Marine Pollution Bulletin 229 (2026) 119738

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Environmental risks from world war shipwrecks: Field-based biomarker evidence from caged mussels in the North Sea

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ARTICLE INFO

Keywords:
2,4,6- Trinitrotoluol (TNT)
Mytilus edulis
Biomarkers
Shipwrecks
Munition
Explosive compounds
Toxicity

ABSTRACT

The two world wars have left a toxic legacy in the oceans, which emerges as a new environmental problem. There are several thousand war wrecks around the world that are still partially or fully loaded with munitions. This issue is common in the waters of the North Sea. Decades of exposure to a saline environment promoted the corrosion of ship hulls and munition casings, allowing the munition compounds to be released. Many of these chemical compounds, especially the most significant explosive in quantity, 2,4,6-trinitrotoluene, better known as TNT, are toxic and threaten the marine environment and its organisms. While most studies on biological effects work with very high concentrations not found in the environment, this study focuses on field trials. To investigate the risk to the environment from wrecks loaded with munitions, experiments were carried out with blue mussels, *Mytilus edulis*, on three wrecks in the North Sea. Mussels were exposed in cages on shipwrecks for several weeks and examined for changes in the enzyme activity involved in detoxification processes, such as the Antioxidant-Defense System, and changes in the accumulation of metabolites. Significant differences can be seen in the activity of the enzymes involved in detoxification processes. There are different rates of accumulation of metabolic end products in the lysosomes of the mussel digestive gland, indicating negative impacts emanating from munitions-laden wrecks. The findings from the cage experiments underline the need for monitoring munitions remains and polluting shipwrecks in the oceans in the future to find the most effective solution for marine ecosystems worldwide.

1. Introduction

80 years after the end of the Second World War and over 100 years after the end of the First World War, both wars have left a heavy legacy. There are still an estimated 1.6 million tons of conventional munition present in German waters in the North Sea and Baltic Sea. Most of the munition was deliberately dumped at sea, especially after the Second World War (Böttcher et al., 2011; HELCOM, 2013). Following the Second World War, an estimated 300,000 tons of munitions were disposed of in the Baltic Sea and around 1.3 million tons in the German North Sea via "authorized disposal of hazardous ammunition by dumping at sea or demolition", which was performed from 1946 onwards (Böttcher et al., 2011). As part of the disposal measures, all types of munition were disposed, from individual cartridges for rifles to sea mines, bombs and torpedoes (Aker et al., 2012). A considerable amount of munition was also dumped in the waters of the North and Baltic Seas after the First World War. In addition, munitions also entered the seas through active warfare, exercises, or being jettisoned by returning bombers (Brenner et al., 2014). Most of these munitions are spread across seven dumping areas and 14 contaminated areas in the North Sea. However, in addition to deliberately dumped munitions, which is the vast majority, there are also ship and aircraft wrecks still carrying munition (Beck et al., 2018;

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https://doi.org/10.1016/j.marpolbul.2026.119738
Received 23 January 2026; Received in revised form 8 April 2026; Accepted 9 April 2026
Available online 17 April 2026

Böttcher et al., 2011; Monfils, 2005). The North Sea was the scene of many naval battles, especially during the First World War, and Bellamy (1991) described the North Sea even as a "wet grave". The recently finished research project "North Sea Wrecks – An Opportunity for Blue Growth: Healthy Environment, Shipping, Energy Production and -transmission"(NSW), led by the German Maritime Museum (DSM) came to the conclusion that at least 680 wrecks with a military background lie within the Belgian, Dutch, German, Danish and Norwegian waters, as well as the respective Exclusive Economic Zones (EEZ's), of the North Sea. Of these, there are at least 120 wrecks in German EEZ and territorial waters, 100 in Belgian waters, and at least 250 in Danish waters (see: https://www.dsm.museum/en/museum/exhibitions/north-sea-wrecks-exhibition/the-legacy-of-two-world-wars/what-quantities-of-wrecks-and-munitions-remain-in-the-north-sea). Wrecks of warships were often still partially or fully loaded with munitions at the time of their sinking. These wrecks including the remaining munition can pose a major threat to e.g. shipping, the construction of offshore facilities and the installation of underwater cables. Accidents involving munitions from the two World Wars occur from time to time. In the years between 1945 and 2014, at least 418 people died in the waters of the North Sea and Baltic Sea due to contact with munition and at least 680 were injured (Nehring, 2015).

The chemicals contained in the munitions pose again an "invisible" risk to the marine environment and are considered "slow disasters" (Knowles, 2014; Liboiron et al., 2018). Many of the substances in conventional munition are potentially hazardous and have a high ecotoxic potential. Munition compounds are often mutagenic, carcinogenic, and toxic to reproduction; therefore, they are classified as CMR substances (Böttcher et al., 2011). Nitroaromatic compounds such as 2,4,6 – trinitrotoluene (TNT), 2,4 - dinitrotoluene (2,4 DNT), and 1,3 - dinitrobenzene (1,3-DNB) are of particular concern to the marine environment (Gledhill et al., 2019), since the majority of munitions contain these chemicals. At the time of dumping or sinking, a large proportion of the explosive compounds were still in metal casings, which initially prevented the chemicals from interacting with the marine environment. However, years of storage in saltwater led to progressive corrosion of these containers, resulting in the release of the chemicals contained within the munitions (Beck et al., 2019).

In general, it is quite difficult to predict the current state of the munition after such a long time under water accurately. It varies from intact to completely corroded metal casings, with warfare agents being partially exposed to completely dissolved (Beddington and Kinloch, 2005). In addition to saltwater exposure, the progress of corrosion also depends on the varying hydrographic parameters such as oxygen content, near-bottom currents, and temperature (Koch, 2009). Furthermore, munition casings may also get damaged by fishing gear, construction works, or ship anchors, making it exceedingly difficult to estimate the condition of the munition. Corrosion, however, will continue to progress, promoting the introduction of the munition chemicals into our seas (Beck et al., 2019; Beddington and Kinloch, 2005; Strehse et al., 2017). Once exposed, explosives react depending on their chemical properties and the physico-chemical parameters of the surrounding environment. While some explosive compounds, such as ammonium nitrate, decompose within a few minutes, others have a much lower water solubility and, consequently, a much longer retention time in the marine ecosystem (Juhasz and Naidu, 2007). The most important explosive, 2,4,6-trinitrotoluene (TNT), with an estimated production volume of 800,000 tons throughout the Second World War, has a water solubility of 130 mg/L at 25 °C for distilled water and is one of the chemical substances that can persist in the marine environment for longer times (Johnson et al., 1994). TNT particles dissolve slowly, promoting long-term environmental retention (Darrach et al., 1998; Juhasz and Naidu, 2007). Once dissolved, TNT adsorbs to sediments but is also repeatedly detected in the water column (Böttcher et al., 2011). Biological transformation is the main degradation pathway of TNT in both water and sediment. TNT is reduced to 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) (Fig. 1) by bacteria via nitroductase (Juhasz and Naidu, 2007; Rosen and Lotufo, 2007). Furthermore, TNT may also be transformed photochemically into 2-ADNT and 4-ADNT in the water column (Fig. 2) (Lima et al., 2011; Lotufo et al., 2013).

The toxic properties of TNT were first noticed after the First World War in human medicine. Many of the workers employed in TNT factories developed gastritis, dermatitis, stomach cancer, aplastic anemia, or toxic hepatitis (Johnson et al., 1994; Lima et al., 2011; Lotufo, 2011). With a lethal concentration of 14.8 mg/KG body weight in humans, TNT is classified as highly toxic when ingested orally and is considered a potential human carcinogen (Böttcher et al., 2011). TNT is mainly absorbed through the skin and metabolized in the liver by nitroreductase to 2-ADNT and 4-ADNT and excreted via the kidneys (Johnson et al., 1994; Lima et al., 2011). The toxic effects of TNT and its derivatives, as well as other explosive compounds used in the two world wars, on marine organisms and the marine environment have been investigated in various studies (Nipper et al., 2001; Nipper et al., 2009; Wexler, 2014; Schuster et al., 2021; Strehse et al., 2017; Strehse and Maser, 2020). The acute toxicity of TNT for marine organisms is generally reported in the low mg/L range, with LC_{50} values typically between 0.5 and 10 mg/L depending on the species and life stage (Johnson et al., 1994; Nipper et al., 2001; Wexler, 2014. Exposure to the contaminants used in munitions is often a stress factor for marine organisms. The first response to an increased concentration of contaminants in the environment is a generic anti-stress response. Some organisms, such as mussels, can adapt to increased environmental concentrations of pollutants by actively changing their behavior, such as closing their shells and reducing the filtration rate (Kramer et al., 1989; Redmond et al., 2016, Schuster et al., 2021). However, when the concentration of pollutants is maintained over a longer period, these simple defense mechanisms can no longer adequately protect an organism, and biochemical changes, such as enzyme activities or an interruption of a normal metabolism, result. This increases energy costs for detoxification and reduce the energy available for vital processes such as growth or reproduction. Mussels, especially blue mussels, are excellent indicator organisms and have been used for decades to study pollution in marine ecosystems (Brenner et al., 2014; Farrington et al., 2016). In 2017, Strehse et al. demonstrated for the first time that blue mussels *Mytilus edulis* can also be used as sensitive biomonitoring systems for TNT, by showing the accumulation of TNT and its metabolites in transplanted caged mussels in the Kolberger Heide, a dumping site of conventional munitions in the Baltic Sea. Schuster et al. (2021) showed in laboratory experiments that exposure to TNT in mussels leads to metabolic disturbances expressed in the accumulation of metabolic end products in the lysosomes of the digestive glands. Due to their sessile properties and their ability to filter large quantities of the surrounding water, their biological response can be linked to the pollution load measured at a certain location.

Many studies dealing with the biological effects of explosives are carried out in the laboratory, under exposure concentrations that rarely occur in the environment (Schuster et al., 2021). There is, however, a lack of information on real, in situ levels of energetic components to create informed lab experiments to assess the hazardous potential of e.g. sunken warship loaded with munition. In addition, most exposure experiments take place over a few days and can therefore only indicate short-term reactions of organisms to pollutants. Yet, pollutants attack an organism at different levels. While the first reactions can often be seen at the molecular level, exposure to pollutants over months or years can cause damage at the organ level, depending on the pollutant. As a result, seemingly non-lethal doses of chemicals can compromise an organism over time. One of the first reactions to environmental pollution is activating the antioxidant defense system (ADS). An important component of the cellular ADS against reactive oxygen species (ROS) is various enzymatic antioxidants that can eliminate these oxygen radicals by converting them into less toxic compounds (Chainy et al., 2016;

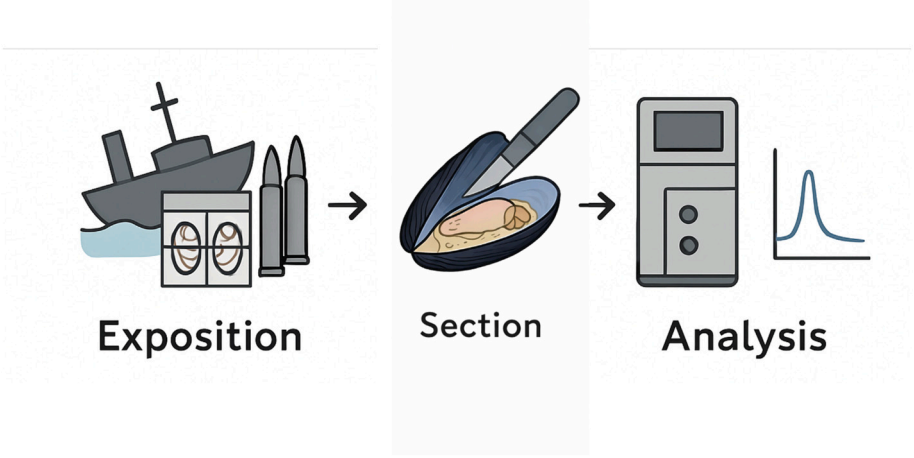

Fig. 1. Schematic overview of the workflow for investigating munition residues in *Mytilus edulis*. Following exposure of mussels to areas potentially contaminated by sunken munitions, tissue sampling is performed, including the mantle, digestive gland, and gills. Finally, the samples are analyzed to determine contaminant concentrations and to assess potential biological effects in the mussels.

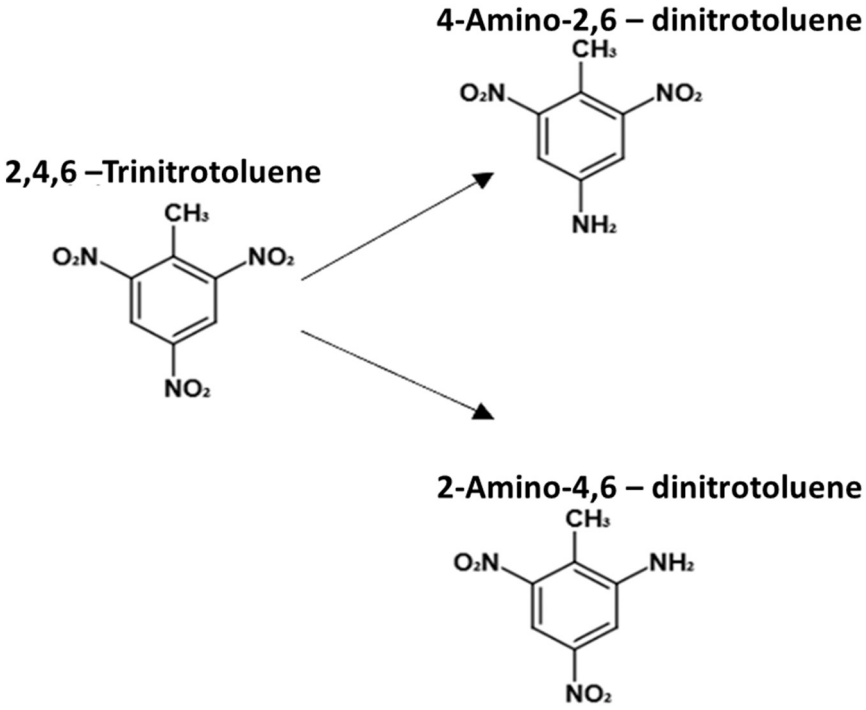

Fig. 2. Structural formula of 2,4,6-trinitrotoluene (TNT) and its two main metabolization products 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT). Structural formulas were created using a structural formula generator.

Ighodaro and Akinloye, 2019; Viarengo and Nott, 1993). Enzymes such as catalase, glutathione S-transferase and superoxide dismutase are some of the enzymes that are part of the ADS (Chainy et al., 2016; Ighodaro and Akinloye, 2019; Viarengo and Nott, 1993; Yu, 1994). Studies show that oxidative stress alters the enzyme activity of catalase and glutathione S-transferase (Bhagat and Ingole, 2016; Valon et al., 2013; Viarengo et al., 2007). If the ADS can no longer neutralize the increasing ROS concentrations successfully, irreversible damage occurs in an organism. Oxidative stress negatively affects the organism by damaging lipids, proteins, and DNA (Livingstone, 2001; Viarengo et al., 2007).

Studies show a link between the increased accumulation of lipofuscin, an aging pigment, in the lysosomes of the digestive gland of mussels and in the liver of fish and oxidative damage caused by various anthropogenic pollutants (Au, 2004; Krishnakumar et al., 1994; Shaw et al., 2019), Exposure to pollutants can also lead to pollutant-induced lipidosis (unwanted fat build up), triggered by lysosomal autophagy of lipid droplets (Krishnakumar et al., 1994; Lowe, 1988; Moore, 1988). This can be observed by the pathological accumulation of lipids in the vacuolar system of cells (Da Ros et al., 2011; Krishnakumar et al., 1994; Lowe, 1988; Moore, 1988, 1991; Viarengo et al., 2007). In addition to the accumulation of metabolites, the energy status of an organism can provide information about its health status, for example, by quantifying the glycogen content (Ansaldo et al., 2006). Pollutant exposure leads to increased energy consumption through the activation of defense mechanisms, which consequently leads to a reduction in stored glycogen. Glycogen content is used in environmental monitoring as changes in content correlate with the degree of chemical exposure (Ansaldo et al., 2006).

Both in vivo and in vitro studies showed the formation of ROS after

exposure to TNT. Therefore, TNT and its metabolites are suspected to cause oxidative stress in exposed organisms (Adomako-Bonsu et al., 2024). Schuster et al. (2021) already demonstrated the response of multiple biomarkers listed above to dissolved TNT in laboratory settings. In this study, a multi-biomarker approach was carried out on caged mussels to investigate the biological effects of shipwrecks from the First and Second World Wars. To this end, common mussels (*Mytilus edulis*) were exposed in cages for several weeks directly on war wrecks in the North Sea in order to estimate pollutant effects of dissolved explosive emanating from the shipwrecks and to fill missing gaps in the literature.

We hypothesize that even environmentally relevant concentrations of explosive compounds (ECs), in the range of ng/L to a few μg/L, can exert measurable negative effects on marine organisms. Specifically, we expect that caged mussels (*Mytilus edulis*) exposed directly on WWII and WWI wrecks will show detectable cellular responses, such as changes in lysosomal metabolism, activation of antioxidant defense systems, or accumulation of stress-related biomarkers, even at these low concentrations.

Furthermore, our study aims to fill gaps in the existing literature by focusing on field-based investigations under environmentally realistic conditions. Unlike most previous laboratory studies that employed high, short-term exposure concentrations, we consider longer exposure durations at low, environmentally relevant concentrations. This approach allows us to evaluate whether subtle, sub-lethal effects occur at the cellular level, providing a more accurate picture of chronic stress responses in marine organisms exposed to munition-derived contaminants.

2. Material and methods

2.1. Origin and handling of test organisms

Mussels (*Mytilus edulis*) for the exposure at SMS MAINZ and UC-30 were collected on the island of Sylt in the North Sea, on 21.03.2023, a few days before the expeditions started. The water temperature on the isle of Sylt was 4 °C.After the collection, mussels were transported cool and dry in a cool box to the laboratory where mussels were measured using a vernier caliper. Only specimens of 5–7 cm total length were kept. They were kept in natural sea water at constant temperature like measured at the sampling site until the expedition started. Onboard mussels were distributed in different cages for in situ deployment. For the KW58 study, mussels were collected at 5 m depth from a pylon in the Belwind offshore windmill farm. Mussels were kept in natural seawater until deployment the next day.

2.2. Study sites

In order to investigate the biological effects of wrecks loaded with munitions on *M. edulis*, experiments were carried out on the three named wrecks. When selecting the wrecks, care was taken to ensure that (1) the wrecks had been identified beyond doubt, (2) historical sources about the ship biography regarding the armament, cargo etc. and the sinking scenario were available, (3) wreck biography as comprehensive as possible is available and (4) the wrecks were easily accessible for divers. After considering the criteria, the wrecks of the SMS MAINZ in German Waters, KW58 HENDRICUS in Belgian waters, and the UC30 in Danish waters were chosen (Table 1) (Fig. 4). It should be noted that due to a lack of information about a ship, which is often caused by an incomplete wreck biography and sometimes imprecise ship biographical details, a more or less reliable estimation about the amount of preserved munition and explosives could only be given for the time of the ship's sinking (Grassel, 2022). The initial planning for the experiment included a reference area, at the natural reserve at Borkum Riffgrund. A lander equipped with mussel was placed there, however, got lost and samples could not be taken.

2.2.1. KW58 HENDERICUS

The KW58 HENDERICUS was a Dutch fishing vessel (motorized herring logger) (Fig. 3a) that played a brief role at the end of World War II. Seized by the German Kriegsmarine, she was used to transport munitions, though probably not fully modified for the task. Measuring 30.39 m in length and 6.68 m in width, the HENDERICUS possessed a cargo carrying capacity of 103.82 net register tonnage. Her service came to an end on the night of November 27, 1944, when she sank in the central part of the Belgian North Sea. Today, the wreck of the KW58 HENDERICUS rests partially buried at a maximum depth of 29 m. Evidence of her function remains visible on her deck, where some of the hazardous cargo, specifically mines and mortars, can still be found. The exact quantity of munitions still on board the sunken vessel, however, remains unknown (Termote and Termote, 2009).

2.2.2. SMS MAINZ

The SMS MAINZ was a light cruiser of the Kolberg-class (Bergmann et al., 2024) (Fig. 3b). The ship was part of the 4th torpedo boat fleet and deployed from August 1914 to protect the German Bight (Hildebrand et al., 1981). The ship had a length of 130 m and was equipped with twelve quick loading guns (cal. 10.5 cm), five torpedoes (type C/06) and two machine guns (cal. 8 mm). In August 1914, the SMS MAINZ fought in the Battle of Heligoland Bight, where she received many grenade hits and a torpedo hit. The ship finally sank after several of the German sailors were saved by a British destroyer. Today, the wreck lies at a depth of around 30 m. At the time of sinking, an estimated amount of 1.500–3.400 kg of explosives within the remaining munition were still on board the ship (Grassel, 2022).

2.2.3. UC-30

The UC30 was a type UC II double-hull submarine of the German Imperial Navy and capable of laying mines (Fig. 3c). It was part of the 1st squadron in Heligoland (Jakobsen, 2018). The UC30 had a total length of 51,12 m and could carry 18 mines (type UC200). The boat was also armed with seven torpedoes (type G6 or G7) and a small quick-loading gun (cal. 8.8 cm). In March 1917, the submarine was sent through the English Channel to Ireland, where it was ordered to lay mines. Due to an engine failure, the boat had to turn back without succeeding its mission. On its way back to Heligoland UC30 ran aground a sea mine in a British minefield off Jutland, presumably on April 20th, 1917. Today the wreck lies at a depth of ca. 24 m, about 10 km off the Danish coast. At the time of sinking, it can be estimated that the submarine was carrying around 3.120–4.812 kg of explosives within the remaining munition.

Table 1
Historical and technical data of the selected shipwrecks.

Wreck	Class	Munition cargo	Time of sinking	Munition left [kg]	Position
KW58 HENDERICUS	Fishing vessel	Mines, Mortars, artillery shells	27.11.1944	unknown	51°23.'N 02°35.'E
SMS MAINZ	Light cruiser Kolberg class	Artillery shells cal. 10.5 cm, torpedoes	28.08.1914	1.500–3.400	54°01.'N 06°43.'E
UC-30	UC II type minelaying submarine	Mines, torpedoes, artillery shells 8.8 cm	21.04.1917	3.120–4.812	55°51.'N 07°95.E

Fig. 3. Historical photographs of the wrecks examined in this study. a. KW58 HENDERICUS (copyright IJmuider Zee- en Havenmuseum) b. SMS MAINZ (copyright archive German Maritime Museum); c. UC30 (copyright Deutsches U-Boot Museum Cuxhaven-Altenbruch).

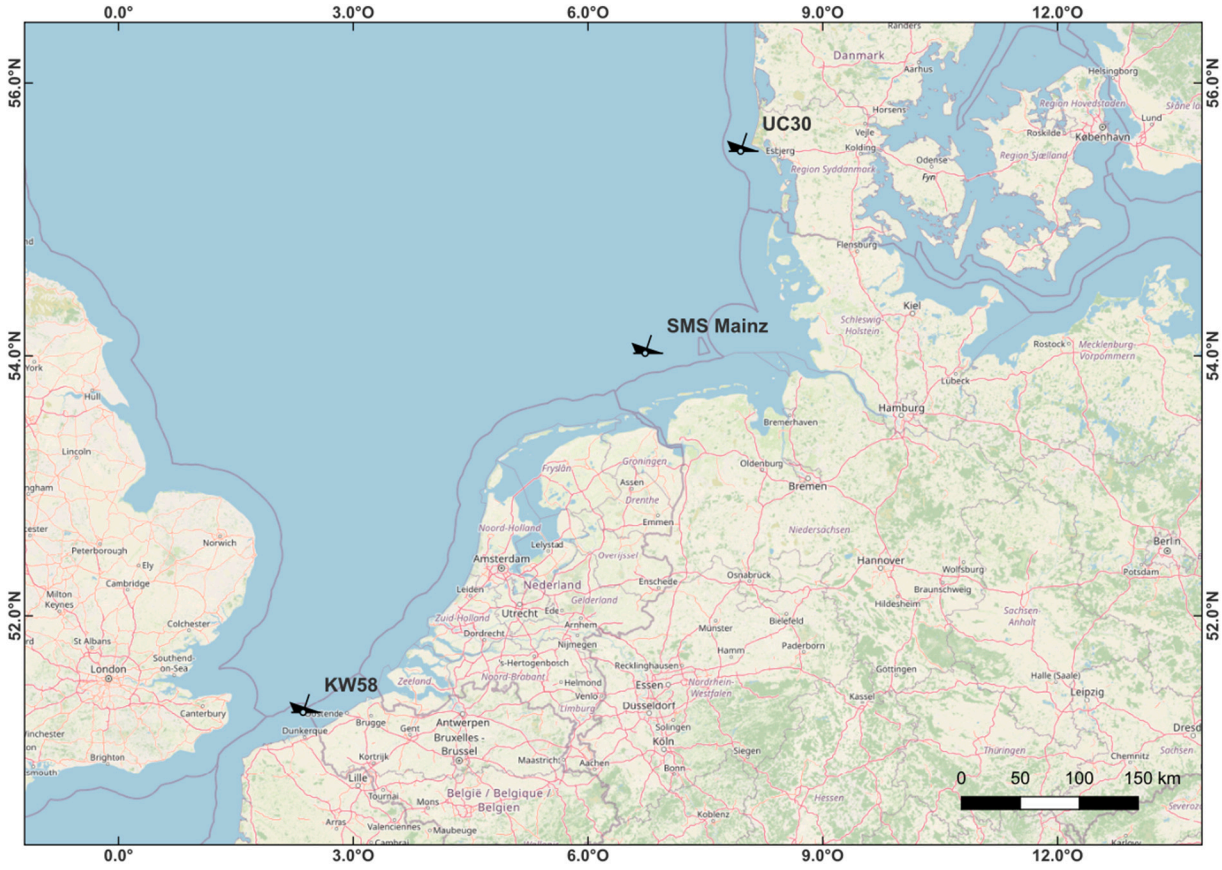

Fig. 4. Map of the North Sea indicating the locations of the investigated ship wreck sites (ship symbols).

2.3. Experimental setup

After selecting the wrecks, three suitable locations were identified on each ship for deployment of the exposure cages. On SMS MAINZ and KW58, two cages were deployed at each location using remote-operated lander systems: one cage containing approximately 50 mussels and a second cage housing two passive samplers to monitor dissolved TNT concentrations in the surrounding water. The cages were positioned as close as possible to the wreck at the bow, midship, and stern. At KW58, one mussel cage was placed adjacent to visible munition remnants.

On UC-30, the same experimental setup was applied at three mine wells located in the stern section of the wreck. In contrast to SMS MAINZ and KW58, the cages at UC-30 were attached directly to the wreck structure rather than deployed on landers. The mussel cages were positioned directly above exposed explosive material, while the second cages contained the passive samplers for TNT measurements.

After a minimum exposure period of 11 weeks, the deployments were recovered. At SMS MAINZ, retrieval was carried out using the research vessel Uthörn. One lander could not be recovered and was therefore excluded from further analyses. The mussels were dissected immediately on board and subsequently frozen. At KW58, recovery was conducted using the research vessel Simon Stevin; mussels were dissected directly in the onboard laboratory and shock frozen thereafter. At UC-30, recovery was performed with the assistance of the Danish Navy using a rigid inflatable boat. Following retrieval, the mussels were transported directly to the museum facilities, where they were dissected without delay and subsequently shock frozen.

2.4. Water and sediment sampling

To assess the occurrence and concentrations of explosive-related compounds in the marine environment, water and sediment samples were collected at all sampling sites. Water samples were obtained using a CTD probe (Sea-Bird; conductivity, temperature, depth). Sampling was

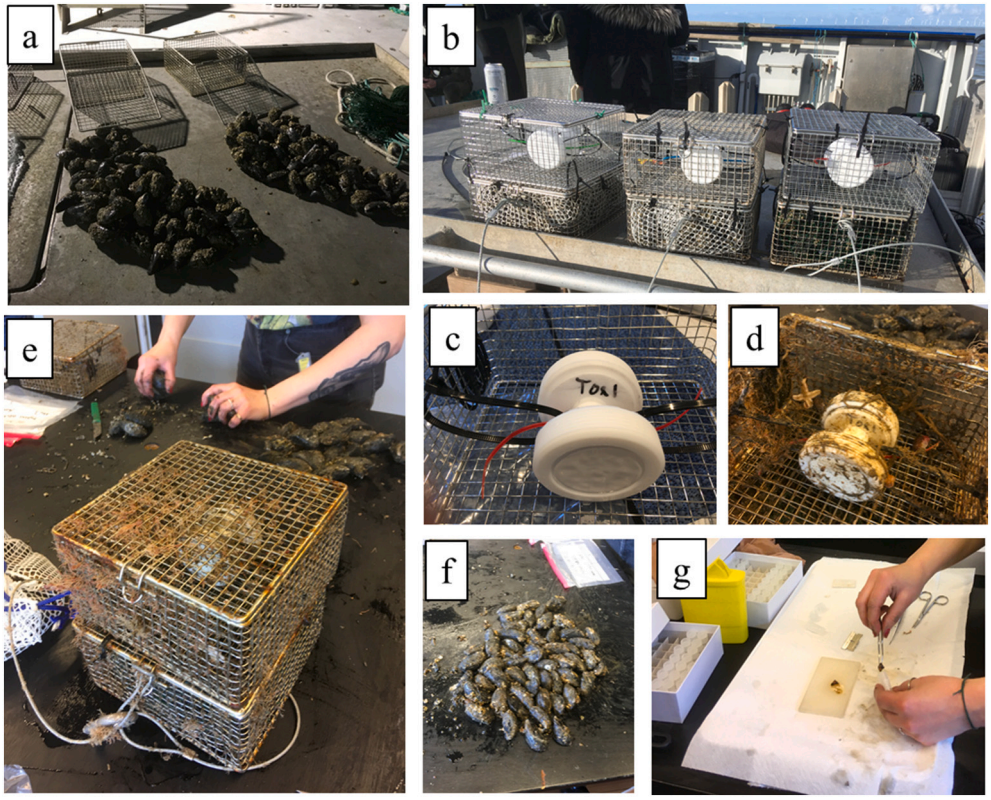

Fig. 5. Photos from deployment (30 March 2022) and recovery (5 July 2022) of mussels and passive samplers at UC30. a) Cages with mussels are prepared onboard RV Aurora. b) Fully packed mussel cages (bottom) and passive sampler cages (top). c) Passive sampler prior to deployment. d) Passive sampler upon retrieval 14 weeks later. e-f) Mussels retrieved from the cages. g) Mussel dissection.

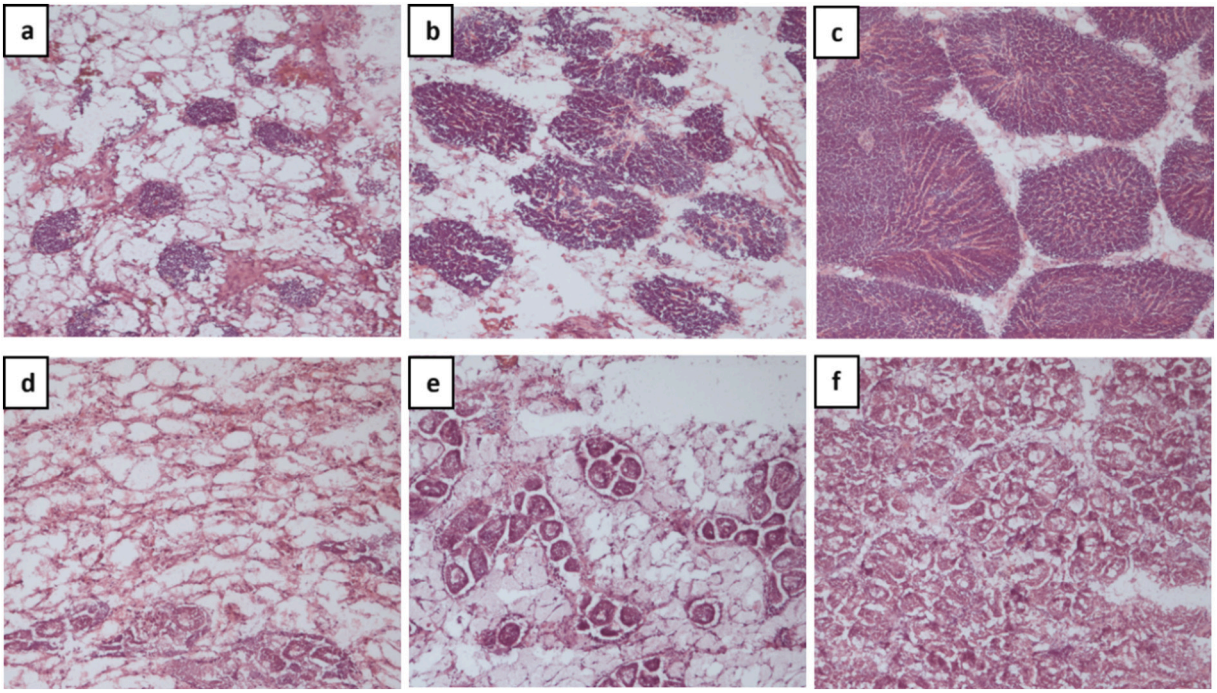

Fig. 6. Images of mussel gonad tissue samples stained with hematoxylin-eosin to determine sex and gonad status (100-fold magnification). Pictures show examples of the classifications: Spawning/recovering (**a**/**d**), growing/ premature male (**b**) and female (**e**), and mature male (**c**) and female (**f**) gonads.

conducted at defined water depths (seafloor, 5 m above the seafloor, 10 m above the seafloor, and surface). At KW58, water samples were only taken at depth (3 m above the seafloor). For each depth, two samples of 1 L each were measured using a graduated beaker, transferred into sterile infusion bags, spiked with 200 μL of a 250 ng/mL solution of $^{13}C^{15}N$-TNT in acetonitrile (ACN), and connected to CHROMABOND

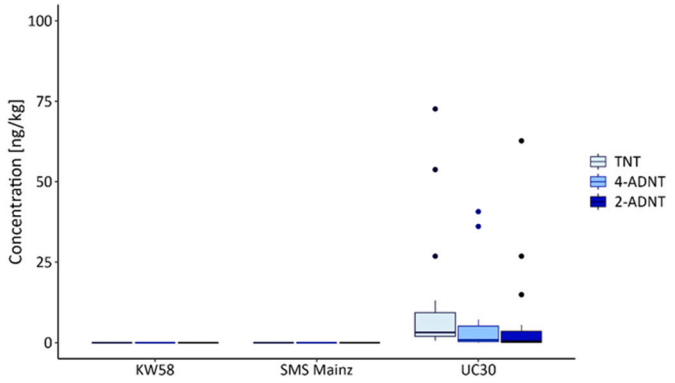

Fig. 7. Concentrations in the water surrounding the wrecks. Water samples at the KW58 wreck site were taken at a distance of 50 m to the wreck, while samples at the SMS MAINZ and UC30 were taken directly at the ships hull. Data were visualized using standard boxplots generated in RStudio with the package ggplot2, where the box represents the interquartile range (IQR), the horizontal line indicates the median, whiskers extend to 1.5 × IQR, and potential outliers are displayed as individual points.

Easy solid phase extraction (SPE) columns (80 µm, 3 mL, 200 mg). The bags were then suspended on board to allow the water column to pass through by gravity.

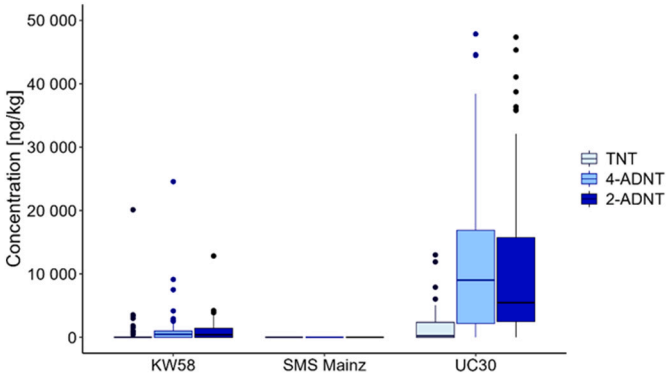

Fig. 9. *Mytilus edulis* tissue concentrations. No EC were detected in the mussels exposed to the SMS MAINZ. Data were visualized using standard boxplots generated in RStudio with the package ggplot2, where the box represents the interquartile range (IQR), the horizontal line indicates the median, whiskers extend to 1.5 × IQR, and potential outliers are displayed as individual points.

Sediment samples were taken near the wrecks as well as in the reference area using a Van Veen grab sampler. At each site, several sediment samples were collected (check Table 2). From each grab, sediment was transferred into zip-lock bags and stored at −20 °C until further processing.

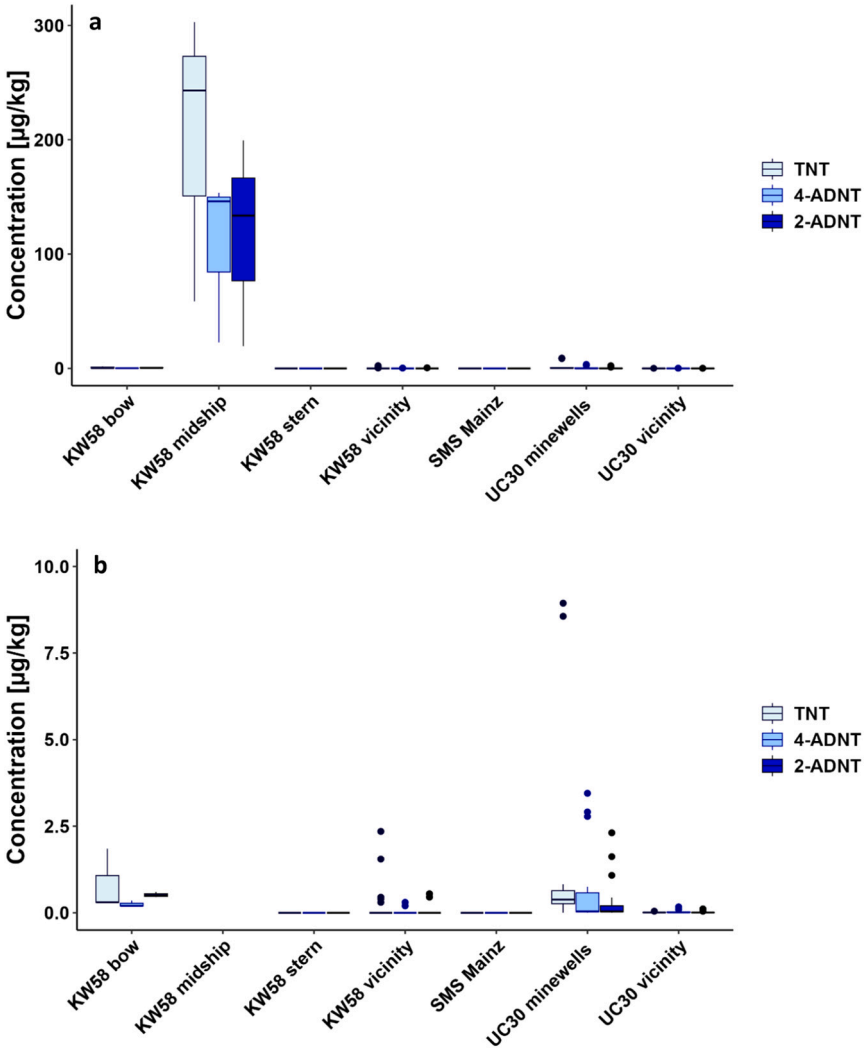

Fig. 8. Sediment concentrations in the surroundings of the wrecks. Data were visualized using standard boxplots generated in RStudio with the package ggplot2, where the box represents the interquartile range (IQR), the horizontal line indicates the median, whiskers extend to 1.5 × IQR, and potential outliers are displayed as individual points.

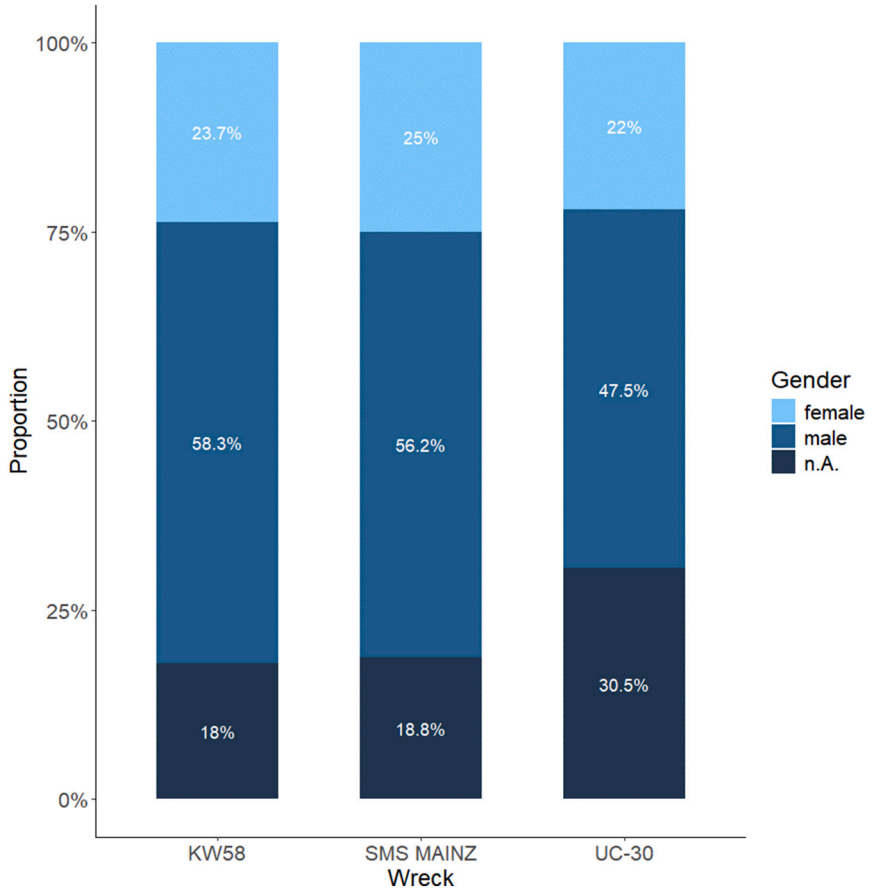

Fig. 10. Percentage distribution of the sexes of *Mytilus edulis*.

2.5. Chemical analysis

2.5.1. Sample preparation

Water samples were prepared according to Bünning et al. 2021. SPE-Columns were thawed, placed on a solid phase extraction vacuum chamber, dried in a mild vacuum (0.5 h) and eluted with 4 mL ACN. Eluates were concentrated to 1000 µL in a rotary vacuum concentrator, given to 1.5 mL amber vials and stored at −20 °C.

Sediments were tawed, transferred to single use beakers, frozen again (−20 °C) and lyophilizated for 18–24 h until full dryness. Ten grams of sediment were mixed with 25 mL ACN and 200 µL of an 250 ng/mL 13C15N-TNT-Solution in ACN as internal standard, vortexed for one minute, sonicated at room temperature for 1 h and centrifuged for 0.25 h at 4100 rpm/10 °C. Supernatants were transferred to 250 mL beakers, made up with 125 mL 18.2 MΩ water and transferred to 3 mL SPE-columns in a mild vacuum. After drying, columns were eluted with 4 mL ACN, eluates were concentrated to 1000 µL in a rotary vacuum concentrator, given to amber 1.5 mL vials and stored at −20 °C.

Mussel samples were prepared by the protocol for lyophilizated mussel tissue, published in Bünning et al., 2021. Mussels were tawed, deshelled, homogenized, frozen to −20 °C and lyophilizated overnight. After determination of the dry weight, 0.5 g (if available, otherwise the whole amount) were weighted into 15 mL tubes and made up with 5 mL ACN and 40 µL of 250 ng/mL 13C15N-TNT-Solution in ACN as internal standard. Samples were vortexed for 30 s, sonicated at room temperature for 0.25 h and centrifuged for 0.25 h. Supernatants were transferred to 50 mL tubes, made up with 30 mL ultrapure water and 3 mL SPE-columns in a mild vacuum. After drying, columns were eluted with 4 mL ACN, eluates were concentrated to 1000 µL in a rotary vacuum concentrator, given to amber 1.5 mL vials and stored at −20 °C.

2.5.2. Sample analysis by GC–MS/MS

Samples were analyzed with a Thermo Scientific TRACE 1310 gas chromatograph, coupled to a TSQ 8000 EVO triple quadrupole mass spectrometer with electron ionization source was used in secondary reaction monitoring (SRM) mode, as described in Bünning et al., 2021. All samples were measured twice. Standard dilutions of the analytes to be examined in acetonitrile were used for external quantification. The recovery of TNT was calculated using the internal standard $^{13}C^{15}N$-TNT. It was 71.7 ± 23.3% for water, 94.9 ± 12.4% for sediment, and 83.4 ± 28.7% for mussels. The TNT peak areas were corrected accordingly. Matrix specific detection and quantification limits (Table 3) were determined with spiked blank samples according to EUR 28,099 EN.

2.6. Mussel dissection

Following the exposure period, mussels were carefully retrieved from their deployment cages. Upon collection, each specimen was visually inspected for mortality and overall condition. Only healthy, intact mussels were selected for further processing. To prevent tissue degradation and analyte loss, the mussels were immediately transported to the laboratory under cooled, controlled conditions, ensuring minimal stress and maintaining sample integrity. In the laboratory, mussels were dissected under clean conditions to avoid cross-contamination between specimens and tissue types (Fig. 5) (Binder et al., 2025). Tissue samples were prepared in the laboratory for the following biomarkers: histological conditions, activity of the enzymes catalase (CAT) and gluthation s-transferase (GST), lipofuscin assessment (LIP), neutral lipids (NL) and glycogen (GLY). Dissection tools were thoroughly cleaned and, where appropriate, rinsed with ultrapure water between samples. The mantle, digestive gland, and gills were carefully excised from each individual, following standardized protocols for bivalve tissue sampling. To

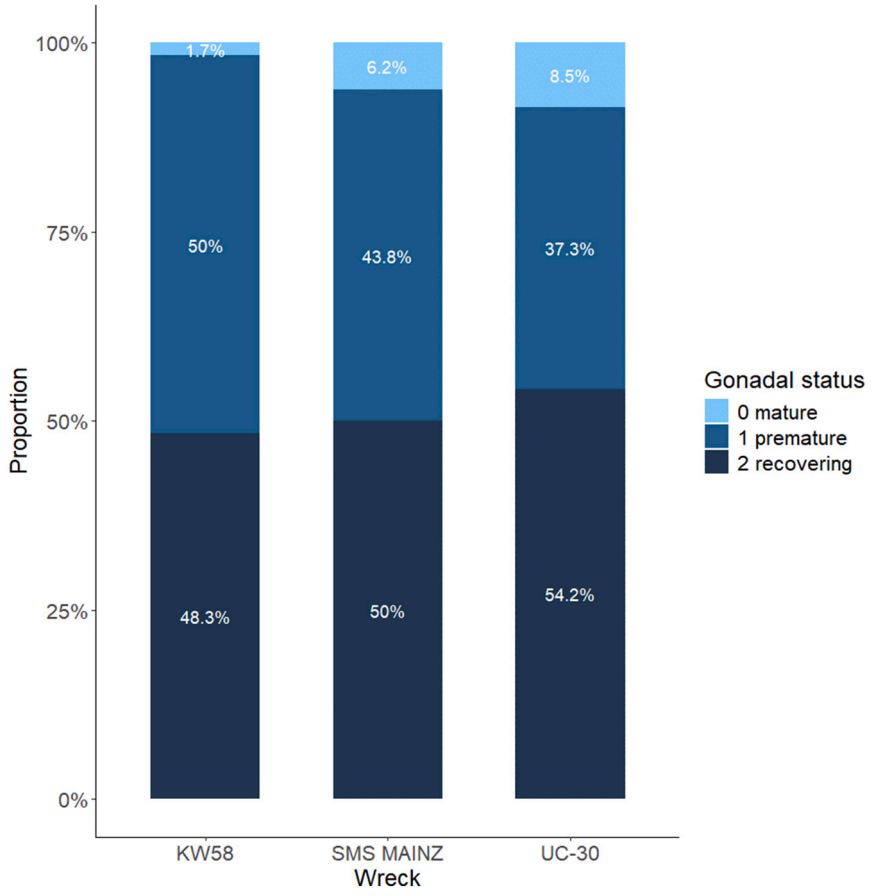

Fig. 11. Comparison of the percentage distribution of gonadal status in *Mytilus edulis*. A distinction was made between recovering, premature, and mature.

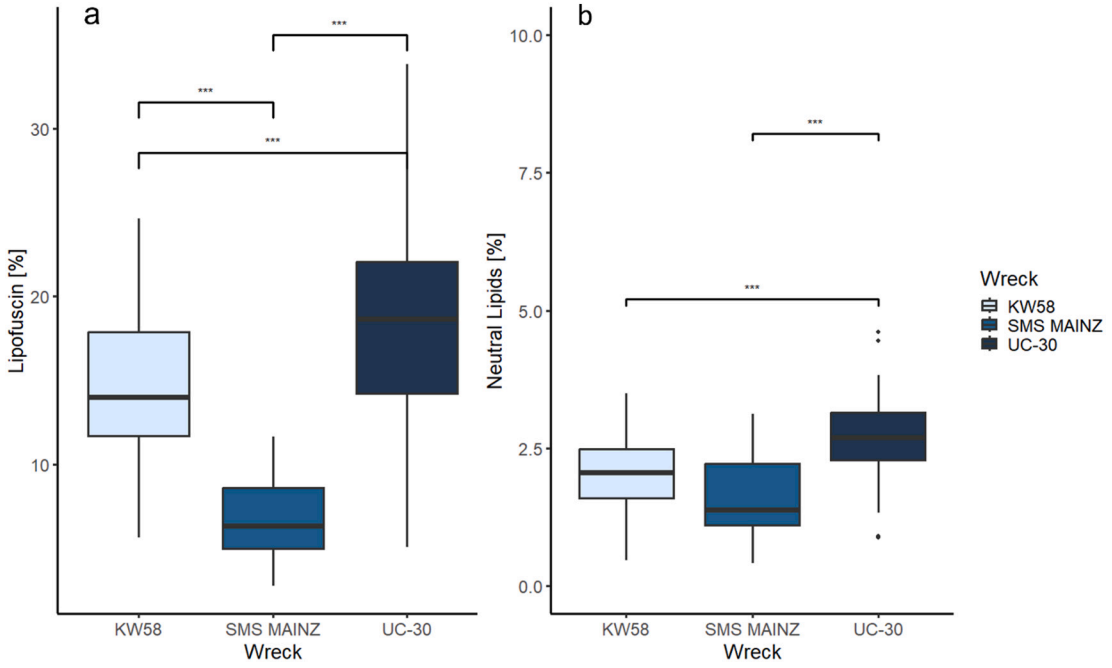

Fig. 12. Accumulation of metabolites determined in tissue cells of the digestive gland in mussels. The accumulation is represented by the percentage of covered area in the liver tissue. The used biomarkers were (a) lipofuscin content and (b) neutral lipids. Data were visualized using standard boxplots generated in RStudio with the package ggplot2, where the box represents the interquartile range (IQR), the horizontal line indicates the median, whiskers extend to 1.5 × IQR, and potential outliers are displayed as individual points. Horizontal brackets show which groups are compared with each other. Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS = not significant.

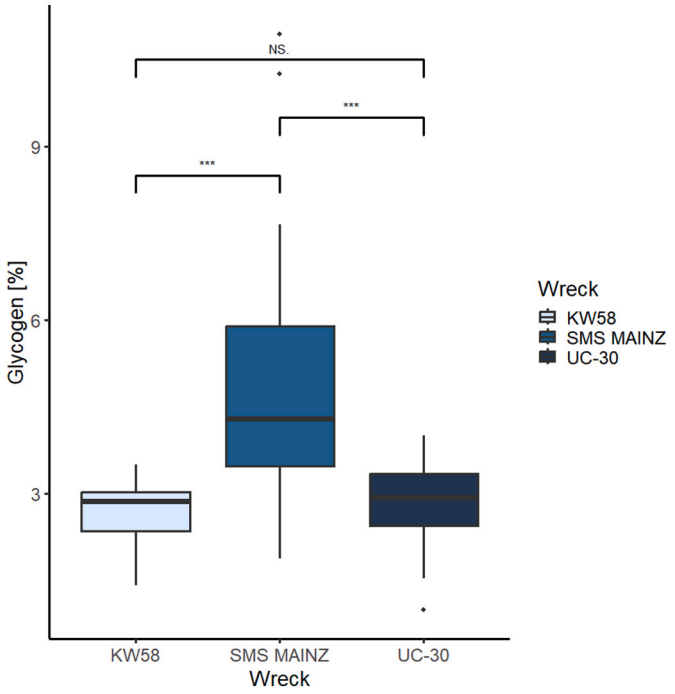

Fig. 13. Comparison of the glycogen content in percent in the digestive gland from mussels exposed at the different war wreck sites. NS shows a non significant difference. Data were visualized using standard boxplots generated in RStudio with the package ggplot2, where the box represents the interquartile range (IQR), the horizontal line indicates the median, whiskers extend to 1.5 × IQR, and potential outliers are displayed as individual points. Horizontal brackets show which groups are compared with each other. Significance levels: * p < 0.05, ** p < 0.01, *** $p < 0.001$, NS = not significant.

preserve tissue integrity and halt all biochemical and chemical processes, all tissues were immediately shock-frozen in liquid nitrogen.

For transport, frozen tissue samples were stored in dry shippers to maintain cryogenic conditions and prevent thawing. Upon arrival at the laboratory, samples were transferred to an ultra-low temperature freezer at −80 °C, where they were maintained until further processing. This protocol ensured the preservation of chemical contaminants and biological markers, minimizing any degradation or alteration prior to analysis.

Using the length and weights, the condition index (CI) was calculated using the following formula (Schuster et al., 2021):

$$CI: \frac{weight\ soft\ body\ [g]}{weight\ shell\ [g]} * 100$$

This index offers a general indication of the animals' overall fitness. However, it's important to mention that a CI could not be obtained for the blue mussels exposed at the SMS MAINZ, as rough seas made it impossible to use the weighing scale.

2.7. Histochemical biomarkers

2.7.1. Cryostat sectioning of mantle and digestive gland samples

For histochemical biomarker analysis, cryostat sections were prepared by adhering frozen mantel and digestive gland tissue samples to pre-frozen aluminum holders (Thermo Scientific™ Richard-Allan

Table 2
Number of samples analyzed.

Wreck	Sediment (aliquots each)	Water (aliquots each)	Mussels
KW 58	11 (3)	2 (1)	60
SMS MAINZ	6 (2)	3 (3)	32
UC30	15 (1)	5 (3)	76

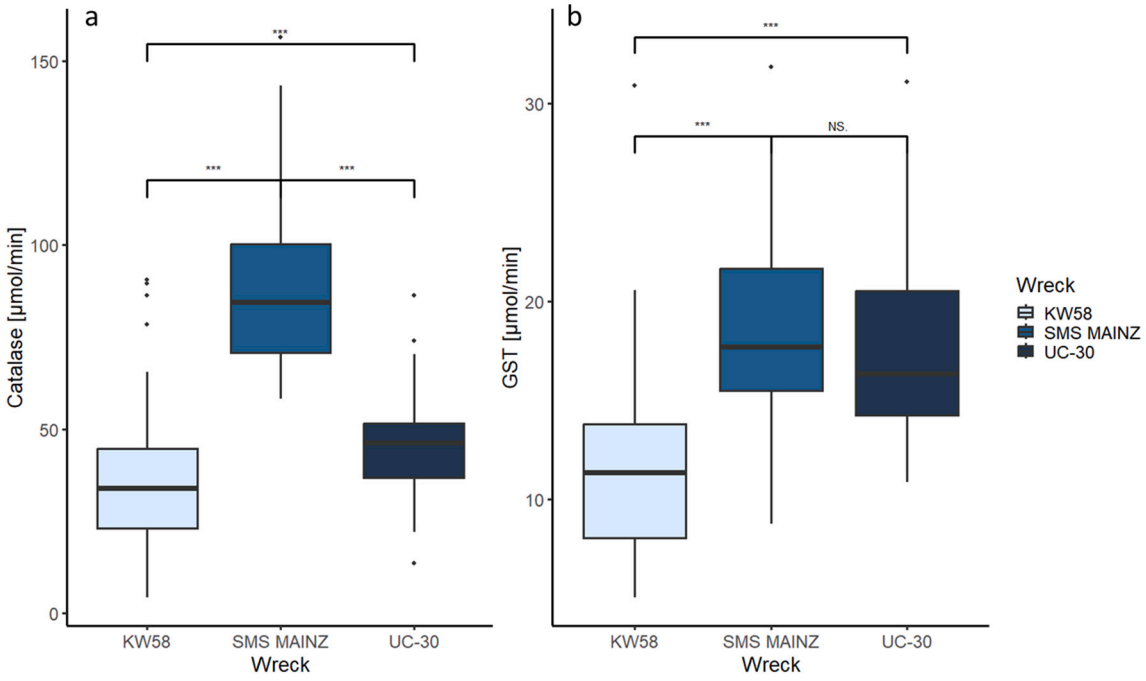

Fig. 14. Enzyme activity in µmol/min in the digestive gland of blue mussels (**a**) Catalase (CAT) activity of digestive gland cells. (**b**) Glutathione-S-transferase (GST) activity of digestive gland cells. Data were visualized using standard boxplots generated in RStudio with the package ggplot2, where the box represents the interquartile range (IQR), the horizontal line indicates the median, whiskers extend to 1.5 × IQR, and potential outliers are displayed as individual points. Horizontal brackets show which groups are compared with each other. Significance levels: * $p < 0.05$, ** $p < 0.01$, *** p < 0.001, NS = not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Matrix specific limits of detection and quantification for the analyzed samples.

	Water		Sediment		Mussels	
	LOD [ng/L]	LOQ [ng/L]	LOD [ng/kg]	LOQ [ng/kg]	LOD [ng/kg dw]	LOQ [ng/kg dw]
TNT	0.09	0.30	10	33	200	680
4-ADNT	0.06	0.19	6	18	50	170
2-ADNT	0.06	0.20	6	20	40	140

Scientific™ Neg-50™) for tissue sectioning. After trimming, tissue sections of 10 μm were prepared using a cryotome (Thermo Scientific™, Cryo Star NX70) at a chamber/knife temperature of −25/ -23 °C. One double section was made per sample and transferred onto a microscopic slide. Sections were stored at −80 °C until staining for lipofuscin, neutral lipids, glycogen, and hematoxylin was performed.

2.7.2. Hematoxylin staining for gender determination and degree of maturity

Sections of mantel tissue were used to visualize gender and status of maturity. A staining was carried out using a hematoxylin-eosin mixture. Tissue sections were fixed in Baker's Formol for 15 min. Afterward, they were immersed three times in Milli-Q for 4 min each time, and then subsequently stained with Gill's hematoxylin for 15 s. After staining, sections were rinsed under running tap water for 20 min and immersed in aqua bidest for 1 min. Tissue sections were stained in an eosin-phloxin solution for 30 s and dipped six times in 80% alcohol. The air-dried sections were mounted in Euparal mounting medium.

2.7.3. Lipofuscin assessment in digestive gland tissue

Lipofuscin (LIP) was determined by using the Schmorl's reaction according to Pearse (1985), modified by Brenner et al. (2014). Cryostat sections were fixed in 4% Baker's formalin for 15 min, rinsed in Milli-Q water, and stained in a 1% aqueous ferric chloride/potassium ferricyanide solution (1:1) for 20 s. The stained sections were washed in 1% acetic acid for 2 min and rinsed under tap water for 10 min. Finally, the sections were rinsed 3 times in Milli-Q water and mounted in Euparal after a short drying period.

2.7.4. Neutral lipid assessment in digestive gland tissue

The accumulation of neutral lipids (NL) in digestive gland tissues was determined using the Oil-Red-O method (ORO) after Lillie and Ashburn (1943), modified by Brenner et al. (2014). First, duplicated sections were fixed in Baker's formalin for 15 min, dipped 15 times in Milli-Q water, and followed by washing in 60% triethylphosphate for 1 min. Afterward, the sections were stained for 15 min in an Oil-Red-O solution containing 1% Oil Red O and 60% Triethylphosphate (the solution was pre-cooked for 5 min and filtered hot and cold once) and rinsed again for 30 s in 60% Triethylphosphate. Finally, the stained sections were rinsed with Milli-Q water, dried, and mounted using Kaiser's glycerine-gelatine.

2.7.5. Glycogen assessment in digestive gland tissue

The duplicate sections for the assessment of glycogen (GLY) content in tissue of the digestive gland were stained using the Perjodic-Acid-Schiff (PAS) method based on Culling et al. (1974). Duplicate sections were fixed for 10 min in Carnoy's fixative, rinsed with Milli-Q water, placed in 1% periodic acid, and rinsed in Milli-Q water again. Sections were then stained for 5 min in Schiff's reagent and rinsed with Milli-Q water. Stained sections were rinsed in different ethanol solutions (50%, 70%, 80%, 96%, 100%) for 1 min each and cleaned with Applichlear until no more color arose in the water. After a short drying period, the stained sections were mounted with Euparal.

2.7.6. Microscopic analysis of cryotome sections

To determine the concentrations of lipofuscin, glycogen, and neutral lipid in the digestive gland of mussel tissue, a computer-assisted image analysis program (camera: MRc, ZEISS; software: AxioVision SE64 Rel. 4.9, ZEISS) combined with a light microscope (Zeiss, Axioskop) at 400-fold magnification was used. For each staining method and sample, 3 black-and-white pictures were taken and analyzed. Image analysis was conducted on selected areas of the image for lipofuscin and glycogen. Suitable areas for image analyses were areas with intact tissue and visible tubules of the digestive gland tissue. Intensity and area of staining was assessed by the image analysis software and is displayed in relation to the area of staining in the chosen part of tissue.

Sex and gonadal status were determined using the same light microscope and camera as described earlier. Gonadal status was separated into three categories according to Schuster et al. (2021): (1) spawned, (2) premature, and (3) mature gonads. Pictures showing examples of the different categories are presented in Fig. 6. Numbers of individuals assigned to a specific category were counted and displayed as percentages.

2.8. Enzyme activities in digestive gland tissue

2.8.1. Tissue homogenization

Tissue homogenization is based on Ahvo (2020a) for the digestive gland. Homogenization was conducted with the Precellys® 24 homogenizer (Bertin Technology). To do so, tissues were transferred in 2 mL vials of the precellys lysing kit Tissue homogenizing CK Mix (Cat.: P000918-LYSK0-A) and filled with buffer. Digestive gland tissue was diluted in 100 mM potassium phosphate buffer (pH 7.0) in a ratio of 1:10 (tissue in mg, buffer in μl). Tissues were always kept on ice. Sample homogenization was executed at 4 °C, 5000 rpm, 2 × 15 s, with 15-s breaks between shakings. Subsequently, samples were centrifuged for 20 min at 4 °C at 10000 *g* using a Sigma centrifuge 3 K18. Supernatants were transferred into 1.5 mL Eppendorf tubes and kept on ice until subsequent enzyme analysis.

Enzyme activity was determined by mass spectrometric measurements using a microplate reader (Infinite 200, TECAN) in combination with i-control software 2.0. Samples were measured over time using 96-well UV microplates for CAT and GST. Leftover extracts were frozen at −20 °C until total protein determination, according to Bradford (1976), using a bovine serum albumin standard (BSA, Sigma A-6003).

2.8.2. Catalase activity

Catalase (CAT) activity in digestive gland tissue was measured based on Claiborne (1985/2018) and modified by Ahvo (2020b). The enzymatic rate was determined as a decrease in absorbance in a mixture at 240 nm containing a final concentration of 10 mM H_2O_2.

2.8.3. Glutathione S-transferase activity

The measurement of glutathione S-transferase (GST) activity in digestive gland tissue was based on Habig et al. (1974) and modified by Ahvo (2020c). GST activity was visible as absorbance increased at 340 nm in a mixture of 2 mM GSH (glutathione) and 1 mM CDNB (1-chloro-2,4 dinitrobenzene) in Dulbecco's buffer.

2.8.4. Determination of proteins in extract and calculation of activity per protein

Enzyme activity was determined from the change in absorbance over time according to the Beer–Lambert law. The slope of the linear absorbance curve (dA/dt) was converted into a reaction rate using the molar

extinction coefficient (ε) and the optical path length (d), and normalized to the reaction and sample volumes (Eq. 1). Activity was expressed in units per milliliter (U/mL, where U = μmol/min). Specific activity (U/mg protein) was calculated by normalizing enzyme activity to the protein concentration of the extract (Eq. 2).

Protein concentrations were determined according to Bradford (1976) using a bovine serum albumin standard (BSA, Sigma A-6003). Absorbance was measured at 590 nm using non-UV microplates.

$$\text{Activity in extract}: \left[\frac{U}{ml}\right] = \frac{\text{slope}\left[\frac{1}{\text{min}}\right] * \text{vol well}[\text{mL}]}{\text{molar coefficient}\,\varepsilon\left[\frac{1}{\left(\frac{mol}{l}\right) * cm}\right] * \text{d}[\text{cm}] * \text{vol sample}[\text{mL}]} \quad (1)$$

$$\text{Activity per protein}: \left[\frac{U}{mg\ protein}\right] = \frac{\text{activity in extract}\left[\frac{U}{\text{mL}}\right]}{\text{protein content}\ [\text{mg/mL}]} \quad (2)$$

2.9. Statistical analysis

Statistical analysis was carried out using RStudio 4.2.3. (R Core Team, 2025) Data were first tested for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene's test. If assumptions of normal distribution and homogeneity of variance were met, differences between mean values of blue mussels among wreck sites were assessed using one-way ANOVA. If these assumptions were violated, the rank-based nonparametric Kruskal–Wallis test (Kruskal and Wallis, 1952) was applied as a robust alternative that does not require normally distributed data. The Dunn–Bonferroni test was used as a post hoc procedure following Kruskal–Wallis, whereas pairwise comparisons were conducted as post hoc tests following ANOVA. For all tests, significance levels were set to $p < 0.05$. Boxplots and bar charts were generated using the package "ggplot2", and significance levels were added to the figures using "ggsignif".

3. Results

3.1. Results of chemical analysis

EC could only be detected in the water at the wreck of the UC30 (Fig. 7). TNT was detected at up to 72.6 ng/L, 4-ADNT at up to 40.7 ng/L, and 2-ADNT at up to 62.7 ng/L. In the samples of SMS MAINZ, taken at different positions and at different depth around the wreck, no EC could be detected. Also, no EC were found in the two water samples taken at the wreck of KW 58.

Concentrations in the sediment varied greatly between the different stations at the KW 58 wreck site (Fig. 8 a,b) Concentrations of 151.6 ± 104.0 μg/kg TNT, 107.5 6 ± 60.0 μg/kg 4-ADNT, and 117.5 6 ± 74.3 μg/kg 2-ADNT have been found in the direct vicinity of the anti-tank mines midship on the deck of the KW58, while much lower concentrations were found at the bow (0.8 ± 0.7 μg/kg TNT, 0.25 ± 0.07 μg/kg 4-ADNT, 0.52 ± 0.06 μg/kg 2-ADNT) and below the limit of quantification at the stern. In the surrounding seafloor at distances between 33 and 89 m to the cargo hold of the KW58 TNT was only detected in some of the samples at concentrations below 2.5 μg/kg TNT, 0.3 μg/kg 4-ADNT, 0.55 μg/kg 2-ADNT. At the UC30, concentrations varied greatly between samples taken directly at the wreck in the minewells (3.11 ± 1.29 μg/kg TNT, 1.77 ± 1.31 μg/kg 4-ADNT, 0.95 ± 0.81 μg/kg 2-ADNT) and in the seafloor around the wreck (0.01 ± 0.01 μg/kg TNT, 0.04 ± 0.05 μg/kg 4-ADNT, 0.02 ± 0.03 μg/kg 2-ADNT). No TNT or metabolites could be detected in the sediment taken at the SMS MAINZ.

In all but 3 of the 59 Mussels from the Wreck of UC 30 and in 51 of the 60 mussels from KW 58 could TNT or its metabolites be detected (Fig. 9). 0.6 ± 2.7 ng/g dry weight (dw) of TNT, 0.5 ± 1.4 ng/g dw of 4-ADNT, and 0.4 ± 1.0 ng/g dw of 2-ADNT were measured in the mussels exposed at the wreck of KW58. The concentrations in the mussels from UC30 were considerably higher at 1.4 ± 2.3 ng/g dw (TNT), 15.6 ± 13.4 ng/g dw (4-ADNT), and 15.2 ± 15.0 ng/g dw (2-ADNT). No TNT, 4-ADNT or 2-ADNT was found in the mussels exposed at SMS MAINZ.

3.2. Morphological parameters

Upon retrieval, the mussels were first assessed for mortality. No mortalities were observed at any of the wreck sites, and no significant differences in mortality rates were detected between the wrecks.

The distribution of the sexes is shown in Fig. 10 in percentage. At all wreck sites, the sex of the mussels could be clearly determined in more than 65% of the animals (KW58 82.00%; SMS MAINZ 81.25%, UC-30 69.49%). Of the identifiable sexes, the proportion of male mussels was higher than the proportion of females on all wrecks. In all wrecks, at least 66% of the identifiable mussels were male. The highest percentage of male mussels (71%) was found on the wreck of KW58. The sex distribution in general did not differ across any of the sites examined.

The animals were divided into three different stages according to their gonadal status. The distribution of the stages did not differ between the wrecks examined (Fig. 11). Most of the mussels examined were recovering and premature (at least 66% of the animals being in this status), with the most significant proportion of animals in the premature status on all wrecks. The smallest proportion of animals were mature. For the following analyses, the mussels could be compared with each other because the influence of the gonadal status and sex ratio was comparable on all wrecks.

3.3. Histochemical biomarkers

3.3.1. Metabolites - lipofuscin and neutral lipids

The accumulation of metabolic end products is shown in Fig. 12. Significant differences in the accumulation of lipofuscin in the lysosomes of the digestive gland of mussels ($F(2): 64.03$; $p < 0.001$), with F denoting the test statistic and the number in parentheses representing the degrees of freedom, were detected between the different wrecks (Fig. 12a). The mussels exposed at the SMS MAINZ showed the lowest accumulation of LIP, with an average of 6.80 +/− 2.49%. In contrast, the LIP accumulation on the two other wrecks, KW58 and UC-30, was significantly higher. With a value of 18.34 +/− 6.13%, LIP accumulation at UC-30 was almost three times as high as in the animals at SMS MAINZ. Similar results can also be seen in the accumulation of neutral lipids (Fig. 12b), where significant differences ($F(2, 135): 20.02$, $p < 0.001$) between the wrecks also occured. While the mussels at SMS MAINZ had the lowest accumulation of neutral lipids with a value of 1.68 +/− 0.75%, the mussels at UC-30 had almost twice the amount of neutral lipids with an average of 2.72+/− 0.75%.

3.3.2. Accumulation of glycogen

There were significant differences ($HF(2): 38.87$,$p < 0.001$) in the accumulation of glycogen between the wrecks examined (Fig. 13). The glycogen accumulation did not differ between the mussels at UC-30 and KW58. With an enrichment of 2.87 +/− 0.63 (UC-30) and 2.67 +/− 0.53 (KW58), the mussels showed a significantly reduced storage of the primary energy supplier glycogen in the lysosomes of the midgut gland. In comparison, the mussels at the SMS MAINZ had almost double the amount of glycogen stored in their midgut glands, with an average value of 4.93 +/− 2.14.

3.4. Enzyme activity

The comparison of the biochemical biomarkers is shown in Fig. 14.

The enzyme activity differed significantly between the different shipwrecks examined. With a catalase activity of −88.63 ±22.52 μmol/min, was significantly higher in the mussels exposed at SMS MAINZ than in the mussels exposed in cages at KW58 and UC-30 (HF(2): 72.48; $p < 0.001$). At 36.47 +/− 19.37 μmol/min, the animals exposed at KW58 Hendericus had only around 50% of the activity of the animals at SMS MAINZ and the comparatively lowest catalase activity.

The results of the glutathione S-transferase show a similar result (Fig. 14b). The activity differs significantly between the wrecks examined (HF(2): 47.03; $p < 0.001$). As with the catalase, the mussels at KW58 show the lowest GST activity (11.83 +/− 4.72 μmol/min). The mussels at SMS MAINZ and UC-30 show significantly higher GST values but do not differ significantly from each other.

4. Discussion

The widespread occurrence of submerged historical shipwrecks, particularly those from past World Wars, poses a significant and often underestimated threat to marine ecosystems. Acting as continuous point sources of pollution, these wrecks slowly release a complex mixture of contaminants into the surrounding environment (Maser et al., 2023), with potentially devastating consequences for marine life. This study delves into the specific impacts observed in marine organisms exposed to such wrecks, focusing on key biomarkers that reveal the extent of environmental stress. Wrecks can be a source of many pollutants depending on their cargo, armament, propulsion and the circumstances of sinking (Landquist et al., 2013; Michel et al., 2005). The wrecks investigated for this study were either coal driven (KW58, SMS MAINZ) or used diesel as fuel (UC-30). To keep the engine running, lubricants and greases based on mineral oil derivates were used for both coal-fired or diesel-fueled vessels. Steel ships of this age were protected against corrosion and fouling by covering them with lead or copper containing coatings. Wooden ships like KW58 would be treated with coal tar or creosote. Overall, all three of the investigated wrecks are certainly a point source of a pollutant mixture of different compositions, most probably unique for a specific wreck. However, they all share the common presence of munition filled with toxic explosives. To gather information about all the potential contaminants, present on each ship at the time of sinking is difficult but for the remaining munition, however, investigations using archive information and battle reports were done to estimate the amount of munition still on the wrecks at least until the time of sinking. In case of UC-30 and KW58 parts of the remaining munition was seen by divers. However, a quantitative assessment of the remaining munitions by the divers was not possible, since most of the munition is silted up and not visible. No munitions have been observed by divers on the SMS MAINZ, though these likely remain deeper within the wreck. Based on archival data and the visual confirmation in situ; we assume that munitions are the dominant source of pollution at KW58 and, assuming that all remaining diesel is fully gone; at UC-30. In contrast, explosive compounds are probably not the main part of the overall pollution mixture at SMS MAINZ given her size and low observed concentrations. Next to the total amount of remaining munitions, the corrosion status and their physical integrity play important roles with regard to their leaking potential and environmental impact. In case of the three wrecks investigated for this study, major differences in the munition status were seen. The mines on UC-30 are in direct contact with seawater, which has led to heavy corrosion. The metal casing of most mines is fully or partly gone, resulting in direct contact of TNT with seawater. By contrast, most of the munition load of the KW58 is covered with sediment, most probably preventing the effective dissolution of explosives at that wreck. At SMS MAINZ, the munition status is unknown since munition was not observed, and no EC could be detected in any of the samples by chemical analysis. Future investigations are, however, recommended to screen the matrices, water, sediment, and biota tissue for the full set of pollutants potentially deriving from a wreck.

Although dumping operations ended in the 1970s, sunken munitions still pose an ongoing risk to marine ecosystems across the globe. One of the most polluted ecosystems, regarding munition, is the North Sea. In addition to the deliberate post-war dumping of munitions, the North Sea was a major theater of war during both world wars. While the ammunition in the dumping areas in the North Sea is covered under several meters of sediment, which has a strong influence on the release of explosive compounds, the munition on many wrecks is still exposed, and explosives can constantly enter the marine environment through the corroding of the casings (Beck et al., 2018) The adverse effects of explosives used in munition, like TNT, hexogen, or tetryl have been demonstrated in various studies. During the Second World War, studies were already carried out on workers from TNT factories, which showed the toxic effects on the human body (Lima et al., 2011). In studies on mammals, like the hispid cotton rat (*Sigmodon hispidus*), pronounced anemia was found in animals exposed to TNT (Reddy et al., 2000). In addition, changes were seen in the liver of animals fed corn oil spiked with TNT. In addition, numerous studies that show the adverse effects of explosives such as TNT on humans and terrestrial organisms (Johnson et al., 1994; Lima et al., 2011), some studies have shown that marine organisms also ingest, metabolize and accumulate explosives (Ballentine et al., 2014; Lotufo et al., 2009; Rosen and Lotufo, 2007). Nipper et al. (2001) showed that various marine organisms, such as algae, shrimps, sea urchin embryos, or redfish, show adverse effects at concentrations of 0.26 mg/L and above. Few studies have investigated chronic toxicity though. In addition, many studies have occurred under laboratory conditions and work with concentrations that rarely occur in the natural environment. Studies investigating the real-world environmental impact of shipwrecks are still lacking. Schuster et al. (2021) found a change in behavior in a laboratory experiment with *Mytilus edulis* spp. They showed that mussels exposed to high TNT concentrations closed their shells significantly more often and for longer periods to reduce filtration rates. In addition, Schuster et al. found an altered accumulation of certain metabolic end products in response to different TNT concentrations. Here, we aimed to expand our knowledge of shipwreck pollution by investigating the effects of TNT pollution in shipwrecks at the molecular level. While it sometimes takes years of exposure to a pollutant to trigger observable changes in the tissue, the first reactions in organisms can often be seen at the enzyme level, even after short periods of exposure.

The metabolization of TNT is prooxidative and generates unstable metabolites that promote the production of reactive oxygen species, ROS (Adomako-Bonsu et al., 2024). The toxic effects of pollutants often depend on their ability to increase cells' reactive oxygen species (ROS) levels. If ROS production is higher than the antioxidant defense, oxidative stress occurs in the cell, which can trigger, among other things, lipid peroxidation of the membrane (Viarengo et al., 1989; Shields et al., 2021). At the sub-cellular level, pollutants interact with individual organelles, reducing or destroying their function (Ren et al., 2025). Like heavy metals, pollutants accumulate in organisms' lysosomes, representing evolutionary primitive detoxification capacity. Excessive accumulation of pollutants suspends this function, leading to cell damage, tissue dysfunction, and impairment of the organism's "state of health". One consequence of the damage is, for example, the accumulation of lipids and the age pigment lipofuscin in the lysosomes of the organisms. The end products of peroxidation accumulate in the lysosomes as insoluble granules containing auto-fluorescent pigments called lipofuscin. The accumulation of this end product in the lysosomal vacuole system of fish hepatocytes or the digestive gland of mollusks indicates the degree of oxidative stress in the cells. It is related to the degree of lipid peroxidation of the membrane (Viarengo and Nott, 1993). Several studies with mussels show that exposure to pollutants promotes the accumulation of lipofuscin (Virengo et al., 1990; Viarengo and Nott, 1993). In 2021, Schuster et al. showed that mussels exposed to TNT had a higher accumulation of the metabolic end product in the cells of the lysosomes than the mussels treated as a reference. The caged mussels exposed to the wrecks from the First and Second World Wars in this

study show similar results. Lipofuscin accumulation in the mussels also increases with a high amount of ammunition remaining on the ships (Fig. 12a). While the mussels on the SMS MAINZ accumulate an average of 0.33% lipofuscin, the values on the other two wrecks, the KW58 and UC-30, are significantly higher. Importantly, these effects were detected under field conditions at TNT concentrations in the low ng/L range (72.6 ng/L at the SMS MAINZ), which are markedly lower than exposure levels commonly applied in laboratory studies. The observation of comparable subcellular alterations under such low-dose conditions provides evidence that even trace concentrations of munition-derived compounds can cause biologically significant adverse effects.

Most biomarkers that indicate oxidative stress are based on a change in the activity of the ROS defense system, such as an adjustment of specific enzyme kinetics. Some of the enzymes involved in cellular antioxidant defense (AD) are catalase (CAT) and glutathione S-transferase (GST) (Viarengo and Nott, 1993; Yu, 1994). CAT and GST respond early to oxidative stress and consequently show modified activity (Geret et al., 2003; Orbea and Cajaraville, 2006). Catalase is found in all aerobic organisms and removes hydrogen peroxide (H_2O_2) from the cells. In addition, catalase eliminates stress-induced formation of oxyradicals when the pollutant load exceeds the tolerance limit of an organism (Winston et al., 1990). This study also observed an effect on catalase activity in mussels. The mussels on the KW58 and UC-30, the two wrecks that had higher TNT concentrations, showed a significant reduction in activity in response to exposure to the pollutants compared to the mussels exposed to the SMS MAINZ. In addition to catalase, GST also plays a role in the ADS (Sheehan et al., 2001). It is a phase II detoxification enzyme that protects the cell from xenobiotics (Fitzpatrick and Sheehan, 1993; Boryslawskyj et al., 1988). Studies with aquatic invertebrates use an adaptation in GST kinetics as a biomarker for oxidative stress (Geracitano et al., 2004; Cunha et al., 2007; Won et al., 2012). The GST activity of the mussels exposed to the wrecks differed significantly. While the mussels at KW58 showed the lowest activity, the activity at SMS MAINZ and UC-30 was significantly higher and showed no differences between the two. However, a high concentration of environmental pollutants does not always have to be accompanied by significantly increased enzyme activity. The activity of enzymes in response to pollutants often follows a bell-shaped pattern (Viarengo et al., 2007). First, there is an increase in activity due to the activation of enzyme synthesis in response to exposure. Subsequently, a decrease in activity can be observed due to the often inhibitory effect of the pollutants. Due to the bell-shaped response of the enzymes, it is essential to look at other reactions in addition to activity in response to exposure to pollutants. In addition, significant differences in carbonyl reductase expression were observed in mussels deployed at Kolberger Heide, particularly in individuals exposed directly at sites with uncovered explosives (Strehse et al., 2017). Although other contaminants were not analytically quantified at this site, their concentrations are likely comparable between bottom and 1 m above bottom due to the absence of nearby point sources. In contrast, TNT concentrations clearly differed between these exposure levels, with elevated concentrations measured in direct proximity to the propellant. This spatial gradient suggests that TNT may have contributed to the observed differences in carbonyl reductase expression, although the influence of additional, unmeasured contaminants cannot be entirely excluded.

For example, the increased accumulation of lipofuscin in the lysosomes of mussels at KW58 and UC-30 can be explained by the decrease in catalase activity. One response to exposure to xenobiotics, in addition to altered GST activity, is the accumulation of neutral lipids in the lysosomes of the midgut gland, which is promoted by the reduction in GST. The change in lipids in the digestive cells and hepatocytes of organisms leads to the autophagic uptake of unsaturated neutral lipids into the lysosomes (Moore, 1994; Köhler et al., 2002). Neutral lipids are biomarkers for toxically induced lipid metabolism (Lüllmann-Rauch, 1979). In the mussels exposed to the wrecks with the highest TNT concentrations measured, KW58 and UC-30, there was a marked increase in the accumulation of neutral lipids in the lysosomes of the mussels' midgut glands (Fig. 12b).

Even low concentrations of pollutants, and also after a very short exposure time, trigger stress in organisms and lead to the upregulation of defense mechanisms to keep the consequences of exposure as low as possible. The initial consequences of exposure to harmful substances are not necessarily irreversible. If the external circumstances improve, antioxidant defense enzymes, for example, can render free ROS harmless and thus prevent more serious consequences. Mussels caged on wrecks with high amounts of remaining ammunition exhibited reduced glycogen levels in their lysosomes. As glycogen represents the primary energy reserve in bivalves, its depletion limits the energy available for cellular defense mechanisms (Ansaldo et al., 2006). This energetic constraint can impair enzyme kinetics and promote the accumulation of metabolic end products within lysosomes.

At the same time, the mobilization of glycogen to sustain defense responses reduces the energy available for other physiological processes, such as reproduction (Patterson et al., 1999). Accordingly, mussels exposed at UC-30 and KW58 showed significantly lower glycogen levels compared to those exposed at SMS MAINZ (Fig. 13). If exposure to pollutants persists over longer periods, continuous activation of defense mechanisms may further deplete energy reserves, potentially leading to an inability to maintain these protective responses. When defense mechanisms can no longer efficiently repair the damage that has occurred, cell organelles deteriorate, causing irreversible damage. Organisms are then, for example, no longer able to suppress the accumulation of metabolic end products.

While the dumped munitions in the Baltic Sea are exposed, and the munitions casings and boxes are continuously corroding due to years of storage in the salty marine environment and gradually releasing the explosive compounds, the situation in the North Sea is different. The dumped munitions were covered with several meters of sediment in the designated dumping areas. To draw reliable conclusions about munitions dumped in the North Sea, alternative methodological approaches are required. In this context, war-related shipwrecks can play an important role and serve as valuable study sites. Many of the wrecks are still exposed today and slowly corrode further, already or eventually releasing explosives from any remaining munitions. Various studies show that the presence of ECsexplosives in the marine environment has a lasting impact on marine organisms (Rosen and Lotufo, 2007; Beck et al., 2018). In this study, negative effects were observed in mussels exposed at two of the three wrecks from the First and Second World Wars in the North Sea. Notably, these effects were detected at the wreck sites characterized by elevated concentrations of explosive compounds (ECs) in both the surrounding water column and the sediment. In contrast, no ECs were detected in water or sediment samples collected at the SMS Mainz, allowing this wreck to serve as a reference site. Accordingly, mussels exposed at SMS Mainz did not exhibit any adverse biological responses associated with contaminant exposure.

Our results demonstrate that a multi-biomarker approach is essential to capture the complex biological responses to pollutants. Explosive compounds affect marine organisms at multiple levels, triggering a cascade of mechanisms aimed at minimizing damage to the organism. Only once the capacity of these detoxification systems is exceeded, irreversible effects can occur, like the accumulation of metabolic end products in response to explosive compounds in the ammunition. The results of this study provide further evidence that the wrecks of the two World Wars still threaten the marine environment and marine organisms. Studies are, however, still mainly concentrating on the immediate vicinity of munitions and wrecks, while the broader spatial distribution of chemical contaminants coming from wrecks with munitions remains poorly understood. This information is, however, essential for making accurate statements on the consequences of polluting shipwrecks for the marine environment. More comprehensive risk assessments are necessary to develop solutions for dealing with munitions dumped in the world's oceans in the future. Importantly, the concentrations of TNT

measured in the present study were in the low ng/L range, thus substantially lower than effect concentrations frequently applied in laboratory-based acute toxicity studies. However, an increasing body of evidence suggests that chronic exposure to low contaminant levels can induce sublethal and cellular effects that may not be detectable under short-term experimental conditions (e.g., Viarengo et al., 2007). In this context, our findings demonstrate that even low environmental concentrations, when combined with chronic exposure under field conditions, are sufficient to trigger measurable cellular and biochemical alterations. These results support the hypothesis that prolonged exposure to trace levels of munition-derived contaminants can lead to biologically relevant effects at the subcellular level.

5. Conclusion

The findings from this study underscore the pressing environmental threats posed by shipwrecks from both World Wars in the North Sea. These wrecks, many of which contain munitions, release toxic substances such as TNT into the marine environment as they corrode over time. The research, using caged blue mussels (*Mytilus edulis*), highlights measurable biochemical and physiological stress responses in these organisms, including altered enzyme activities, metabolic disruptions, and reduced energy reserves for essential biological processes like reproduction. The study demonstrates that these pollutants trigger oxidative stress, evident through increased lipofuscin,neutral lipid accumulation and a reduced glycogen accumulation in the mussels' digestive tissues and reduced detoxification enzyme activity. The results also correspond with the amount of TNT pollution detected on the wrecks. The findings stress the need for continued monitoring and a comprehensive risk assessment of these underwater hazards to inform strategies for mitigation. This approach highlights the ecological legacy of wartime activities and serves as a model for addressing similar issues in marine ecosystems globally.

CRediT authorship contribution statement

Romina Marietta Schuster: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Franziska Isabell Binder:** Writing – review & editing, Validation, Methodology, Investigation, Data curation, Conceptualization. **Lillian Tabea Hannah Bünning:** Writing – review & editing, Data curation. **Jennifer Susanne Strehse:** Writing – review & editing. **Katrine Juul Andresen:** Writing – review & editing, Resources. **Maarten De Rijcke:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Sven Van Haelst:** Writing – review & editing, Resources. **Uwe Wichert:** Writing – review & editing, Resources. **Philipp Grassel:** Writing – review & editing, Resources. **Edmund Maser:** Writing – review & editing. **Matthias Brenner:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization.

Nagoya conformity statement

Genetic resources collected in-situ in marine areas under Belgian jurisdiction fall only within scope of the national/federal implementation regulations of the Nagoya Protocol and not under any regional decree. Until to date only the sub-national/regional ABS regulation of the Walloon region, but not the national/federal regulations foresee the issuing of permits like the IRCC. Compliance in case of non-commercial research consists only of a notification obligation at the time of publication, which is not triggered when results are being published open-accessibly according to Article 6 §2(1) of the law concerning access to federal genetic resources and the fair and equitable sharing of benefits arising from their use (2023-05-17/13). Further, Denmark does not regulate access to genetic resources within its national jurisdiction according to Denmark's National Focal Point under the Nagoya Protocol. Therefore, no PIC and MAT are required when accessing the genetic resources in Danish waters.

Funding declaration

The research was co-funded by the European Union (European Regional Development Fund) under the North Sea Interreg Programme 2014–2020. Research was conducted, within the framework of the project “North Sea Wrecks - An Opportunity for Blue Growth: Healthy Environment, Shipping, Energy Production and –transmission (NSW).

Declaration of competing interest

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

Acknowledgements

We would like to express our sincere gratitude to the crews of RV Heincke, RV Aurora, and RV Simon Stevin for their outstanding support during the field campaigns and sample collection. We also thank the divers of the Royal Danish Navy for their efforts and support during the sampling at the UC30 site. Likewise, we are grateful to the scientific divers from VLIZ for their active assistance during the exposure operations at KW58 and SMS MAINZ.

We further acknowledge The Fisheries and Maritime Museum in Esbjerg for providing access to laboratory facilities for sample preparation. Our special thanks go to Lars Møller Petersen for facilitating and supporting the involvement of the Danish Navy. We would also like to thank Wyona Schütte and Matt Skelhorn for their contributions to the historical research on KW58 Hendericus. Finally, we thank our student Lucian Fiedler for his assistance with sample processing.

Data availability

The biological data presented in this study are published open access as: Schuster et al. (2025), Accessible at PANGAEA database via: https://doi.org/10.1594/PANGAEA.984199

Water and sediment data presented in this study are published open access as: Bünning et al. (2026), Accesile at PANGAEA database via: https://doi.org/10.1594/PANGAEA.991466

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