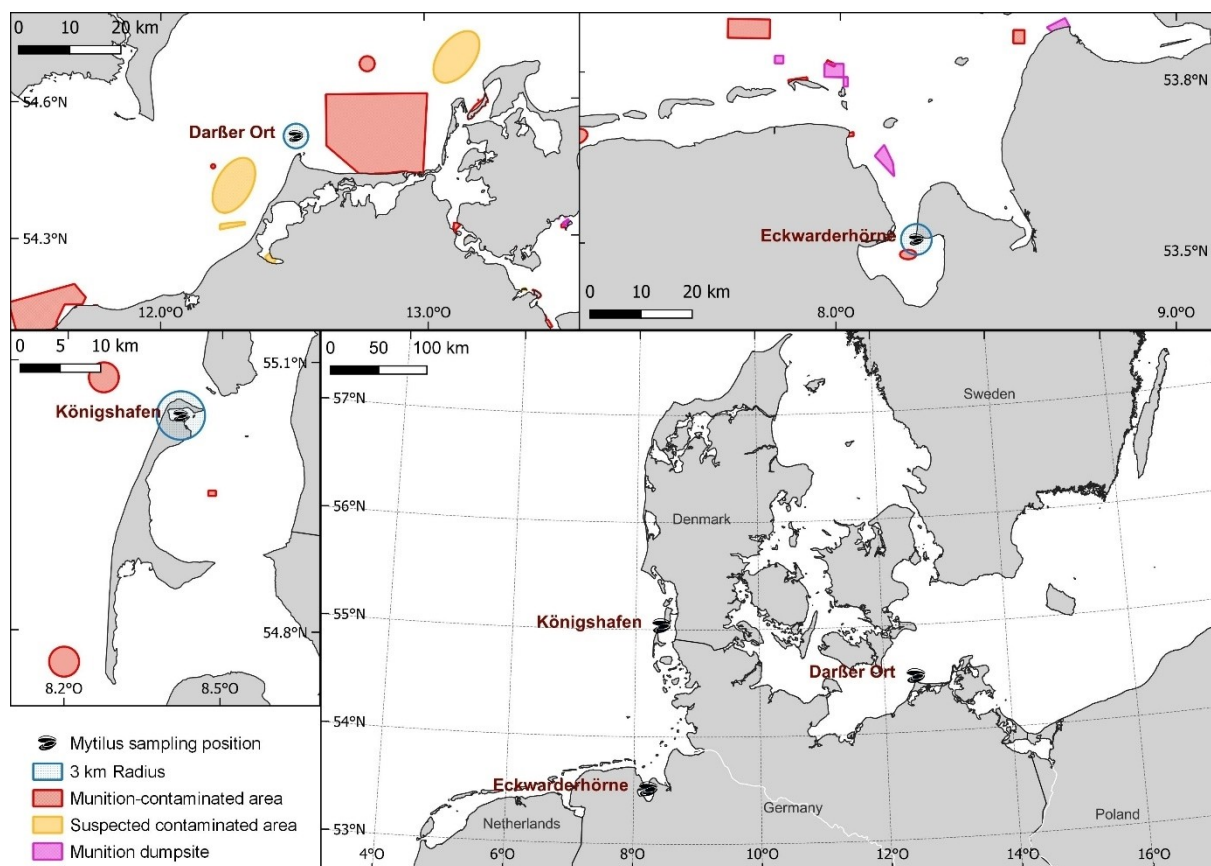


FIGURE 6 Map of the *Mytilus* sampling areas of the Environmental Specimen Bank: “Königshafen” on the island of Sylt (N55_2.3, E8_25.5), “Eckwarderhörne” (N53_31.2, E8_13.8) in the German North Sea, and “Darßer Ort” (N54_29.7, E12_31.6) in the Baltic Sea. The officially known dumpsites, munition-contaminated areas, and suspected areas are shown as colored spaces (data provided by amucad.org). A 3 km radius was drawn around the sampling positions to show their proximity to possible munition deposits.

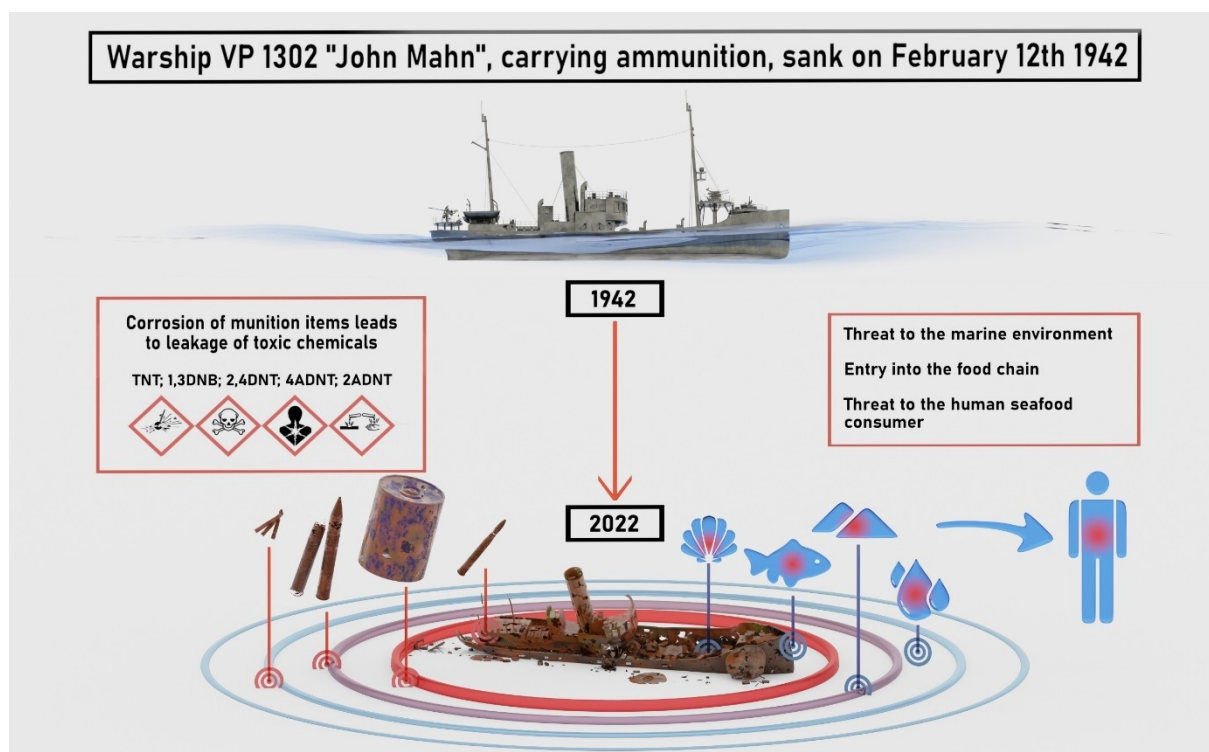


FIGURE 8 V 1302 (formerly „John Mahn”) was sunk in 1942 by an attack of British aircrafts. In 2022 the wreck and its surrounding was monitored for the leakage of energetic compounds from corroding munitions on board. This figure was published as a graphical abstract in [60]; Copyright Elsevier 2023.

integrated into the EU Zero Pollution Ambition and the goals of the Marine Strategy Framework Directive to remove these legacy contaminations from European seas and to avoid long lasting damage to ecosystem and human health.

9 | SUNKEN WAR SHIP WRECKS AS A THREAT TO THE MARINE ENVIRONMENT

Beside the intensive exploitation of the oceans by overfishing and the discharge of hazardous substances by rivers, the seas worldwide are currently threatened by an emerging source of pollution. Several million metric tonnes of all kinds of munitions dumped into the seas during and after the two World Wars (WWI and WWII) are corroding and leaking toxic energetic compounds (such as TNT and its metabolites) [1, 23, 24, 28, 34, 69] and chemical warfare agents [70] into the marine environment.

In addition, several thousand ships containing considerable amounts of munitions and fuels were lost at sea during WWI and WWII [71, 72]; an estimated 7800 or more ships (both military and commercial) and vessels were sunk during WWII [72]. Likewise, it was

common practice to dump redundant munitions together with the ships that carried them to the dump-site after the wars. As a result, the seas contain several thousands of wrecks and tons of conventional munitions and chemical warfare agents [28, 71–73].

Shipwrecks have generally been considered hazardous to shipping alone. Hence, their positions and minimum depths were often logged such that wrecks considered to be significantly dangerous were either removed or marked on the nautical charts, and with buoys, to ensure safe navigation. However, as is the case with dumped munitions casings, relevant wrecks that were sunk with munitions onboard have become of research and public interest [71, 72]. A recent report revealed the deterioration of wrecks over time [74]. Thus, in addition to the steel components of the vessels (required for stability of the wrecks), the munition casings on these ships are corroding and releasing their toxic chemicals into the marine environment [23, 24, 75]; thereby posing the same risk as sea-dumped mines, bombs, and torpedo heads to the marine environment and/or the human seafood consumer [76, 77].

To get a deeper inside into the problem of sunken war ships and their significance in environmental issues, a European Interreg North Sea Region research project was started in 2018 called North Sea Wrecks (NSW).

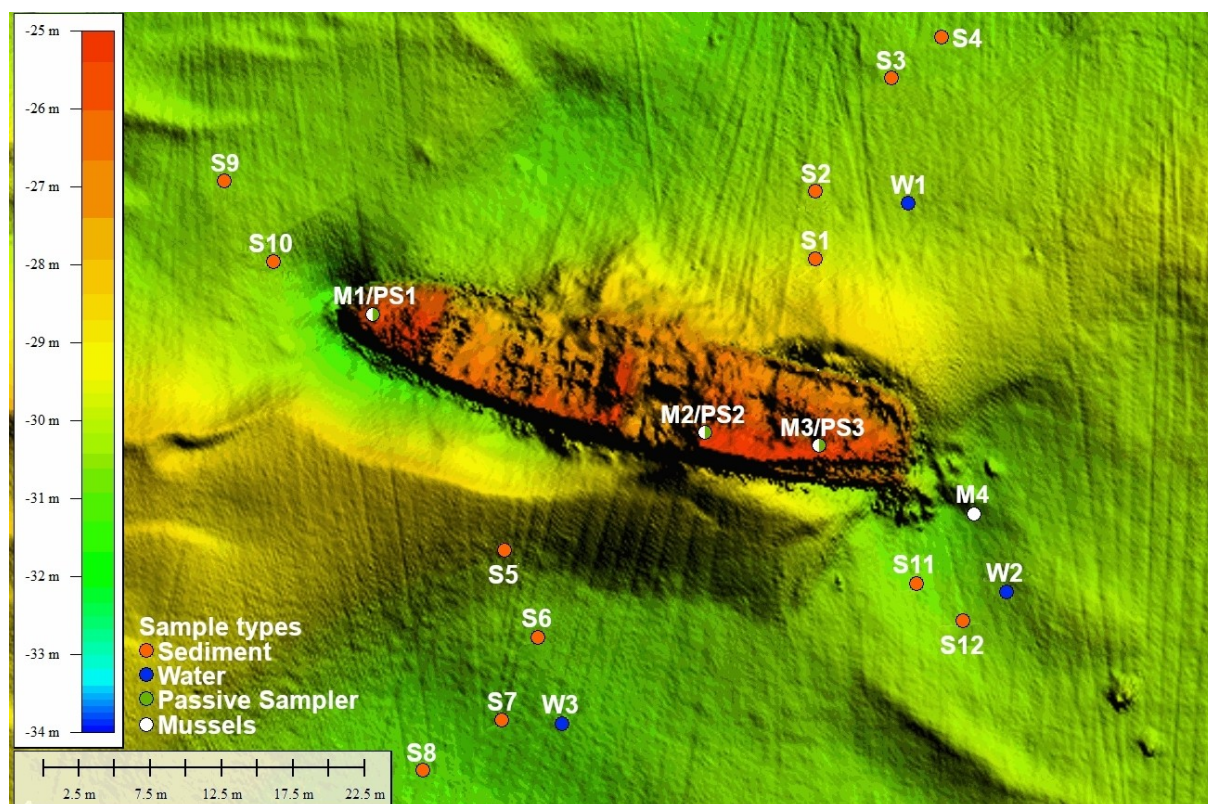


FIGURE 7 A multibeam image of the V 1302 wreck indicating sampling stations. Three water samples (W1–W3) and 12 sediment samples (S1–S12, collected in a cross-shaped pattern around the wreck) were obtained from the wreck site. Three nets containing mussels (M) and passive samplers (PS) were placed along a transect of the entire length of the wreck and labelled as M1/PS1–M3/PS3. The last net M4 was recovered with only the mussels inside, missing the passive sampler. This figure was published as Figure 4 in [60]; Copyright Elsevier 2023.

This project is a cooperation between research organizations in Belgium, the Netherlands, Germany, Denmark, and Norway, where each country is investigating several wrecks within their territorial waters and/or EEZ. The main focus munitions that are still onboard the sunken wrecks and their possible impact on the marine environment. The criteria for selection were the availability of historical information about the ship and the sinking scenario at site, the accessibility of the wreck to divers, and the suspected amount of munitions items onboard at the time of sinking. Based on these criteria the “Vorpostenboot V 1302” (civilian name John Mahn) at the Belgian coast was selected (Figures 7 and 8).

In the course of the project, the V 1302 wreck site and surrounding areas were mapped in great detail by scientific divers and a multibeam echosounder. Water and sediment samples were taken in a cross-shaped pattern around the wreck (Figure 7). To assess a possible entry into the marine food chain, caged blue mussels were exposed at the wreck, and fish (pouting), a sedentary species that stays locally at the wreck, were caught.

All samples were analyzed for the presence of TNT and derivatives thereof by GC-MS/MS analysis. As a

result, evidence was provided that sunken warship wrecks emerge as a point source of contamination with nitroaromatic energetic compounds leaking from corroding munitions cargo still on board (Figure 8). Not only these energetic substances were found in bottom water and sediment samples around the wreck, but also in the caged mussels as well as in fish living at the wreck. Fortunately, so far, the concentrations found in mussel flesh and fish filet were only in the one-digit ng per gram range thus indicating no current concern for the human seafood consumer. However, in the future the situation may worsen as the corrosion continues. From this study, it is proposed that wrecks should not only be ranked according to critical infrastructure and human activities at sea, but also to the threats they pose to the environment and the human seafood consumer [60].

10 | CONCLUSION

Submerged munitions from World War I and II are threatening all human activities in the oceans through

unwanted explosions. Furthermore, another important danger is that the munition shells are corroding, thereby leaking toxic energetic compounds (EC) into the marine environment. EC like 2,4,6-trinitrotoluene (TNT), its conversion products (2-ADNT, 4-ADNT, DANT) and other nitroaromatics are known for their toxicity and carcinogenicity. As for the risk assessment, numerous studies have measured EC like TNT and its metabolites in seawater, sediments, and marine organisms (reviewed in [6], [34], [37]).

Recently, Beck et al. [75] provided a comprehensive study on the accumulation of EC in field-collected biota. In the southwest Baltic Sea, EC were detected in more than 98% of organisms (median 1 ng/g). In most cases, concentrations of TNT and its transformation products ADNT and DANT were significantly higher in biota from the munitions dump site compared with other locations [75]. Generally, the concentrations of these compounds were low and, as far as available, no quality standards or limits were exceeded. Toxicity studies suggest that chemical components of munitions are unlikely to cause acute toxicity to marine organisms in this area.

However, there is increasing evidence that EC can have sublethal and chronic effects in aquatic biota, especially in organisms that live directly on the sea floor or in subsurface substrates. In more detail, they could have a lethal effect on the development of fish eggs, fish larvae and young fish. Furthermore, Strehse et al. [60] found an upregulation of carbonyl reductase gene in the blue mussel (*Mytilus* spp.) upon exposure to TNT, which is indicative for the fact that the mussels suffer from oxidative stress (Chapter 6).

Unfortunately, since EC may enter the marine food chain and directly affect human health upon consuming contaminated seafood, EC accumulation along marine trophic levels may be of relevance with regard to human food safety. However, as of today the consumption of seafood may generally be considered as being safe from today's point of view (Chapter 5).

ACKNOWLEDGMENTS

This work was financially supported by the German Federal Ministry of Education and Research (Project: "UDEMM" 03F0747B); by the Cluster of Excellence "The Future Ocean"; by the Schleswig-Holstein Ministry of Energy, Agriculture, the Environment, Nature and Digitisation (MELUND); by the State Government Schleswig-Holstein, by the German Environment Agency (UBA) with the FuE project "Trends und Daten zur Verbreitung von Munitionsresten in der Meeresumwelt" (TATTOO) (FKZ 3719 25 2990) and by the Interreg North Sea Region (Project: North Sea Wrecks - An

Opportunity for Blue Growth: Healthy Environment, Shipping, Energy Production and -transmission, J-No.: 38-2-13-18). Open Access funding enabled and organized by Projekt DEAL.

All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data may be requested via the authors.

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How to cite this article: E. Maser, T. Hartwig Bünning, J. S. Strehse, *Propellants, Explos., Pyrotech.* **2024**, 49, e202300181.
<https://doi.org/10.1002/prop.202300181>