

**Dumped munitions: Effects, metabolism and detection of  
explosive compounds and chemical warfare agent-related  
chemicals in fish from the Baltic Sea**

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## Abstract

Dumped munitions in the marine environment, a legacy mainly originating from World Wars I and II, constitute a contemporary environmental problem affecting marine biota, maritime infrastructure and economy, and humans. The disposal mainly took place in the aftermath of the war as legal practice until the 1970s and environmental consequences played a minor role in this situation. Nowadays, due to the permanent exposure of the munition shells to seawater, many shells are corroded and the corrosion will continue in the future. Where shells are broken, the compounds they contain leak into the marine environment leading to exposure of marine organisms, including fish. This risk posed by dumped munitions exists at various locations around the world, often in coastal waters.

With hundreds of thousands of tons of dumped munitions, both conventional explosives and chemical warfare agents (CWAs), the Baltic Sea is an important area of investigation. Conventional munitions contain organic explosive compounds such as the nitroaromatic 2,4,6-trinitrotoluene (TNT) and the nitramines hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). CWAs are part of chemical weapons (CW), which were also dumped on large scale in the Baltic Sea and often contain sulphur mustard (Mustard Gas) or arsenic-based warfare agents like Clark I and II and Adamsite. Both compounds from conventional munitions and chemical warfare agents are known to cause toxic effects in humans and wildlife. However, toxicity studies of nitroaromatic compounds in fish are mainly limited to their acute effects and the toxicity of CWAs in fish is completely unknown.

To date, information on the distribution and condition of munitions is scarce. Most of the countries affected lack coordinated action by political stakeholders to develop a management concept for the future. In order to provide solutions for these unsolved challenges, the international DAIMON project (Decision Aid for Marine Munitions), involving research institutions from countries bordering the Baltic Sea, aimed to establish a scientific basis for decisions on the future management of dumped munitions in the sea. This also includes improving our understanding of the effects and fate of munition compounds in fish, which are an essential part of the marine environment. This thesis was carried out in the framework of the DAIMON project and focused mainly on TNT as a frequently occurring component of conventional munitions. It investigated: 1) the genotoxicity of nitroaromatic compounds in fish, 2) the metabolism of munition

compounds in fish liver and 3) the detection of munition compounds in fish from the Baltic Sea.

In the first study, zebrafish embryos (*Danio rerio*) were used to determine the genotoxicity of TNT and its degradation products (2-amino-4,6-dinitrotoluene, 2-ADNT and 4-amino-2,6-dinitrotoluene, 4-ADNT) in an *in vivo* exposure scenario. As genotoxicity caused by TNT has not yet been demonstrated in fish, the evidence of such effects is crucial for discussing long-term effects in fish exposed to dumped munitions. The genotoxic effects were evaluated with the alkaline comet assay. Both TNT and the degradation products tested caused significant genotoxic effects. Together with existing studies demonstrating the mutagenicity and carcinogenicity of nitroaromatic compounds, this study showed that there is considerable evidence that fish exposed to dumped munitions are likely to face long-term health effects.

Since the compounds leaking from the munitions are subject to biotransformation processes as soon as they are taken up by fish, the metabolism is of great importance for understanding the fate of munition compounds and enabling their detection in fish. The second and the third study investigated the metabolism of TNT and CWA-related phenylarsenic chemicals in fish from the Baltic Sea, using an *in vitro* metabolism approach with liver tissue from dab (*Limanda limanda*) and cod (*Gadus morhua*). Rapid biodegradation of TNT and phenylarsenic chemicals by liver enzymes was observed, and sophisticated analytical techniques (high performance liquid chromatography-mass spectrometry, HPLC-MS) were used to identify different biotransformation products. These can serve as target compounds for monitoring a munition-related contamination of fish from the Baltic Sea. In these studies, the *in vitro* approach using fish liver tissue proved to be a useful tool to study the metabolism of munition compounds.

The fourth study aimed to verify the results of the *in vitro* experiment and to obtain a sound overview of the contamination status of fish likely to be exposed to dumped munitions in the Baltic Sea. For this purpose, dab from a munition dumpsite in the western Baltic Sea were sampled. Their bile was analyzed for explosive compounds by HPLC-MS and 48% of 115 dab specimen analyzed from the dumpsite contained at least one explosive compound, including HMX, RDX, TNT, 2-ADNT and 4-ADNT. In contrast, a similarly large sample from a nearby reference site did not contain explosive compounds. This study demonstrated that a considerable proportion of flatfish caught in the vicinity of a munition dumpsite in the Baltic Sea is contaminated with explosive compounds, some of which are known to be genotoxic, mutagenic and carcinogenic.

The present thesis clearly demonstrates that nitroaromatic compounds related to conventional munitions damage the DNA in exposed fish. Together with other known ad-

verse effects, this could affect fish health in the vicinity of munition dumpsites. However, the mechanism of action and associated thresholds need to be clarified. Furthermore, this thesis shows that munition compounds in fish undergo rapid biotransformation processes and that metabolites are important for detection. Explosive compounds originating from conventional munitions are found in flatfish from a dumpsite in the Baltic Sea. The results indicate the need for monitoring munition compounds in the affected marine areas in order to properly assess the current state of contamination and to develop a management concept for munition dumpsites, especially in the light of a changing environment.





## Zusammenfassung

Versenkte Munition in der Meeresumwelt, die hauptsächlich aus dem Ersten und Zweiten Weltkrieg stammt, stellt ein aktuelles Umweltproblem dar. Dieses betrifft den Menschen, das Leben im Meer sowie die maritime Infrastruktur und Wirtschaft. Die Entsorgung der Munition fand als legale Praxis hauptsächlich in der Nachkriegszeit bis in die 1970er Jahre statt. Dabei spielten die Umweltfolgen eine untergeordnete Rolle. Heutzutage sind aufgrund der andauernden Exposition der Munitionshüllen im Meerwasser viele Hüllen korrodiert und die Korrosion wird in Zukunft fortschreiten. Wo Hüllen beschädigt sind, treten die enthaltenen Verbindungen in die Meeresumwelt aus und führen zur Exposition von Meeresorganismen, einschließlich Fischen. Diese Gefahr, die von versenkter Munition ausgeht, besteht an verschiedenen Orten auf der ganzen Welt, oft in Küstengewässern.

Mit mehreren hunderttausend Tonnen versenkter Munition, sowohl konventioneller Sprengstoffe als auch chemischer Kampfstoffe (CWAs), ist die Ostsee ein wichtiges Untersuchungsgebiet. Konventionelle Munition enthält organische Sprengstoffverbindungen wie den Nitroaromaten 2,4,6-Trinitrotoluol (TNT) und die Nitramine Hexahydro-1,3,5-trinitro-1,3,5-triazin (RDX) und Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocin (HMX). CWAs sind Bestandteil chemischer Waffen (CW), welche ebenfalls in großem Umfang in der Ostsee verklappt wurden und oft Senfgas oder arsenhaltige Kampfstoffe wie Clark I und II und Adamsit enthalten. Sowohl Verbindungen aus konventioneller Munition als auch chemische Kampfstoffe sind bekannt dafür, dass sie toxische Effekte bei Menschen und Tieren verursachen. Toxizitätsstudien zu nitroaromatischen Verbindungen in Fischen beschränken sich jedoch hauptsächlich auf ihre akuten Wirkungen und die Toxizität von CWAs in Fischen ist weitestgehend unerforscht.

Bis heute gibt es wenig Informationen über die Verteilung und den Zustand der versenkten Munition. In den meisten betroffenen Ländern mangelt es an koordinierten Maßnahmen der politischen Akteure, um ein Managementkonzept für die Zukunft zu entwickeln. Um Lösungen für diese Herausforderungen zu finden, verfolgte das internationale DAIMON Projekt (Decision Aid for Marine Munitions) unter Beteiligung von Forschungseinrichtungen der Ostseeanrainerstaaten das Ziel, eine wissenschaftliche Grundlage für Entscheidungen über das zukünftige Management von versenkter Munition im Meer zu schaffen. Dazu gehört auch ein verbessertes Verständnis über die Auswirkungen und den Verbleib von Munitionsverbindungen in Fischen, die ein wesentlicher Bestandteil der Meeresumwelt sind. Diese Arbeit wurde im Rahmen des DAI-

MON Projekts durchgeführt und konzentrierte sich hauptsächlich auf TNT als häufig vorkommende Komponente konventioneller Munition. Dabei wurden 1) die Genotoxizität von nitroaromatischen Verbindungen in Fischen, 2) der Metabolismus von Munitionsverbindungen in der Fischleber und 3) der Nachweis von Munitionsverbindungen in Fischen aus der Ostsee analysiert.

In der ersten Studie wurden Zebrafischembryonen (*Danio rerio*) verwendet, um die Genotoxizität von TNT und seinen Abbauprodukten (2-Amino-4,6-dinitrotoluol, 2-ADNT und 4-Amino-2,6-dinitrotoluol, 4-ADNT) in einem *in vivo* Expositionsszenario zu bestimmen. Da die, durch TNT verursachte, Genotoxizität bei Fischen noch nicht gezeigt werden konnte, ist der Nachweis solcher Effekte wichtig für die Ermittlung langfristiger Effekte bei Fischen, die versenkter Munition ausgesetzt sind. Die genotoxischen Effekte wurden mit dem alkalischen Comet-Assay untersucht. Sowohl TNT als auch die getesteten Abbauprodukte verursachten signifikante genotoxische Effekte. Zusammen mit Studien, welche die Mutagenität und Kanzerogenität von nitroaromatischen Verbindungen belegen, erbrachte diese Studie bedeutende Hinweise darauf, dass Fische langfristige gesundheitliche Schäden erleiden können, wenn sie versenkter Munition ausgesetzt sind.

Da die aus Munition austretenden Verbindungen Biotransformationsprozesse durchlaufen sobald sie von Fischen aufgenommen werden, ist der Metabolismus von großer Bedeutung, um das Schicksal der Munitionsverbindungen zu verstehen und ihren Nachweis in Fischen zu ermöglichen. Die zweite und die dritte Studie untersuchten den Metabolismus von TNT und CWA-bezogenen phenylarsenhaltigen Chemikalien in Fischen aus der Ostsee. Dafür wurde eine *in vitro* Methodik mit Lebergewebe der Kliesche (*Limanda limanda*) und des Kabeljaus (*Gadus morhua*) verwendet. Es wurde ein schneller biologischer Abbau von TNT und phenylarsenhaltigen Chemikalien durch die Leberenzyme beobachtet und mithilfe spezieller analytischer Methoden (Hochleistungs-Flüssigkeitschromatographie-Massenspektrometrie, HPLC-MS) konnten verschiedene Biotransformationsprodukte identifiziert werden. Diese können als Zielverbindungen für die Überwachung einer munitionsbedingten Belastung von Fischen aus der Ostsee dienen. In diesen beiden Studien erwies sich die *in vitro* Methodik mit Fischlebergewebe als nützliches Instrument, um den Metabolismus von Munitionsverbindungen zu erforschen.

Die vierte Studie zielte darauf ab, die Ergebnisse des *in vitro* Experiments zu bestätigen und einen fundierten Überblick über den Kontaminationszustand von Fischen zu erhalten, die wahrscheinlich versenkter Munition in der Ostsee ausgesetzt sind. Zu diesem Zweck wurden Klieschen aus einem Versenkungsgebiet in der westlichen Ostsee beprobt. Ihre Galle wurde mittels HPLC-MS auf Sprengstoffverbindungen hin

untersucht. 48% der 115 analysierten Klieschen des Versenkungsgebietes enthielten mindestens eine Sprengstoffverbindung, darunter HMX, RDX, TNT, 2-ADNT und 4-ADNT. Im Gegensatz dazu konnten in einer ähnlich großen Stichprobe, die aus einem nahen Referenzgebiet stammte, keine Sprengstoffverbindungen nachgewiesen werden. Diese Studie zeigte, dass ein beträchtlicher Anteil der Plattfische, die in der Nähe einer Munitionsdeponie in der Ostsee gefangen wurden, mit Sprengstoffverbindungen kontaminiert ist, von denen einige bekanntermaßen genotoxisch, mutagen und karzinogen sind.

Die vorliegende Arbeit zeigt deutlich, dass nitroaromatische Verbindungen aus konventioneller Munition die DNA in exponierten Fischen schädigen. Zusammen mit anderen bekannten schädlichen Effekten könnte dies die Gesundheit von Fischen in der Nähe von Munitionsdeponien beeinträchtigen. Der Wirkungsmechanismus und die davon abhängenden Schwellenwerte müssen jedoch noch erforscht werden. Darüber hinaus zeigt diese Arbeit, dass Munitionsverbindungen in Fischen schnelle Biotransformationsprozesse durchlaufen und die Metabolite für den Nachweis von Bedeutung sind. Sprengstoffverbindungen, die aus konventioneller Munition stammen, werden in Plattfischen aus einem Versenkungsgebiet in der Ostsee nachgewiesen. Die Ergebnisse zeigen, dass ein Monitoring der Munitionsverbindungen in den betroffenen Meeresgebieten notwendig ist, um den aktuellen Stand der Belastung richtig einzuschätzen und ein Managementkonzept für Versenkungsgebiete zu entwickeln, insbesondere im Hinblick auf eine sich verändernde Umwelt.



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## Abbreviations

10-M-5 <i>H</i> -PA-10-O	10-methyl-5 <i>H</i> -phenarsazinine-10-oxide
1,3-DNB	1,3-dinitrobenzene
1,4-DNB	1,4-dinitrobenzene
2-ADNT	2-amino-4,6-dinitrotoluene
2,4-DANT	2,4-diamino-6-nitrotoluene
2,4-DNT	2,4-dinitrotoluene
2,5-DNT	2,5-dinitrotoluene
2,6-DANT	2,6-diamino-4-nitrotoluene
3,5-DNBA	3,5-dinitrobenzoic acid
4-ADNT	4-amino-2,6-dinitrotoluene
ADNTs	Amino-dinitrotoluenes
BSC	Black Sea Commission
CW	Chemical weapon
CWA	Chemical warfare agent
DA	Clark I
DANTs	Diamino-nitrotoluenes
DM	Adamsite
DMM	Discarded munition material
DM[ox]	10-hydroxy-5 <i>H</i> -phenarsazinine-10-oxide
DMSO	Dimethyl sulfoxide
DPA[ox]	Diphenylarsinic acid
EIC	Extracted ion chromatogram
EU	European Union
GC	Gas chromatography
GES	“Good Environmental Status” according to the Marine Strategy Framework Directive
GSH	Glutathione

## Abbreviations

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GST	Glutathione- <i>S</i> -transferase
HCD	Higher-energy collisional dissociation
HELCOM Convention	Convention on the Protection of the Marine Environment of the Baltic Sea Area administered by the Helsinki Commission
HESI	Heated electrospray ionization
HMX	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC	High performance liquid chromatography
iAs	Inorganic arsenic
MeOH	Methanol
MDPAO	Methyldiphenylarsine oxide
MPAA	Methylphenylarsinic acid
MS	Mass spectrometry
MSFD	Marine Strategy Framework Directive
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance spectroscopy
OSPAR Convention	Oslo-Paris Convention for the Protection of the Marine Environment in the North-East Atlantic
PDCA	Phenyldichloroarsine
PDCA[ox]	Phenylarsonic acid
RDX	Hexahydro-1,3,5-trinitro-1,3,5-triazine
SAM	<i>S</i> -adenosyl methionine
TAT	Triaminotoluene
TNB	1,3,5-trinitrobenzene
TNT	2,4,6-trinitrotoluene
TPA	Triphenylarsine
TPA[ox]	Triphenylarsine oxide
UNEP-MAP	United Nations Environment Programme - Mediterranean Action Plan
UXO	Unexploded ordnance

## Contribution of Authors

1. **D. Koske, N. I. Goldenstein, and U. Kammann. Nitroaromatic compounds damage the DNA of zebrafish embryos (*Danio rerio*). *Aquatic Toxicology*, 217:105345, 2019. ISSN 0166-445X. doi: 10.1016/j.aquatox.2019.105345**

Author Contribution: Daniel Koske and Ulrike Kammann discussed and designed the study together. Daniel Koske carried out the acute toxicity test, the comet assay and microscope work as well as processing of all data. Nadine Goldenstein carried out concentration measurements with HPLC-MS. Daniel Koske wrote the manuscript and all authors contributed with helpful comments before the submission of the manuscript.

2. **D. Koske, N. I. Goldenstein, T. Rosenberger, U. Machulik, R. Hanel, and U. Kammann. Dumped munitions: New insights into the metabolism of 2,4,6-trinitrotoluene in Baltic flatfish. *Marine Environmental Research*, 160:104992, 2020a. ISSN 0141-1136. doi: 10.1016/j.marenvres.2020.104992**

Author Contribution: Daniel Koske and Ulrike Kammann designed the study together. Daniel Koske carried out the enzyme activity and protein measurements, the *in vitro* metabolism experiment, the processing of the data and wrote the manuscript. Nadine Goldenstein and Ulrike Machulik carried out the analytical work with HPLC-MS. Timothy Rosenberger performed enzyme activity measurements. Reinhold Hanel and Ulrike Kammann provided insightful ideas into the discussion of the *in vitro* metabolism results. All authors contributed with helpful comments before the submission of the manuscript.

3. H. Niemikoski, D. Koske, U. Kammann, T. Lang, and P. Vanninen. Studying the metabolism of toxic chemical warfare agent-related phenylarsenic chemicals *in vitro* in cod liver. *Journal of Hazardous Materials*, 391:122221, 2020a. ISSN 0304-3894. doi: 10.1016/j.jhazmat.2020.122221

Author Contribution: Hanna Niemikoski and Daniel Koske designed the study, carried out *in vitro* metabolism experiments and wrote the manuscript together. Hanna Niemikoski performed analytical work with HPLC-HRMS and NMR. Thomas Lang organized and conducted sampling of livers. Ulrike Kammann and Paula Vanninen contributed to the discussion of the results. All authors contributed with helpful comments before the submission of the manuscript.

4. D. Koske, K. Straumer, N. I. Goldenstein, R. Hanel, T. Lang, and U. Kammann. First evidence of explosives and their degradation products in dab (*Limanda limanda* L.) from a munition dumpsite in the Baltic Sea. *Marine Pollution Bulletin*, 155:111131, 2020b. doi: 10.1016/j.marpolbul.2020.111131

Author Contribution: Daniel Koske, Katharina Straumer, Thomas Lang and Ulrike Kammann designed the study together. Daniel Koske and Katharina Straumer validated the data and wrote the manuscript together. Nadine Goldenstein performed analytical work with HPLC-MS. Katharina Straumer, Nadine Goldenstein and Thomas Lang collected bile samples. Reinhold Hanel, Thomas Lang and Ulrike Kammann contributed to the discussion of the results. All authors contributed with helpful comments before the submission of the manuscript.

# 1. General Introduction

## 1.1. Pollutants in the marine environment and how to manage them

Marine ecosystems are affected by human activities worldwide, and pollutants, often originating from human sources, are found in oceans and estuaries around the world (Halpern et al., 2008). For decades and centuries, pollutants have been deliberately and unintentionally, directly and indirectly introduced into the marine environment. This marine pollution coincides with the “Anthropocene” epoch and has its origins in the industrial revolution in the eighteenth century. It has accelerated throughout history with steady population growth (Crutzen, 2006). According to Kennish (1997) and Clark (1992), the contamination of the marine environment is driven by six main inputs; riverine inputs, nonpoint source runoff, direct pipeline discharge, atmospheric deposition, offshore inputs and discharge from ships. Accordingly, the greatest impact of marine pollution can be expected to occur in coastal areas where human influence is great and where estuaries enhance the input of pollutants (Kennish, 1997; Robinson et al., 2017; Webster et al., 2011). Nevertheless, sea-based activities such as shipping, mariculture, offshore activities, dredging or dumping contribute to marine pollution (Tornero and Hanke, 2016).

Based on the intrinsic properties and the presence of the pollutants in the marine environment, influenced by various factors such as bioavailability or remobilization from sediments, marine biota take up the pollutants and are thus subject to threats and adverse effects (Borchardt et al., 1988). The types of pollutants present may change over time, but persistent pollutants can be found almost everywhere in marine ecosystems and within the marine food web (Jepson and Law, 2016; Walker and Livingstone, 2013). Popular contaminants that pose a risk to the ecosystem including marine biota are petroleum hydrocarbons, halogenated hydrocarbons, metals and metalloids, radionuclides, nanomaterials and litter, especially plastics (Kennish, 1997; Newman, 2014).

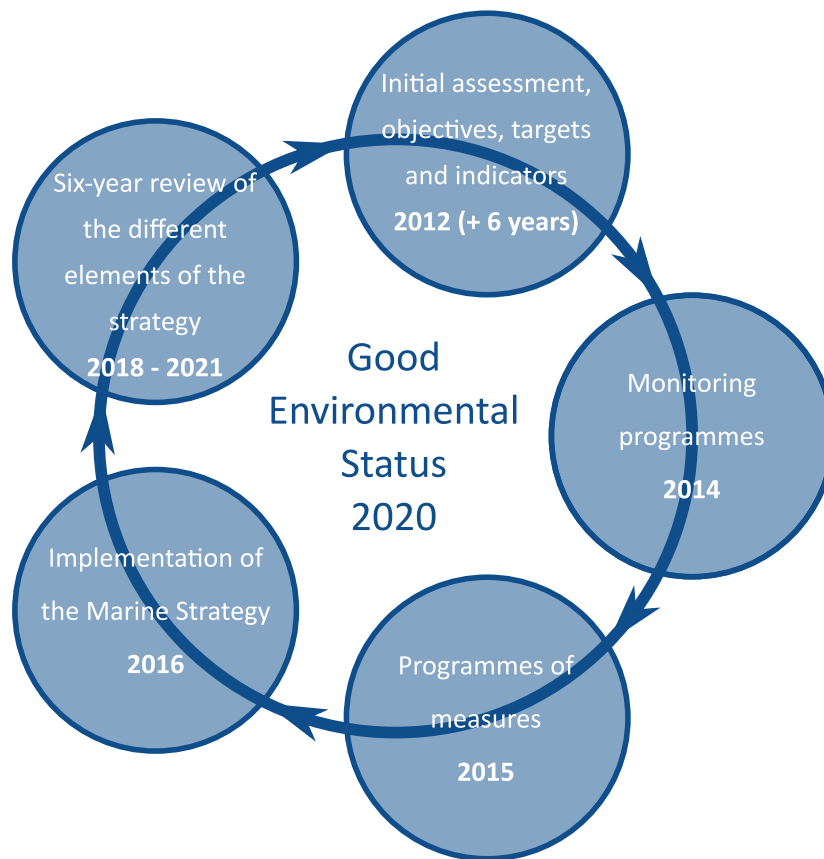
For decades, there has been a political consensus that these hazardous substances in the marine environment require a coordinated environmental policy. Regional sea conventions for the different marine regions in Europe exist among others for this purpose.

Four regional sea conventions exist in Europe which are: The Oslo-Paris (OSPAR) Convention for the Protection of the Marine Environment in the North-East Atlantic, the Convention on the Protection of the Marine Environment of the Baltic Sea Area administered by the Helsinki Commission (HELCOM), the Convention for the Protection of Marine Environment and the Coastal Region of the Mediterranean administered by the United Nations Environment Programme - Mediterranean Action Plan (UNEP-MAP) and the Convention for the Protection of the Black Sea administered by the Black Sea Commission (BSC). The individual commissions, whose members are the respective bordering states, have the task of implementing the relevant convention for the protection of the marine regions.

Different marine legislations exist worldwide, such as the Oceans Act in the USA and Canada, or the National Water Act in South Africa, all aiming to assess the environmental status as an integrated parameter at the ecosystem level (Borja et al., 2008; Ricketts and Harrison, 2007). As part of the need for coordinated environmental policy measures and integrated assessment, the European Union (EU) established the Marine Strategy Framework directive (MSFD) in 2008, a regulatory framework for necessary measures to protect the marine regions in Europe.

The Marine Strategy Framework Directive (MSFD) (European Commission, 2008) was adopted in 2008 in order to achieve or maintain the so called “good environmental status” (GES) of the marine environment in Europe until the year 2020. The MSFD operates as a legislative instrument at European level and obliges each Member State to develop individual integrated marine strategies aiming at: preserving the marine environment, avoiding its deterioration and restoring the environment in areas that have been adversely impacted (European Commission, 2008). According to the MSFD (2008) the GES is defined as an “*environmental status of marine waters where these provide ecologically diverse and dynamic oceans (...) and the use of the marine environment is at a level that is sustainable (...)*”.

The keystones for the implementation of the MSFD are the marine strategies in each Member State, which had to be developed starting with the adoption of the directive and are reviewed every six years. The marine strategy includes an initial assessment of the current environmental status and a definition of the GES for national waters, the establishment of monitoring programmes and programmes of measures needed to achieve or maintain the GES and the operation of the programmes according to the MSFD (Figure 1.1). The European Commission is obliged to evaluate and publish a report on assessing the programmes of the Member States every six years, starting in 2018 (European Commission, 2018).



**Figure 1.1.:** Steps for the development of the marine strategies by the Member States within the Marine Strategy Framework Directive (adapted from European Commission (2019)).

The GES is specified in 11 qualitative descriptors of the MSFD, including contaminants in the sea (D8) and contaminants in seafood (D9). The European Commissions' 2018 report concluded that, despite the measures taken by the Member States, they are not fully adequate to prevent the pressures on the marine environment. Therefore, the achievement of the GES for all 11 descriptors in all European regions by 2020 seems unlikely (European Commission, 2018). However, even if the goals of the MSFD are not fully met by 2020, the implementation of environmental directives has already led Europe to a more coordinated and integrated marine management (Borja et al., 2010).

Within the descriptors, various popular pollutants are considered in the MSFD, but there are certain pollutants in the marine environment that are not part of the directive. For political, historical or even scientific reasons, dumped munitions in the sea and the chemicals contained therein are not considered in the MSFD. However, this issue has gained attention in the EU and especially in Germany in recent years and

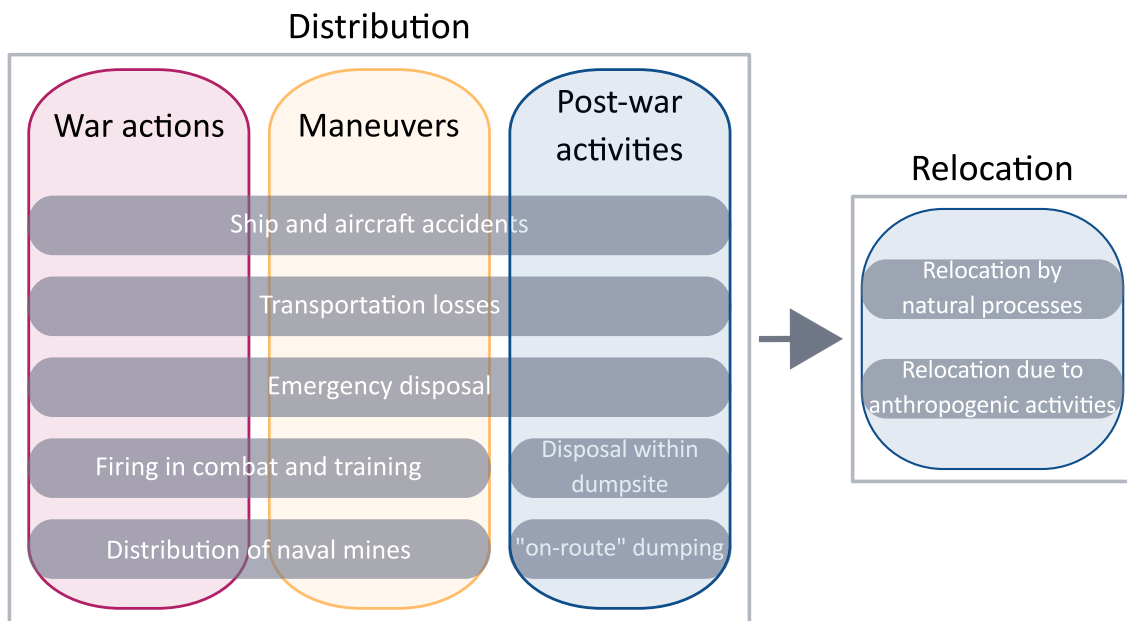
several scientific research projects have been funded. In Germany, the 2019 Conference of Environmental Ministers in Hamburg (2019) requested the Federal Government to investigate the contamination of fishery resources with munition compounds and to conduct a screening of the contamination in German waters. The subject of contamination, effects and fate of dumped munitions in the marine environment is therefore a highly relevant, applied scientific topic addressed in this thesis.

## **1.2. Dumped munitions in the sea – a special case of marine pollution**

Considering the spatial distribution of organic pollutants, it is evident that these substances can be detected worldwide, although higher concentrations are found in the vicinity of more inhabited areas (Giesy and Kannan, 2001; Laflamme and Hites, 1978). These pollutants are emitted from innumerable sources around the world and their distribution pathways are diverse, including distribution through the air. Dumped munitions are also considered as marine pollution on a global scale (Beck et al., 2018), with the difference that most munitions have been dumped intentionally, at certain locations and within relatively short periods of time. Therefore, the environmental consequences associated with dumped munitions often occur at local scale and need to be investigated accordingly. With regard to dumped munitions, a general distinction is made between conventional munitions, which mainly contain explosives, and chemical weapons (CW), which contain chemical warfare agents (CWAs). This distinction is also made at several points in this thesis.

The dumping of munitions after the war is, along with activities during the war and maneuvers, the main route through which munitions enter the marine environment. Due to natural and anthropogenic processes, the location of dumped munitions may change over the years, which often makes the location of dumped munition objects difficult (Figure 1.2).





**Figure 1.2.:** Possible entry pathways of munition in the sea. Figure adapted from Böttcher et al. (2011).

The disposal of munitions in the sea has been considered a safe and legal practice until the 1970s, when it was banned by the adoption of the Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter (London Convention) in 1972 (Carton and Jagusiewicz, 2009). In the year 2020, the London Convention has 87 contracting states (International Maritime Organization, 2020). Nowadays, different kinds of munitions are found in the marine environment. On the one hand, discarded munition material (DMM) which is munition that has been mainly disposed from stockpiles. On the other hand, unexploded ordnance (UXO) which is explosive munition prepared for action but not detonated at the time of deployment (Aker et al., 2012; Beck et al., 2018). Unlike DMM, UXO pose a higher potential for detonation because their fuses have been armed and are often intact (Carton and Jagusiewicz, 2009). However, both DMM and UXO contain explosive compounds.

The data on the quantities of dumped munitions are subject to great uncertainty and for many regions they are completely missing. Estimates for German waters are about 1.6 million tonnes of conventional munitions in North Sea and Baltic Sea (Böttcher et al., 2011). For Norwegian waters, estimates range around 200,000 tonnes or more of conventional munitions (Rossland et al., 2010; Voie and Mariussen, 2017). Dumped munitions are found worldwide, for instance in the northeast and the southwest Pacific, the Atlantic, the Mediterranean, the North Sea and the Baltic Sea (Beck et al., 2018). Most of the munitions are located in coastal waters, which were severely affected in

World Wars I and II. But even today, military activities still contribute to the munitions burden in the sea. The US Navy used Vieques Island in Puerto Rico as a military training area from the World War II until 2003, and rough estimates of the amount of bombs and other explosives that were dropped on the Island range up to 85,000 tonnes (Porter et al., 2011).

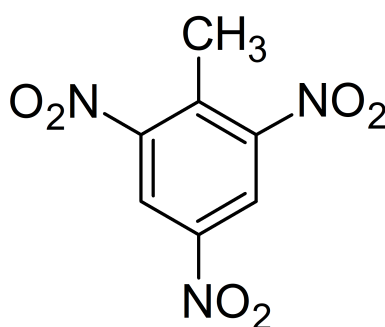
Today, dumped munitions raise international awareness in various areas as the marine environment is being used more and more intensively by the growing economy. There is a direct risk for fishermen who lift the munitions on board with their nets or when munitions are washed up on the beach (Missiaen et al., 2010; Missiaen and Henriët, 2002). The clearance of areas to be used for the construction of wind farms, pipelines or cables is very expensive, as is the clearance of traffic routes (Bełdowski et al., 2016b; Paetzl, 2002).

The release of explosive compounds into the environment is caused by different factors. The exposure of munition shells to the saltwater results in corrosion and breaching of the casings (Rosen et al., 2018; Voie and Mariussen, 2017), which ultimately leads to the release of explosive compounds into the environment. In addition to corrosion, blast-in-place detonations of conventional munitions can spread the munition material across a large area because the munition only partially detonates. Blast-in-place detonations are often used to disarm UXO if they pose a threat at the location where they were found (Pennington et al., 2008). Taylor et al. (2004) estimated that up to 44% of the explosives contained in the intact munitions can remain in pieces on the seafloor after a low order detonation. The exposure caused by different processes, its consequences for the environment and the fate of the munition compounds in organisms have not been clearly understood, which is what this thesis is focusing on.

### **1.3. Explosives used in conventional munitions, their toxicity and environmental fate**

In general, conventional munitions contain various explosive compounds or incendiary mixtures. The most common groups include nitroaromatics, nitramines, nitrate esters and nitrophenols (Craig and Taylor, 2011; Lotufo et al., 2013). Additionally to the explosive compounds, they usually consist of shells made of metals, and plasticizers, metals and stabilizers are contained in the explosive and propellant formulations (Liebezeit, 2002; Tornero and Hanke, 2016).

Military explosives are categorised as primary or secondary explosives. Primary explosives are often termed “initiating explosives” because they are used to detonate secondary explosives due to their high susceptibility for detonation. Common primary explosives are lead azide and lead styphnate (Lotufo et al., 2013). Secondary explosives are less susceptible for detonation and used as main charge for conventional munitions. Common secondary explosives include the nitroaromatic 2,4,6-trinitrotoluene (TNT) and the nitramines hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). As these substances were used in large quantities as explosives in the last century, they are found in dumped munitions in the marine environment. This thesis focuses mainly on the secondary explosive TNT (Figure 1.3), as this is the most frequent explosive present in dumped munitions.



**Figure 1.3.:** Generic structure of 2,4,6-trinitrotoluene (TNT).

TNT was used in World War I and World War II and continues to be used as an explosive in recent times. Usually, it is mixed in explosive formulations in military munitions with other explosives such as RDX, HMX and aluminium powder (e.g. “Composition B” a mixture of RDX, HMX and TNT, or “Schießwolle 39” a mixture of TNT, ammonium nitrate, aluminium powder and hexanitrodiphenylamine) (Böttcher et al., 2011; Lewis et al., 2009; Lotufo et al., 2013). TNT appears as a yellow powder at room temperature, is soluble in water (130 mL at 25 °C) and has a relatively low octanol/water-partition coefficient ( $\log K_{OW}$ ) of 1.6 (Lotufo et al., 2013; Ro et al., 1996). It is prone to undergo photolysis in freshwater and seawater, depending on the temperature, the salinity and the dissolved organic matter (Luning Prak et al., 2017).

The few studies that investigated the contamination of biota with explosive compounds at munition dumpsites identified intact explosives as well as degradation products. Biota sampled at Vieques Island in Puerto Rico contained explosive compounds like TNT, 1,3,5-trinitrobenzene (TNB), 1,3-dinitrobenzene (1,3-DNB) or RDX (Porter et al., 2011). Explosive compounds in fish samples were also detected in starry flounder,

*Platichthys stellatus*, from Jackson Park Housing Complex, USA (Lotufo et al., 2017) and in goatfish from “Ordnance Reef”, Hawaii (Lotufo et al., 2017; University of Hawaii, 2014).

Stable isotope experiments investigating the fate of TNT in marine ecosystems showed that TNT undergoes rapid degradation and simultaneously its degradation products accumulate in the water column, sediments, particulate matter and biota (Smith et al., 2015). The accumulation of contaminants in organisms depends on the contaminant characteristics, the organism and the surrounding environmental conditions. Physico-chemical conditions, such as water solubility or log  $K_{OW}$ , affect the degree of possible uptake, biotransformation and elimination of the contaminant by the organism. The organism itself, including its behaviour, ecology, age and sex, influences the accumulation to a great extent. For example, the lipid content of an organism influences the accumulation of lipophilic contaminants. Lastly, environmental conditions such as salinity, pH or temperature also determine the availability of the pollutant for accumulation (Newman, 2014). These are parameters that are not least affected by climate change. Taking these points into account, TNT exposure experiments with fish showed a rapid biotransformation and subsequent detection of TNT degradation products in fish, which implies the low bioaccumulation potential of TNT (Belden et al., 2005; Mariussen et al., 2018; Ownby et al., 2005). In contrast, the degradation products of TNT accumulate to a greater extent and play a crucial role in understanding the environmental fate of explosive compounds in the marine environment.

The toxicity of TNT has been extensively studied in humans, particularly among workers in munition production plants, and a relationship has been demonstrated between TNT exposure and the development of anaemia and carcinoma (Mallon et al., 2014; Sabbioni et al., 2007; Yan et al., 2002). Several *in vitro* studies have also shown a correlation between the exposure to TNT and mutagenicity in bacteria (George et al., 2001) and genotoxicity in mammalian cells (Honeycutt et al., 1996; Kennel et al., 2000). The acute toxicity of TNT in marine species, especially fish, has also been studied (Lotufo et al., 2010b; Talmage et al., 1999), but no studies investigated the genotoxicity of explosive compounds in fish. However, information on possible genotoxic effects in fish exposed to explosive compounds is essential to identify possible adverse long-term effects. This knowledge is also valuable for future marine management of munition dumpsites. For this reason, this thesis investigates the genotoxicity of nitroaromatic compounds in fish, in order to gain knowledge about the consequences of dumped munitions on fish health and long-term effects such as the carcinogenic potential of TNT.

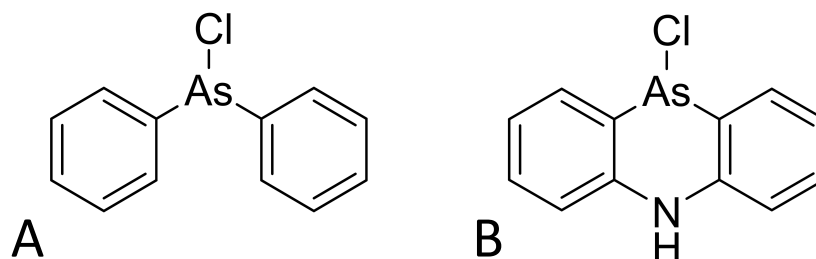
## 1.4. Chemical weapons and chemical warfare agents

Chemical weapons (CW) equipped with chemical warfare agents (CWAs) were produced on a large scale during World War I and II. After the adoption of the Geneva Protocol in 1925 prohibiting the use of CW in battle, the first major CW dumping took place in the 1920s in the English Channel. After World War II, when large quantities of CW remained in German stockpiles, they were disposed by the Allied armies in different ways in accordance with the Potsdam Agreement (Bełdowski et al., 2017, 2016a; Szarejko and Namieśnik, 2009). In the following years, thousands of tonnes of CW were dumped until this practice was prohibited in the 1970s (Carton and Jagusiewicz, 2009). Disposal at the sea was chosen by the Allied armies as a fast and efficient method of demilitarization, especially immediately after the end of World War II under time pressure and was also considered more cost-efficient than disposal on land (HELCOM, 2013). Approximately one million tonnes of CW, mainly originating from both World Wars, were dumped in the oceans, corresponding to 300 suspected dumpsites worldwide, as in the Atlantic, the Pacific and the Indian Oceans, the Black, the Baltic, the Mediterranean and the North Seas (Bełdowski et al., 2017).

A closer look at European waters shows that after World War II, major parts of CW containing thousands of tonnes of CWAs were dumped in the Baltic Sea (50 000 t) and the Skagerrak (170 000 t) (HELCOM, 2013; Paka and Spiridonov, 2002), including entire ships loaded with CW that were sunk in the Skagerrak (Bełdowski et al., 2016a; HELCOM, 1995). Even if most of the CW were dumped in designated dumping areas such as the Norwegian trench, the Bornholm Basin or the Gotland Basin, it is not known how many CW were dumped on the way to these areas (Bełdowski et al., 2017). Furthermore, the ships were not equipped with modern navigation technologies at the time of dumping, which contributes to the uncertainties regarding the position of the dumped CW (HELCOM, 2013).

Regarding intact CW, CWAs are the main component, but they also contain primary and secondary explosives responsible for the detonation of the CW. However, CWAs have also been dumped in containers without fuses. In Europe, most of the dumped CW contain sulphur mustard (Mustard Gas) or arsenic-based warfare agents like Clark I and II, Adamsite and Lewisite (Bełdowski et al., 2017; HELCOM, 2013). Similar to the shells of conventional munitions, corrosion in seawater leads to the release of the contained CWAs into the marine environment. It is likely that the corrosion increase of the years, even though it always depends on the prevailing conditions and the material of the shells (Höher et al., 2019). The release of CWAs from dumped munition materials has been demonstrated by detecting CWA-related compounds in sediments

from different dumping areas (Amato et al., 2006; Briggs et al., 2016; Niemikoski et al., 2020b; Tørnes et al., 2006). This thesis focuses on compounds related to organoarsenic warfare agents such as Clark I and Adamsite (Figure 1.4), which were also dumped in the Baltic Sea and the Skagerrak.



**Figure 1.4.:** Generic structure of Clark I (A) and Adamsite (B).

Clark I (diphenylchloroarsine) and Adamsite (10-chloro-5,10-dihydrophenarsazine) were designed as riot control agents in World War I (Szinicz, 2005) and are classified as CWAs by the Chemical Weapons Convention. Clark I and Adamsite are relatively poorly soluble in water, but exhibit bioaccumulation capabilities according to their  $\log K_{OW}$ . Adamsite in particular is expected to be more persistent in the environment due to its long dissipation half-life (HELCOM, 2013; Sanderson et al., 2008). Under aqueous conditions, Clark I and Adamsite undergo hydrolysis and oxidation (Haas et al., 1998), resulting in oxidation products that may occur in the marine environment. The oxidation product of Clark I, diphenylarsinic acid, and its biotransformation products are known to be more toxic than their parent compound, causing adverse cerebral effects in humans, as well as promoting liver carcinogenesis in rats (Ishii et al., 2004, 2017; Ochi et al., 2006; Wei et al., 2013).

## 1.5. Fate of munition compounds in fish – the key for monitoring

The effects and the degradation of conventional munition compounds in organisms have been studied since the 1940s (Bueding and Jolliffe, 1946), particularly driven by the occupational exposure of workers in munition factories. At that time, the threat to the marine environment caused by dumped munitions was not subject to scientific investigations and research focusing on the implications for the marine environment

developed later. Looking at marine management in the EU, dumped munitions and their hazardous components are not included in any of the descriptors of the MSFD as a mandatory parameter for monitoring. Therefore, they are not subject to management activities. Nevertheless, the issue has received more attention in the recent past and dumped munitions are considered as an important point source of contamination in descriptor 8. The Joint Research Centre of the European Commission therefore recommends that the Member States identify and remediate hotspots, such as munition dumpsites, so that the achievement of the GES is not jeopardized (Law et al., 2010). As part of the national implementation process of the MSFD, Germany has submitted a factsheet to the European Commission in 2016 containing measures for dealing with dumped munitions in the sea, including the development of suitable methods to investigate explosive compounds in the marine environment (BLANO, 2016a,b). These measures generally apply to both conventional munition compounds and chemical warfare agent-related compounds, even though the focus in German waters lies on conventional munition compounds, as these are by far the major contamination problem.

In order to develop suitable methods for the detection of explosive compounds in the marine environment, specifically in fish, target compounds are needed. These target compounds have to be selected considering the fate of explosive compounds in fish. Due to various transformation processes (Beck et al., 2018; Monteil-Rivera et al., 2009), it is unlikely to describe the contamination of fish with explosive compounds by focusing on the parent compounds such as TNT or RDX. Therefore, the focus must be on the investigation of degradation products of explosives in fish, which assigns a key role to the biotransformation in the organism.

Biotransformation describes the metabolic transformation of foreign compounds, such as xenobiotics, in organisms and is able to dramatically change their biological and chemical activity and thus the effects of the compound to the organism. A vast number of different enzymes, including cytochrome P450 monooxygenases or glutathione transferases, are involved in biotransformation processes of xenobiotics (Stegeman and Hahn, 1994). These transformation processes can generally be distinguished into oxidation, reduction and hydrolysis reactions (phase I) and conjugation reactions (phase II), which ultimately aim to make the compound more hydrophilic and thus facilitate its elimination (Parkinson, 2001). The reduction of the nitro groups is an important step during the biodegradation of TNT, which is observed in bacteria, fungi and higher organisms, leading to the formation of amino-dinitrotoluenes (2-ADNT, 4-ADNT) and further to di-amino-nitrotoluenes (2,6-DANT, 2,4-DANT) (Juhasz and Naidu, 2007; Monteil-Rivera et al., 2009). So far, further reduction to triaminotoluene (TAT) has

only been observed under anaerobic conditions (Hawari et al., 1998; Preuss et al., 1993). The nitro group reduction of TNT is most likely catalyzed by a cytochrome P450 reductase found in the liver, as shown in *in vivo* experiments with rats by Shinkai et al. (2016). Furthermore, the intestinal microflora is considered to be important for the reduction of nitro groups due to its anaerobic environment and may be responsible for the genotoxicity of certain nitroaromatic compounds (Mirsalis and Butterworth, 1982; Parkinson and Ogilvie, 2008).

Considering the biotransformation of TNT in fish, the accumulation of ADNTs or DANTs has been observed in experimentally exposed species, like channel catfish, *Ictalurus punctatus* (Belden et al., 2005; Ownby et al., 2005), and Atlantic salmon, *Salmo salar* (Mariussen et al., 2018). It was also shown that biotransformation products accumulate to a greater extent in *Ictalurus punctatus* than TNT itself. However, parts of the extractable biotransformation products remained unidentified (Ownby et al., 2005). Despite various experimental exposure studies and a few studies that investigated the contamination status of fish in the marine environment (Lotufo et al., 2017; Porter et al., 2011; University of Hawaii, 2014), so far no study has been conducted to examine the contamination of fish from the Baltic Sea, which contains heavily polluted munition dumpsites. Information on the contamination status of fish is essential to better assess the impact of munition dumpsites on the marine environment in the Baltic Sea. For this purpose, this thesis investigates the metabolism and the detection of explosive compounds in dab (*Limanda limanda*), a flatfish species commonly found in the Baltic Sea.

The fate of CWA-related phenylarsenic compounds in the marine environment and particularly in organisms is poorly understood. The research on their metabolism in organisms has focused only on diphenylarsinic acid in the context of contaminated drinking water in Japan (Ishii et al., 2004). However, a recent study has shown that diphenylarsinic acid has been detected in marine biota samples caught in the vicinity of dumped CWAs (Niemikoski et al., 2017). This indicates that marine organisms take up CWA-related phenylarsenic chemicals from dumped munitions, a source of contamination that has not been considered so far. In order to identify CWA-related phenylarsenic chemicals in fish, research on the fate, specifically on the biotransformation of these chemicals in marine biota is necessary and is part of this thesis.



### **1.5.1. Bioanalytical approaches to investigate the fate of explosive compounds**

In order to specifically study the metabolism of explosive compounds such as TNT in fish, experimental approaches allowing repetitive studies of biotransformation under controlled conditions are required. *In vivo* and *in vitro* experiments are commonly used to meet this requirement, with advantages and disadvantages conversely. Investigating the metabolism of a certain compound *in vivo* represents the entire biotransformation process inside a living organism, but it is usually associated with sophisticated experiments and a high administrative burden in terms of animal welfare. In contrast, *in vitro* experiments usually offer a faster, more cost-efficient, and a reproducible way to map parts of biotransformation processes in living cells. For this purpose, the liver in fish is a suitable tissue, as it is known to contain a large number of important biotransformation enzymes, as in most vertebrates (Stegeman and Hahn, 1994). *In vitro* metabolism experiments have also been used to study the biotransformation of TNT (Leung et al., 1995; Shinkai et al., 2016) and CWA-related compounds (Halme et al., 2015) using rat liver homogenates. Although common biotransformation enzymes, as cytochrome P450 or glutathione *S*-transferase, also occur in most other vertebrates, there are variations in the composition of the enzymes depending on the species, the tissues and their respective substrate specificity (Stegeman and Hahn, 1994).

Fish liver has not yet been used to study the *in vitro* biotransformation of munition compounds, which implies uncertainty regarding their metabolism. In this thesis the biotransformation of TNT with dab (*Limanda limanda*) liver was investigated *in vitro* and additionally the biotransformation of CWA-related phenylarsenic compounds with cod (*Gadus morhua*) liver was studied using the same methodology.

### **1.5.2. Chemical analytical methods to identify explosive compounds**

In general, when investigating the toxicity or fate of chemicals in organisms, analytical methods are required to quantify the chemicals used for exposure or to identify degradation products. This also applies to munition compounds when studying their toxicity and fate in fish.

Various analytical methods are used to identify explosive compounds in laboratory or environmental samples, often involving solvent extraction, separation by high performance liquid chromatography (HPLC) or gas chromatography (GC) (Appel et al.,

2018; Ek et al., 2006) and detection by UV-VIS (US Environmental Protection Agency, 2007). In order to improve the identification of compounds, mass spectrometry (MS) using different types of ionization is more frequently applied in recent studies and achieves a high sensitivity (Gledhill et al., 2019; Mariussen et al., 2018; Rapp-Wright et al., 2017; Xu et al., 2014). It should be mentioned that the comparison of concentrations of explosive compounds in environmental samples from different studies is difficult because the methods often differ in the extraction procedure and in the analytical method itself, or because the matrices examined are not comparable with regard to their chemical properties (Beck et al., 2018).

## **1.6. Aims and objectives of the thesis**

This thesis was carried out within the framework of the international project DAIMON (Decision Aid for Marine Munitions), which aimed to provide a basis for decisions on the future management of dumped munitions in the sea. Future decisions should take into account the most recent scientific findings in order to facilitate targeted management. As one component for the development of a future management of dumped munitions, this thesis concentrates on the effects of dumped munitions on marine organisms and fish in particular. Three main topics are addressed: 1) The toxicity, 2) the metabolism and 3) the detection and identification of explosive compounds in fish from the Baltic Sea. The topic of the metabolism of munition compounds was further investigated by studying the metabolism of CWA-related phenylarsenic compounds in fish. The following research questions were addressed in four chapters, as described below:

### **1.6.1. Genotoxicity of nitroaromatic compounds**

In order to assess the risk posed by dumped munitions to the marine environment, and in particular to fish, toxicity thresholds are necessary which relate a specific effect in the organism to a certain aqueous exposure concentration of explosive compounds. To obtain a comprehensive overview of the risk, data on acute toxicity, but also on cytotoxicity, mutagenicity and genotoxicity of explosive compounds are important. Several studies provide threshold values for the acute toxicity of explosive compounds in fish (Liu et al., 1983; Lotufo et al., 2010b; Talmage et al., 1999). Data on the genotoxicity of TNT in fish are lacking in general and studies with mammalian cells have produced partly contradictory results on the genotoxicity of TNT (Ashby et al., 1985; Honeycutt

et al., 1996; Kennel et al., 2000; Lachance et al., 1999). To fill this gap, the genotoxicity of TNT and two of its degradation products was investigated by exposing zebrafish embryos (*Danio rerio*), a common model fish species in ecotoxicology (Strähle et al., 2012), *in vivo* and detecting DNA strand breaks in single cells.

Objective 1: *Do 2,4,6-trinitrotoluene and its primary degradation products cause genotoxic effects in fish and are there differences in the level of effect?*

### **1.6.2. Metabolism of compounds from dumped munitions**

The detection of compounds originating from conventional munitions or CWAs in fish requires knowledge about the biotransformation of the parent compounds in the specific fish species. Since nitroaromatic compounds are known to be rapidly degraded in the marine environment (Ballentine et al., 2015; Belden et al., 2005; Lotufo et al., 2009; Rosen and Lotufo, 2007), biotransformation products are also of great interest when investigating the total body burden of these explosive compounds in fish. The same applies to phenylarsenic CWAs, as they are prone to undergo hydrolysis in the water (Haas et al., 1998; Missiaen et al., 2010) and may be subject to further biotransformation reactions in fish. Therefore, understanding the biotransformation of compounds from dumped munitions is crucial to monitor contamination in the marine environment or to detect any contamination in seafood. Biotransformation reactions of compounds related to conventional munitions and CWAs were studied *in vitro* using two fish species common in the Baltic Sea, dab (*Limanda limanda*) and cod (*Gadus morhua*).

Objective 2: *Which biotransformation products of 2,4,6-trinitrotoluene and chemical warfare agent-related phenylarsenic chemicals can be found in fish from the Baltic Sea, using *Limanda limanda* and *Gadus morhua* as common species, and could be used for monitoring purposes?*

### **1.6.3. Detection of explosive compounds in Baltic flatfish**

Only few studies have investigated the contamination of wild fish with explosive compounds in the vicinity of munition dumpsites, showing great differences in the contamination, which make it difficult to extrapolate to other locations (Lotufo et al., 2017; Porter et al., 2011; Rosslund et al., 2010; University of Hawaii, 2014). Despite the fact that some heavily polluted munition dumpsites are present in the Baltic Sea, no investigation has yet been carried out to detect explosive compounds in a common fish

species in the Baltic Sea. Information on whether fish in the Baltic Sea are contaminated with explosive compounds and to what extent this is the case is valuable for a targeted management of dumped munitions in the future. To this end, dab (*Limanda limanda*) were sampled in the vicinity of a known munition dumpsite and a reference site in Kiel Bight and explosive compounds were detected in their bile.

Objective 3: *What is the actual level of contamination with explosive compounds in fish from a munition dumpsite in the Baltic Sea?*

## **2. Nitroaromatic compounds damage the DNA of zebrafish embryos (*Danio rerio*)**

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## 2.1. Abstract

Lethal and sublethal effects of trinitrotoluene (TNT) and its degradation products 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) to zebrafish embryos (*Danio rerio*) were investigated in a 120 h exposure scenario. Lethal concentrations (LC<sub>50</sub>) were 4.5 mg/L for TNT, 13.4 mg/L for 2-ADNT and 14.4 mg/L for 4-ADNT. Embryos exposed to 2-ADNT or 4-ADNT revealed a high proportion of chorda deformations among the surviving individuals. Genotoxicity of the nitroaromatic compounds in zebrafish embryos was investigated by comet assay isolating cells from whole embryos after 48 h *in vivo* exposure. Significant genotoxicity was induced by all three compounds tested, in comparison to the corresponding controls at 0.1 mg/L and 1 mg/L as lowest tested concentrations. The genotoxicity caused by TNT was about three to four times higher than that of 2-ADNT and 4-ADNT. To our knowledge, this is the first study demonstrating the genotoxicity of TNT in fish embryos by *in vivo* exposure. The results are discussed in the context of dumped munition in the marine environment.

**Abbreviations:** TNT, 2,4,6-trinitrotoluene; 2-ADNT, 2-amino-4,6-dinitrotoluene; 4-ADNT, 4-amino-2,6-dinitrotoluene.

## 2.2. Introduction

We are facing a global problem of conventional munition contamination in the marine environment (Beck et al., 2018). The main contaminants are organic explosives containing nitroaromatic compounds such as trinitrotoluene (TNT) (Jenkins et al., 2012). This environmental burden is the consequence of historical improper, intentional disposal of ammunition since World War I as well as leftovers from training and combat operations or wastewater from production facilities (Beddington et al., 2005; Juhasz and Naidu, 2007; Lotufo, 2017; Voie and Mariussen, 2017). Until the 1970s, dumping ammunition at sea was a commonly used disposal method, causing a large-scale contamination of the aquatic environment with conventional munition (Carton and Jagusiewicz, 2009). Considering the German waters, it is estimated that approximately 1.6 million tonnes of conventional munition are still lying on the seafloor in the North Sea and Baltic Sea (Böttcher et al., 2011).

The condition of the munition shells in seawater can deteriorate over time due to corrosion and increase the risk of environmental contamination (Beck et al., 2018; Beddington et al., 2005). Furthermore, during blow-in-place operations, low-order detonations scatter TNT-containing ammunition components across the seafloor, which can then directly enter the water column (Juhasz and Naidu, 2007). The detection of nitroaromatic compounds in marine biota (Gledhill et al., 2019; Strehse et al., 2017) proves their release into the water and the uptake of these substances by marine organisms. Consequently, fish living in contaminated areas are exposed to nitroaromatic compounds. And the question, whether this leads to long-term effects with an impact on fish health, has not yet been clarified.

TNT toxicity has been studied particularly in humans, such as workers occupationally exposed to ammunition, and profound toxicological effects like aplastic anaemia, toxic hepatitis and liver cancer have been identified (Sabbioni and Rumler, 2007; U.S. Department of Health and Human Services and Registry, 1995). Information on environmental toxicity is less frequent, but different studies investigated the acute toxicity of TNT in fish and different species revealed toxicity thresholds between 0.8 mg/L to 7.6 mg/L (Liu et al., 1983; Lotufo et al., 2010b; Nipper et al., 2001; Talmage et al., 1999). Compared to TNT, its primary degradation products 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) (Mariussen et al., 2018) appeared to be less toxic with  $LC_{50}$  values between 6.9 mg/L to 15.1 mg/L (Liu et al., 1983; Lotufo et al., 2010b). Genotoxicity of TNT and its degradation products was investigated using various *in vitro* approaches with *Salmonella* strains (Ahlborg et al., 1988;

George et al., 2001; Spanggord et al., 1982), revealing mutagenicity of TNT and aminodinitrotoluenes (ADNTs). Positive as well as negative genotoxicity was observed in different mammalian cell lines (Honeycutt et al., 1996; Kennel et al., 2000; Lachance et al., 1999). Carcinoma were observed in rats after long-term *in vivo* exposure to TNT (Furedi et al., 1984a), demonstrating the possible severe chronic effects of TNT exposure. Although it is of great interest to gather information on the actual genotoxic risk for fish, the genotoxicity of nitroaromatic compounds has been less well investigated with *in vivo* approaches.

In the absence of toxicological data on the genotoxicity of TNT and its primary degradation products in fish using *in vivo* approaches, we here exposed zebrafish embryos (*Danio rerio*) to different concentrations of TNT, 2-ADNT and 4-ADNT to derive acute toxicity thresholds and to investigate the genotoxic potential of these nitroaromatic compounds in a second step. Therefore, for the first time, we evaluated whether nitroaromatic compounds from dumped munition are genotoxic to fish and discussed the results related to fish health at ammunition dumpsites.



## 2.3. Material and methods

### 2.3.1. Fish embryo toxicity assay

Adult wild type zebrafish (*Danio rerio*) were used for egg production and maintained in 160 L tanks at  $26 \pm 1$  °C in a 14:10 light-dark cycle. Once a week the water was changed and filters cleaned to ensure good water quality. The fish were fed twice a day with commercial dry flakes (Tetra, Germany) and additionally once a week with deep-frozen Chironomidae larvae (Tetra, Germany). Egg collection took place in spawning trays, 1 h after beginning of the light period.

Exposure was performed in polypropylene 96-well plates (2-ADNT, 4-ADNT, 200  $\mu$ L per well) and in glass dishes (TNT, 10 mL per beaker). To account for possible adhesion of ADNTs to the plastic surface, 96-well plates were filled with the corresponding test solution the day before the start of the test to saturate the surface. ISO water (400  $\mu$ M  $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$ , 100  $\mu$ M  $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$ , 155  $\mu$ M  $\text{NaHCO}_3$  and 15.5  $\mu$ M KCl, according to OECD guideline 203, Annex 2 (1992)) was used for dilution of nitroaromatic compounds and all test solutions were aerated overnight. Dimethyl sulfoxide (DMSO, 99.9%) was used as solvent for 2-ADNT and 4-ADNT and solvent concentration in any test solution did not exceed 0.1%. Twenty-four fertilized eggs (< 1.5 h post fertilization, hpf) were exposed to each of the corresponding test solutions, including negative, positive and solvent control. Embryos were incubated for 120 h at  $26$  °C at a 14:10 h photoperiod. To ensure steady exposure, 80% of the test solutions were replaced after every 24 h. At 24 hpf, 48 hpf and 120 hpf, the development of embryos was assessed for sublethal and lethal effects according to Braunbeck and Lammer (2006). To ensure the validity of the tests, 3,4-dichloroaniline (4 mg/L) was used as a positive control and mortality had to exceed at least 30% (OECD, 2013). Mortality in the negative control (ISO water) and solvent control (0.1% DMSO) should not surpass 10% by the end of the test. Seven concentrations of each nitroaromatic compounds were tested on the embryos in the acute scenario; 0.5, 0.82, 1.36, 2.24, 3.68, 6.07, 10 mg/L of TNT and 3.5, 4.86, 6.74, 9.35, 12.98, 18.02, 25 mg/L in case of 2-ADNT and 4-ADNT. TNT was obtained in dissolved form (Fraunhofer ICT, Pfinzthal). The stock solution was prepared using solid TNT (“military grade”) which was dissolved in deionized water at  $60$  °C, protected from light. 2-ADNT and 4-ADNT were purchased as analytical standard (Sigma-Aldrich, Germany).

To perform the comet assay, zebrafish embryos were exposed for 48 h according to the procedure described above. The following concentrations of nitroaromatic compounds

were tested; 0.1, 0.27, 0.73, 2 mg/L of TNT and 1, 2.29, 5.24, 12 mg/L in case of 2-ADNT and 4-ADNT. The actual concentrations of the test solutions were detected by HPLC-MS (see HPLC-MS quantification). Each test (TNT, 2-ADNT, 4-ADNT) was repeated three times for the acute scenario and comet assay. Hydrogen peroxide ( $H_2O_2$ ) was used as positive control for the comet assay as it is known to cause severe DNA damage (Dietrich et al., 2005; Hook and Lee, 2004). For this purpose, 20 of 48 h-old embryos were exposed to 0.35%  $H_2O_2$  solution for 20 min and then used for cell isolation.

### **2.3.2. Cell isolation from zebrafish embryos**

To obtain single cells from zebrafish embryos for performing comet assay, a mechanical isolation procedure according to Kosmehl et al. (2006, 2008) was used with minor modifications. After exposing the embryos for 48 h, 20 surviving embryos of each treatment and control were pooled and immersed in a saturated benzocaine solution prior to isolation for anaesthesia, as no impairment of the results by benzocaine is to be expected (de Miranda Cabral Gontijo et al., 2003). A glass/glass homogenizer (Potter-Elvehjem type, Sartorius, Germany) was used for disintegration of embryos in the presence of 1.3 mL phosphate-buffered saline (PBS). Subsequently, the homogenates were filtered through 70  $\mu$ m filter mesh into 1.5 mL reaction tubes. The reaction tubes were centrifuged at 2000 g for 10 min at 4 °C, resuspended in 500  $\mu$ L PBS and then centrifuged at 1800 g for 7 min at 4 °C. Afterwards, the pellets were resuspended in a final volume of 150  $\mu$ L PBS and used for comet assay and trypan blue assay.

### **2.3.3. Trypan blue assay for cell viability**

Cell viability was assessed by trypan blue exclusion test and only cell suspensions with more than 75% viability were used for the comet assay. To determine the number of viable cells in the samples, 1 part of 0.4% trypan blue was mixed with 1 part of the cell suspension diluted in PBS (Strober, 1997). The mixture was allowed to incubate for 3 min and a haemocytometer was used to count viable and nonviable cells at 200 x magnification.

#### **2.3.4. Comet assay**

The comet assay procedure was carried out according to Singh et al. (1988) with minor modifications according to Kosmehl et al. (2008). Frosted microscope slides (Menzel Gläser, Germany) were cleaned with ethanol (99%) and a first layer of 0.5% normal melting agarose (Serva, Germany) was applied to the surface. Zebrafish cell suspensions were diluted 1:2 (volume) with 0.7% low melting agarose (Serva, Germany), placed on top of the first layer and hardened on ice for 3 min. As a third layer, 100  $\mu$ L of 0.7% low melting agarose was placed on top of the cell suspension and cooled on ice for 3 min for solidification. Two slides per sample were prepared and treated equally during the whole experiment.

The slides were incubated in refrigerated lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% Triton X-100) for 1.5 h in the dark. After lysis, the slides were placed horizontally in cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) to unwind the DNA for 20 min and electrophoresis (22 V, 300 mA) was then performed for 17 min. Finally, the slides were put into neutralization buffer (0.4 M Tris, 0.2 M HCl, pH 7.5) for 20 min and stored in PBS at 4 °C until analysis. Midori Green dye (Biozym, Germany) (40  $\mu$ L) was used to stain DNA and visualize DNA damage under fluorescent light.

The slides were analysed at 200 x magnification using a Zeiss Axioskop 50 microscope with mercury lamp and a Nikon DS-Fi3 camera. For each concentration 80 randomly selected nucleoids (40 from each slide) were measured using CASP (Końca et al., 2003) as image-analysis program and the DNA damage was quantified as “% tail DNA” and “tail moment”.

#### **2.3.5. HPLC-MS quantification of actual concentration of nitroaromatic compounds**

In order to measure the actual concentrations of TNT, 2-ADNT and 4-ADNT in the test solutions, 20  $\mu$ L of each sample were diluted 1:1, 1:2, 1:3 or 1:4 (v/v) with acetonitrile (99.9%) according to their nominal concentrations.

From each solute dilution 5  $\mu$ L were injected on the column in an Agilent 1290 Infinity High-Performance-Liquid-Chromatograph (HPLC) coupled to an AB Sciex QTrap 5500 Triple Quadrupole/Ion-Trap Mass Spectrometer (QQQ-MS). Gradient separation was achieved using an Acclaim Explosives E2 Column (Thermo Fisher Scientific) kept at 22 °C, with eluents A: H<sub>2</sub>O (10 mM ammonium acetate + 2.7 mL acetic acid (99%)),

pH 4) and B: Methanol (99.9%) (10 mM ammonium acetate + 2.7 mL acetic acid) starting at 45% B increasing to 95% B in multiple steps over 45 min keeping a constant flow rate of 0.3 mL/min.

Ionization was conducted in negative atmospheric pressure chemical ionization (APCI) mode. TNT and selected metabolites were detected via multiple reaction monitoring (MRM) mode based on characteristic MS/MS transitions, previously optimized using commercially available standard substances. Abundances of explosives and metabolites were quantified via the internal standard 1,4-dinitrobenzene and corrected for response factors of commercially available standards. Response factors were determined using an external calibration covering the expected range of concentrations. Limits of detection (LOD) and limits of quantitation (LOQ) for TNT detected with MS were 0.01 ng (LOD) and 0.02 ng (LOQ) on column.

### **2.3.6. Statistical analysis**

Statistical analyses were carried out using R Version 3.3.1 (R Core Team, 2019). Effective concentrations and lethal concentrations for acute toxicity of 2-ADNT, 4-ADNT and TNT were derived by applying a four-parameter log-logistic model to the data using the “drc-package” (Ritz and Streibig, 2005). For genotoxicity data, a generalized linear model (GLM) with gamma probability distribution was applied to test for differences in genotoxicity between control and treatments. A Tukey’s all-pair comparison was used to determine which treatments had different genotoxicity compared to the control.

## 2.4. Results

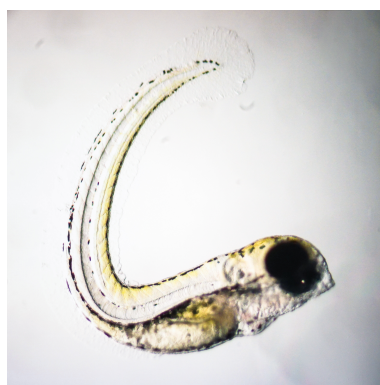
### 2.4.1. Acute toxicity of nitroaromatic compounds

Concentrations of nitroaromatic compounds in stock solutions were measured to confirm the nominal concentrations used in the experiments and are shown in appendix A (Table A.1). Especially, the high concentrations tested for 2-ADNT (18.02 mg/L and 25 mg/L) and 4-ADNT (25 mg/L) contained considerably lower quantities of the corresponding compound. For calculating toxicity threshold values, the measured concentrations of 2-ADNT, 4-ADNT and TNT were used. Survival in all controls (negative control, solvent control) remained higher than 90% after 120 h. The 120 h LC<sub>50</sub> values for 2-ADNT (13.4 mg/L) and 4-ADNT (14.4 mg/L) were around 3 times higher than for TNT (4.5 mg/L) (Table 2.1). Calculated EC<sub>10</sub> values for 2-ADNT (9.2 mg/L), 4-ADNT (10.3 mg/L) and TNT (2 mg/L) were correspondingly lower.

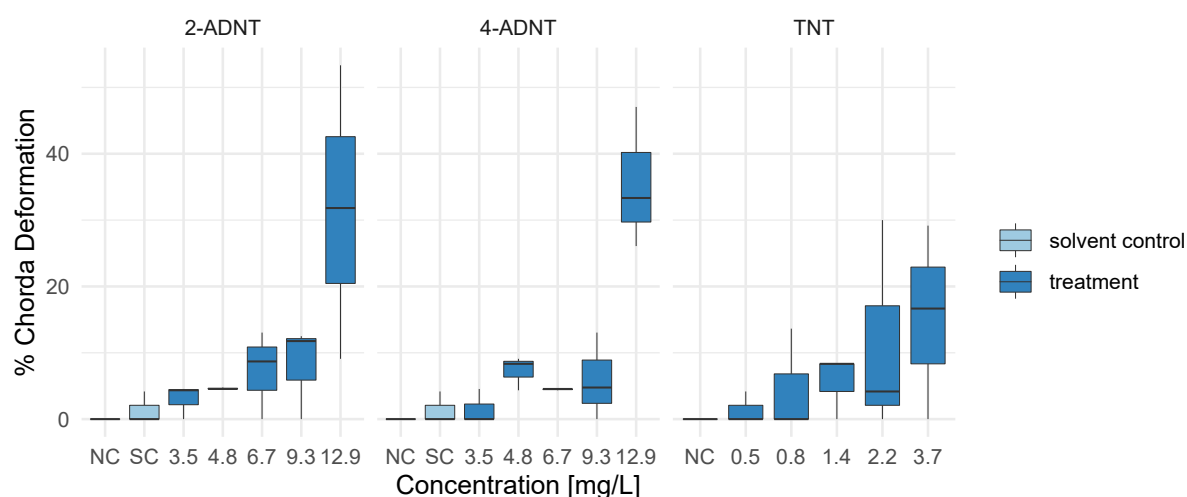
**Table 2.1.:** Acute toxicity of nitroaromatic compounds to zebrafish embryos. Effective concentrations (EC<sub>10</sub>, EC<sub>50</sub>) and lethal concentrations (LC<sub>50</sub>) were calculated using a 120 h exposure scenario. Standard errors (SE) are estimated for the corresponding EC<sub>10</sub>, EC<sub>50</sub> and LC<sub>50</sub>. Number of individuals per replicate, n = 24.

Compound	EC <sub>10</sub> ± SE (mg/L)	EC <sub>50</sub> ± SE (mg/L)	LC <sub>50</sub> ± SE (mg/L)
2-ADNT	9.2 ± 0.5	10.6 ± 0.3	13.4 ± 2.1
4-ADNT	10.3 ± 0.4	12.5 ± 0.2	14.4 ± 0.5
2,4,6-TNT	2.0 ± 0.3	3.3 ± 0.2	4.5 ± 1.2

The examination of embryogenesis during the exposure period revealed in particular chorda deformation of the embryos as a sublethal endpoint (Figure 2.1). For all nitroaromatic compounds tested, increasing proportions of chorda deformations were observed in surviving embryos after 120 h with rising concentration (Figure 2.2). About 30% of surviving embryos exposed to 2-ADNT or 4-ADNT suffered chorda deformations. The highest concentration of TNT considered (3.7 mg/L) led to a mean proportion of chorda deformation of 15% in surviving embryos. In addition, all embryos exposed to TNT exhibited hypo-pigmentation between 48 h and 96 h.



**Figure 2.1.:** Chorda deformation in a zebrafish embryo after exposure to 2-ADNT at 120 hpf.

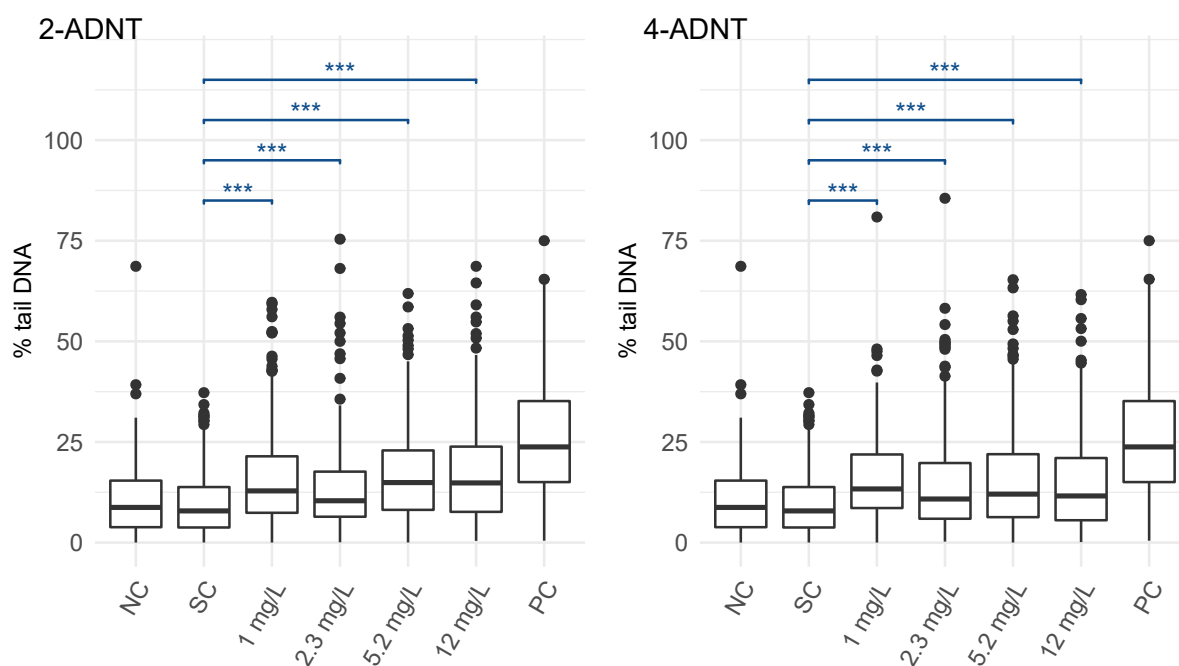


**Figure 2.2.:** Deformation of chorda structure in zebrafish embryos caused by nitroaromatic compounds. The percentage of chorda deformations in living embryos was calculated after 120 h. Only treatments with a mortality rate of less than 50% were considered. NC, negative control (ISO water); SC, solvent control (DMSO).

#### 2.4.2. Genotoxicity of nitroaromatic compounds

The degree of DNA damage caused by 2-ADNT and 4-ADNT in cells from zebrafish embryos was assessed using the comet assay (Figure 2.3). All treatments led to a significant increase of DNA content in the tail (% tail DNA) compared to solvent control and 2-ADNT and 4-ADNT revealed a comparable pattern of DNA damage in zebrafish embryo cells. No clear dose-response relationship was found for both compounds, although the highest treatment resulted in the highest median percentage of tail DNA.

The median tail DNA percentages of the controls ranged from 7% to 8%, whereas already 1 mg/L of 2-ADNT and 4-ADNT caused a median of 12% to 13% tail DNA after 48 h of exposure (Figure 2.3). A significant increase in DNA damage was also observed for the positive control ( $H_2O_2$ ) compared to the solvent control. The HPLC-MS analysis of the freshly prepared ADNT test solutions revealed that the mean measured concentrations exceeded the nominal concentrations by 103% to 134% for 2-ADNT and by 108% to 141% for 4-ADNT (appendix A, Table A.2).

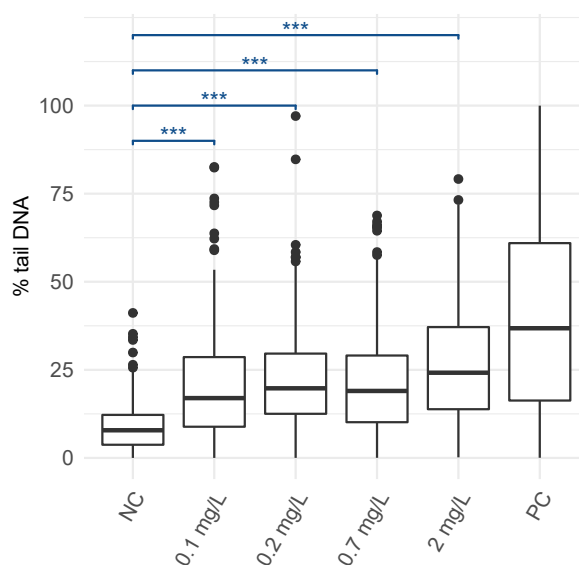


**Figure 2.3.:** Genotoxicity (% tail DNA) in cells from zebrafish embryos after 48 h *in vivo* exposure to 2-ADNT and 4-ADNT. % Tail DNA is given as boxplot showing the median (tick line), the 25<sup>th</sup> and 75<sup>th</sup> percentiles (box), and the lower and upper whiskers extend from the box no further than  $1.5 \times$  inter-quartile range. Treatments contain 3 replicates ( $n = 20$ ) with 80 individual cells each. NC, negative control; SC, solvent control (DMSO); PC, positive control ( $H_2O_2$ ). Significance code, treatments compared to solvent control: \*\*\*  $p < 0.001$  according to Tukey multiple comparisons.

TNT exposure caused higher genotoxicity in zebrafish embryos compared to ADNT exposure in terms of tail DNA content (% tail DNA), even at lower concentrations (Figure 2.4). The lowest tested TNT concentration (0.1 mg/L) led to a median tail DNA content of around 16% which constitutes a significant difference compared to the control. In the tested range from 0.1 mg/L to 2 mg/L TNT, a dose-response relationship was observed and 2 mg/L TNT caused almost a median of 25% tail DNA in single cells

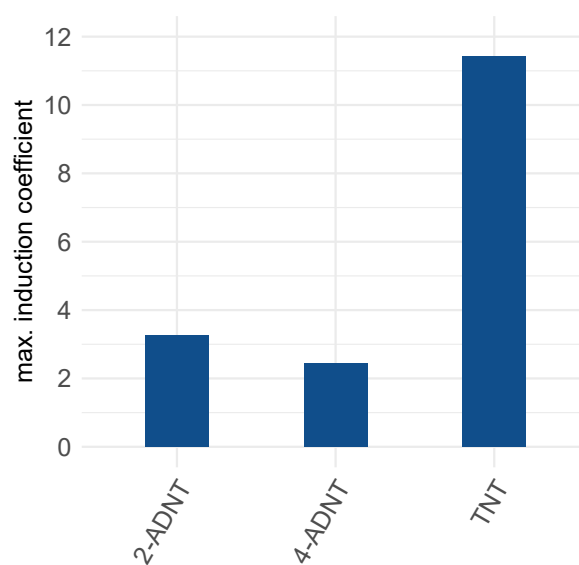
from zebrafish embryos. Chemical analysis of the test solutions confirmed that the mean measured TNT concentrations corresponded to 78% to 120% of the nominal concentrations (Table A.2).

For comparing the different genotoxic potentials of nitroaromatic compounds in the comet assay the maximum genotoxic induction for each compound is shown in Figure 2.5. According to the induction coefficient, genotoxicity in zebrafish embryos induced by TNT is three to four times higher than that of 2-ADNT or 4-ADNT.



**Figure 2.4.:** Genotoxicity (% tail DNA) in cells from zebrafish embryos after 48 h *in vivo* exposure to TNT. % Tail DNA is given as boxplot showing the median (tick line), the 25<sup>th</sup> and 75<sup>th</sup> percentiles (box), and the lower and upper whiskers extend from the box no further than 1.5×inter-quartile range. Treatments contain 3 replicates (n = 20) with 80 individual cells each. NC, negative control; PC, positive control (H<sub>2</sub>O<sub>2</sub>). Significance code, treatments compared to control: \*\*\* p < 0.001 according to Tukey multiple comparisons.





**Figure 2.5.:** Maximum genotoxic induction coefficient of 2-ADNT, 4-ADNT and TNT in cells from zebrafish embryos after 48 h *in vivo* exposure. The genotoxic induction was calculated by dividing the maximum median tail moment of the treatment by the median tail moment of the corresponding control according to Kosmehl et al. (2008).

## 2.5. Discussion

### 2.5.1. Acute toxicity and sublethal effects

In the framework of dumped munition, the aim of this study was to clarify the potential genotoxic threat to fish posed by nitroaromatic compounds. Furthermore, this study was designed to investigate differences in the genotoxic potential of nitroaromatic compounds with respect to their biological transformation in fish.

The acute toxicity experiments with zebrafish embryos revealed an LC<sub>50</sub> of 4.5 mg/L for TNT. This threshold is slightly higher compared to LC<sub>50</sub> values for other fish species like sheepshead minnow (*Cyprinodon variegatus*, 1.7 mg/L, 96 h) (Lotufo et al., 2010b), rainbow trout (*Oncorhynchus mykiss*, 0.8 mg/L, 96 h) (Talmage et al., 1999) or fathead minnow (*Pimephales promelas*, 2.7 - 3.0 mg/L, 96 h) (Liu et al., 1983; Yoo et al., 2006). However, the results indicate a similar sensitivity of zebrafish to TNT compared to other freshwater fish species. Compared to TNT toxicity in freshwater fish, the aquatic invertebrate *Daphnia magna* (LC<sub>50</sub> 11.7 - 11.9 mg/L) (Liu et al., 1983; Martins et al., 2007) is less sensitive. Toxicity experiments with redfish embryos (*Sciaenops ocellatus*) as marine species revealed also a slightly greater tolerance against TNT exposure (EC<sub>50</sub> 8.2 mg/L, 48 h) (Nipper et al., 2001).

Acute toxicity of 2-ADNT (LC<sub>50</sub> 13.4 mg/L) and 4-ADNT (LC<sub>50</sub> 14.4 mg/L) in zebrafish embryos was comparable and the toxicity thresholds were around three times higher than for TNT in the same exposure scenario. These thresholds are also in the same order of magnitude as for other freshwater fish species, with LC<sub>50</sub> values for 2-ADNT (8.6 mg/L, 15.1 mg/L) and 4-ADNT (6.9 mg/L) (Liu et al., 1983; Lotufo et al., 2010b) and emphasize that nitroreduction of TNT decreases its toxicity to fish. In case of ADNT toxicity, freshwater invertebrates like *Daphnia magna* or *Hyalella azteca* seem to be more sensitive (Johnson et al., 1994; Liu et al., 1983; Sims and Steevens, 2008) than zebrafish embryos. The comparable toxicity of 2-ADNT and 4-ADNT in zebrafish embryos observed in this study is consistent with the observation that no relationship was found between acute toxicity in fish and the structure of ADNT isomers (Bailey and Spangford, 1983).

The exposure of zebrafish embryos to nitroaromatic compounds revealed chorda deformations as a conspicuous sublethal effect, especially after ADNT exposure. Same effects were observed in fathead minnow larvae exposed to TNT (Yoo et al., 2006) and also for other nitroaromatic compounds such as nitrophenols (Ceylan et al., 2016). As a further striking effect hypo-pigmentation was obvious in zebrafish embryos exposed

to TNT, suggesting a suppression of melanin synthesis during the embryogenesis (Eum et al., 2016). Hypo-pigmentation was also described in early life stages of zebrafish for nitrophenols (Ceylan et al., 2016) and 2,4-dinitrotoluene (Schmidt et al., 2016), implying that the mode of action of nitroaromatic compounds causes hypo-pigmentation as a sublethal effect. However, hypo-pigmentation of zebrafish embryos was also observed after exposure to other chemicals such as bromophenols and bromoindoles (Kammann et al., 2006), indicating that hypo-pigmentation is not exclusively related to nitroaromatic compounds.

### 2.5.2. Genotoxicity of nitroaromatic compounds

In the present study, TNT, 2-ADNT and 4-ADNT caused significant genotoxic effects in zebrafish embryos exposed *in vivo* for 48 h compared to the corresponding controls at 0.1 mg/L and 1 mg/L as lowest tested concentrations. In accordance to the higher acute toxicity of TNT compared to ADNTs, the genotoxicity quantified using the comet assay revealed the same relationship. Especially in the case of 2-ADNT and 4-ADNT, genotoxicity revealed a hump in the dose-response relationship between the two lowest concentrations tested. A comparable pattern has already been described as a biphasic dose-response curve for genotoxic effects in zebrafish (Boettcher et al., 2011) and might be caused by repair mechanisms induced by higher concentrations (Ehrenberg et al., 1983).

TNT induced three to four times higher genotoxicity in zebrafish embryos than 2-ADNT or 4-ADNT. No further published data exist regarding the genotoxicity of TNT and its primary degradation products in fish. However, mutagenicity of TNT has been shown in several *in vitro* studies testing base-pair substitution and frameshift mutations in *Salmonella* strains or *Vibrio fischeri* (Ahlborg et al., 1988; Frische, 2002; George et al., 2001; Spanggord et al., 1982). Similarly, 2-ADNT and 4-ADNT revealed their mutagenicity in these test systems, with a slightly higher mutagenic potential for 2-ADNT (George et al., 2001; Spanggord et al., 1982). Mutagenicity of TNT was also proved using mammalian cells from Chinese hamster ovaries and also 4-ADNT caused a significant increase in mutation frequency (Kennel et al., 2000). Since negative genotoxicity results for TNT have also been found in mammalian cell lines including enzyme activation (Honeycutt et al., 1996; Lachance et al., 1999), it is obvious that the genotoxicity of TNT is influenced by the cell type and the biological degradation of the compounds. Since whole zebrafish embryos were used for cell isolation in this study, no statement

can be made about the different susceptibility of zebrafish cell types regarding genotoxicity, but a differentiation can be expected. Only a few *in vivo* studies investigated TNT-related genotoxicity especially in higher organisms such as mammals, but urinary bladder carcinomas were found in rats exposed to TNT in a two-year dietary study (Furedi et al., 1984a). In contrast to these findings, short-term exposures of rodents did not confirm the genotoxicity of TNT in the bone marrow or liver (Ashby et al., 1985). Overall, TNT has been classified as a carcinogen in mammalian model organisms and the partially contrasting results in terms of genotoxicity are probably based on the species, tissue or sexes studied (Inouye et al., 2009). The genotoxicity of nitroaromatic compounds in fish in combination with the known mutagenicity of these substances substantially increases the significance of the results of the present study, as the *in vivo* exposure demonstrates the biological relevance for fish.

Since TNT undergoes biotransformation processes in fish (Belden et al., 2005; Ek et al., 2003; Mariussen et al., 2018) the observed genotoxicity cannot only be referred to the tested substances (TNT, 2-ADNT, 4-ADNT) alone. For instance, 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT), a TNT metabolite, caused severe DNA damage to the male reproductive system in rats while TNT did not (Homma-Takeda et al., 2002). 4-HADNT is formed as an intermediate product during the reduction of TNT to 4-ADNT, catalysed by cytochrome P450 (CYP) enzymes (Shinkai et al., 2016). As CYP genes are already expressed in early life stages of zebrafish embryos (Goldstone et al., 2010), 4-HADNT, or similar metabolic intermediates, probably also occurred in zebrafish embryos exposed to TNT. Therefore, the DNA fragmentation observed in the present study appears to be a logical implication of reactive oxygen species generated during TNT reduction (Shinkai et al., 2016) and known to cause DNA damage (Breen and Murphy, 1995). However, also 2-ADNT and 4-ADNT revealed significant genotoxicity and undergo further biotransformation reactions inside the organism. For example nitrobenzoic acids, such as 2-amino-4,6-dinitrobenzoic acid and 4-amino-2,6-dinitrobenzoic acid are formed and have also been shown to be genotoxic and mutagenic in *in vitro* experiments (Grummt et al., 2006). In summary, the genotoxicity of nitroaromatic compounds in fish is probably highly influenced by the rapid metabolism of these substances and should not only be associated with the tested parent compounds.

### 2.5.3. Ecotoxicological relevance

In the larger context of dumped munitions and TNT in the marine environment, the results confirm that fish in dumpsites are possibly subject to threats beyond acute

toxicity. However, only few studies investigated water concentrations of nitroaromatic compounds in the vicinity of dumped munitions and the difficulties in measuring the contamination became apparent (Ampleman et al., 2004; Gledhill et al., 2019; Hoffsommer and Rosen, 1972). Several munition compounds, including TNT, have recently been detected in water samples in the ng/L range from a munition dumpsite in the Baltic Sea (Gledhill et al., 2019). Concentrations in the low µg/L range were measured close to munition compounds in the Caribbean (Porter et al., 2011) (Porter et al., 2011). Despite the fact, that these concentrations are considerably lower than the concentrations that led to genotoxicity in this study (0.1 mg/L and 1 mg/L lowest tested concentrations), marine organisms are in direct contact with munition compounds throughout the water column. Recently, degradation products of TNT were detected in blue mussels (*Mytilus edulis*) exposed in a munition dumpsite in the Baltic Sea (Strehse et al., 2017).

Since we used a freshwater fish for investigating TNT toxicity, the results cannot be fully related to marine fish species, but freshwater toxicity data are often used for extrapolation to saltwater species and can serve as a sound basis for this. When comparing toxicity data between freshwater and saltwater species, the main factors contributing to a different response are biological differences, chemical differences in the medium and differences in the test methodology (Leung et al., 2001; Wheeler et al., 2002). These factors also have an impact on the effects of nitroaromatic compounds in fish, as salinity and temperature influence solubility (Beck et al., 2018, 2019) and consequently the availability of TNT in the water column. Studies comparing the sensitivity of freshwater and saltwater fish to various chemicals revealed that the difference in sensitivity for most substances is within a factor of 5 to 10 (Hutchinson et al., 1998; Wheeler et al., 2002). This relationship is also evident when comparing TNT toxicity data available for marine fish species (Ek et al., 2008; Leffler et al., 2014; Nipper et al., 2001) with freshwater data. Therefore, we consider the results with zebrafish shown in this study to be also significant for the marine environment. However, freshwater systems such as lakes are also known to have been used as ammunition dumpsites (Bernet et al., 2008), thus the freshwater species living there are also likely to be exposed to nitroaromatic compounds.

The comet assay results only cover the genotoxicity occurring after 48 h and prolonged or chronic exposure of fish living in close proximity to dumpsites corresponds to the actual exposure. In order to detect genotoxic effects in wild fish from dumpsites, gills could be an appropriate target tissue as shown in European conger (*Conger conger*) exposed to chemical warfare agents (Della Torre et al., 2010). Elevated nuclear abnormalities have recently been found in erythrocytes used as markers for genotoxicity from

herring, flounder and cod in areas of the Baltic Sea associated with dumped chemical and conventional munitions (Valskienė et al., 2018). Investigations on fish living near a munition dumpsite in the Baltic Sea revealed an increased prevalence of liver tumours (Straumer and Lang, 2019). We therefore see sufficient evidence for genotoxic effects in fish living in munition dumpsites and the associated consequences for fish health.

## 2.6. Conclusion

For the first time, we tested the genotoxicity of nitroaromatic compounds in fish using an *in vivo* exposure approach. In addition to acute toxicity, TNT and its primary degradation products 2-ADNT and 4-ADNT exhibited significant genotoxic effects in zebrafish embryos after short term exposure using the comet assay. The genotoxicity induced by TNT was three to four times higher in comparison to 2-ADNT and 4-ADNT. Based on these results, we demonstrated the relevance of the genotoxic threat posed by TNT and its degradation products to fish and, in combination with the known mutagenicity, see the possibility of long-term effects for fish living in munition dumpsites. For further research, a case study that focus on investigating fish health in dumpsites and thereby addresses the possible chronic consequences for fish might be helpful to evaluate the actual risk for fish.

## 2.7. Acknowledgements

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### **3. Dumped munitions: New insights into the metabolization of 2,4,6-trinitrotoluene in Baltic flatfish**

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### 3.1. Abstract

Livers from dab (*Limanda limanda*), plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*) sampled from the Baltic Sea were used to determine the interaction of flatfish CYP1A enzymes with 2,4,6-trinitrotoluene (TNT) *in vitro*. Competitive inhibition of 7-ethoxyresorufin-O-deethylase (EROD) and 7-methoxyresorufin-O-deethylase (MROD) could be demonstrated for all three flatfish species. The highest inhibition of CYP1A activities was measured in liver samples of flounder resulting in a half maximal inhibitory concentration (IC<sub>50</sub>) of 28.1 μM TNT. Due to their lower inhibition (EROD IC<sub>50</sub> 65.2 μM TNT, MROD IC<sub>50</sub> 40.3 μM TNT), dab liver samples were used to conduct *in vitro* metabolization experiments with TNT. The metabolization of TNT in fish was investigated with post-mitochondrial fractions (PMF) of dab liver as a model system after adding different cofactors. Rapid and time-dependent enzymatic degradation of TNT was observed. The concentrations of 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene increased in the samples over time. Additionally, 2,2,6,6-tetranitro-4,4-azoxytoluene was detected in one sample. The results of this study indicate that *in vitro* experiments are useful to investigate the xenobiotic metabolism of fish under controlled conditions prior to field studies. The metabolites found can serve as target compounds for marine monitoring of TNT contamination in munition dumpsites.



## 3.2. Introduction

Dumping ammunition at sea was a common practice until the 1970s (Carton and Jagusiewicz, 2009) and has been acknowledged as a worldwide problem in recent years (Beck et al., 2018). Dumped munition is increasingly associated with safety concerns in the framework of sea bottom activities like offshore wind parks and submarine cable installation (Sanderson et al., 2014; Tornero and Hanke, 2016) as well as fishing (Missiaen et al., 2010). However, the environmental impact of this possible source of marine pollution has been largely ignored.

The North- and Baltic Sea are major dumping sites for millions of tons of chemical weapons and conventional munition. Most ammunition originates from military operations or munition dumping after World War I and World War II (Böttcher et al., 2011). Due to corrosion of the shells, ammunition conditions deteriorate over time (Voie and Mariussen, 2017), posing the risk of a chronic exposure of entire ecosystems with hazardous substances. The vast majority of the ammunition in the Baltic Sea are conventional explosives containing 2,4,6-trinitrotoluene (TNT) (Böttcher et al., 2011). Due to the high amount of munitions containing TNT in the Baltic waters and the progressive corrosion of the metal shells, TNT is known to already be released into the marine ecosystem (Strehse et al., 2017). However, the effects of long-term exposure of different ecosystem components, including marine fish, to TNT are largely unknown, despite some recent experimental progress:

For instance, TNT at relatively high concentrations was shown to be acutely toxic to fish during short term exposure scenarios (Liu et al., 1983; Lotufo et al., 2010b; Yoo et al., 2006). A possible accumulation of TNT in fish was repeatedly investigated, demonstrating that both, the bioconcentration factor (BCF) and bioaccumulation factor (BAF) with 0.79 mL/g and  $2.4 \times 10^{-5}$ , respectively, were low (Belden et al., 2005; Ownby et al., 2005; Talmage et al., 1999). However, the same publications proved that TNT biotransformation products accumulate in fish to a much greater extent than the parent compound. Therefore, in combination with the rapid degradation of TNT in water (Luning Prak et al., 2017) and in organism, the analysis of TNT degradation products is crucial to assess the environmental impact of exposure with conventional munition.

By investigating the effects of TNT on biotransformation enzymes in European eel, a significant decline in CYP1A activities became obvious (Della Torre et al., 2008). However, no other fish species has been studied so far that is known to naturally

occur in munition dumpsites. Few studies detected the primary TNT metabolites, 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT), in fish following the experimental exposure to TNT (Belden et al., 2005; Ek et al., 2003; Mariussen et al., 2018). Furthermore, a rapid reduction of the nitro groups of TNT in rat microsomes to the intermediate form 4-hydroxylamino-2,6-dinitrotoluene (4-HADNT) and subsequently further to the corresponding amino metabolites have been recorded (Leung et al., 1995; Shinkai et al., 2016).

Since fishing in munition dumpsites is often subject to special permits and generally requires sophisticated infrastructure, *in vivo* studies with fish from these areas are more difficult to realize. In contrast, *in vitro* experiments offer an easy and focused solution to study biotransformation processes in animals and contribute to research with less animal testing. First investigations on TNT metabolism in mammals using *in vitro* experiments were performed in the 1940s, more than 70 years ago (Bueding and Jolliffe, 1946). More recent work used *in vitro* approaches to search for specific enzymes that catalyse the release of nitrite from TNT (Shinkai et al., 2016). An effective way for the analysis of TNT degradation is to use liver tissue, the biotransformation organ, and expose it to TNT and its degradation products.

Common flatfish in the North Sea and Baltic Sea, like dab (*Limanda limanda*), plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*) are particularly prone to munition exposure due to their purely benthic lifestyle. Therefore, we chose flatfish as suitable vertebrate species to study the environmental effects of dumped ammunition. All three species were used in a first step to identify a suitable species for *in vitro* metabolization experiments with TNT. Gaining knowledge about prevalence of TNT metabolites in these species is important for identifying target compounds suitable for the detection of TNT contamination in fish or further monitoring.

Potential monitoring of explosives in fish requires target compounds that are well suited for this purpose. Since environmental contaminants are often degraded in organisms, the parent compound, e.g. TNT, may not be the most promising choice for monitoring contamination. Biotransformation of contaminants, including nitroaromatic compounds, can generally be segregated into oxidation, reduction and hydrolysis processes (Phase I) as well as conjugation processes (Phase II) (Livingstone, 1998; Parkinson, 2001; Stegeman and Hahn, 1994) resulting in a wide range of degradation products. In order to detect these possible candidates for a future monitoring, highly sophisticated methods such as HPLC-MS are needed.

The aim of the present study was to understand the fate of TNT during its metabolization in marine flatfish species present in the dumping areas of conventional munition

in the Baltic Sea. For this purpose, we used a two-step *in vitro* approach. The first step served to select the appropriate sample material for the metabolism experiment based on the enzyme response in the flatfish liver. (1) Enzyme interaction with TNT in three flatfish species was assessed using the example of the CYP1A enzymes 7-ethoxyresorufin-O-deethylase (EROD) and 7-methoxyresorufin-O-deethylase (MROD). (2) In the second step, dab liver fractions were exposed *in vitro* to TNT for different durations and the resulting metabolites were identified and measured with HPLC-MS.

## 3.3. Material and methods

### 3.3.1. Fish liver samples

For *in vitro* assays, specimens of dab (*Limanda limanda*), flounder (*Platichthys flesus*) and plaice (*Pleuronectes platessa*) were selected from bottom trawl catches on board the chartered fishing vessel “Marianne” in September 2016 in Kiel Bight (54 33.567 N - 54 35.973 N, 10 48.652 E - 10 39.666 E). All fish were randomly sorted and examined for length, weight and sex while a small piece of liver was dissected and stored directly at -80 °C in liquid nitrogen for the following experiments.

### 3.3.2. Chemicals

Dissolved 2,4,6-trinitrotoluene (TNT) was supplied by the Fraunhofer Institute for Chemical Technology (ICT), Germany. The TNT stock solution was prepared with solid TNT (“military grade”) which was dissolved in deionised water at 60 °C, shielded from light. Chemicals for enzyme activity measurement and Lowry protein assay were purchased from Merck (Germany), with the exception of dithiothreitol (Sigma-Aldrich, Germany), 7-ethoxyresorufin, 7-methoxyresorufin and nicotinamide adenine dinucleotide phosphate (NADPH) (Biomol GmbH, Germany). Dimethyl sulfoxide (DMSO, 99.9%), acetonitrile (99.9%), water and methanol (MeOH, 99.9%) were purchased from Th. Geyer (Germany). Acetic acid (99%) was obtained from Merck (Germany) and ammonium acetate (NH<sub>4</sub>Ac) was purchased from Sigma Aldrich (Germany). Reference standards of 2,4,6-trinitrotoluene (TNT), 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT), 1,3,5-trinitrobenzene (TNB) and 2,2,6,6-tetranitro-4,4-azoxytoluene (TNAzoxo) were obtained from AccuStandard (USA). 3,5-dinitrobenzoic acid (3,5-DNBA) and 1,4-dinitrobenzene (1,4-DNB) were obtained from Sigma-Aldrich (Germany).

### 3.3.3. *In vitro* assays

#### 3.3.3.1. Enzyme preparation

Liver samples were prepared by following the method of Ronisz and Förlin (1998). The livers (500 mg tissue) were homogenized 1:10 (w/v) in ice-cold Na<sup>+</sup>/K<sup>+</sup>-phosphate

buffer (0.1 M) with 0.15 M KCl, 1 mM EDTA, 1 mM dithiothreitol using a Potter-Elvehjem glass-Teflon homogeniser. The homogenates were first centrifuged at 10 000 g for 20 min and the resulting supernatant was centrifuged at 105 000 g for 1 h in a Beckman Coulter Optima MAX-XP ultracentrifuge to obtain the microsomal pellet. The pellets were resuspended and homogenized in 5 mL of the homogenisation buffer. All steps were carried out on ice and microsomal fractions were stored at -80 °C for further analysis.

The protein content of the microsomal fraction was determined according to Lowry et al. (1951) using a FLOUstar Optima microplate reader from BMG Labtech and bovine serum albumin as standard.

### **3.3.3.2. Experiment I: Enzyme inhibition in flatfish**

The liver microsomes were used to measure 7-ethoxyresorufin (CYP1A1) and 7-methoxyresorufin (CYP1A2) O-deethylase activity (EROD, MROD) according to the method of Burke and Mayer (1974) using 96-well plates as described by Eggens and Galigni (1992) and a FLOUstar Optima microplate reader. The final reaction mixture (200  $\mu$ L per well) contained 180  $\mu$ L of the microsomal fraction diluted 1:90 in Na<sup>+</sup>/K<sup>+</sup>-phosphate buffer (0.1 M, pH 7.5), 10  $\mu$ L of 7 ethoxyresorufin (40  $\mu$ M) or 7 methoxyresorufin (20  $\mu$ M) were used as substrate. DMSO was used as solvent for the different substrates (7 ethoxyresorufin, 7 methoxyresorufin) at a concentration of 0.5% DMSO in all experiments. Microsomal fraction was mixed with aqueous dissolved TNT (0.15 mg/L - 15 mg/L) to investigate inhibition effects on microsomal enzyme activity. For each of the three different flatfish species 8 individual specimens were examined. The catalytic reaction was started by adding 10  $\mu$ L of NADPH (10 mM) to the mixture and the increase in fluorescence (excitation wavelength 544 nm, emission wavelength 590 nm) was recorded at 25 °C.

The microplate reader was calibrated using resorufin as standard. Enzyme activity is expressed as picomole of resorufin per minute per milligram protein (pmol/min/mg). The detection limits for enzyme activity measurements were 1.74 pmol/min/mg (EROD) and 0.69 pmol/min/mg (MROD), respectively.

The mechanism of CYP1A inhibition was investigated using a dab liver sample, exposed to various concentration of 7-ethoxyresorufin (0.016  $\mu$ M - 4  $\mu$ M) and different TNT concentrations (0 mg/L - 7 mg/L). In two replicates per concentration kinetic parameters  $V_{\max}$  and  $K_m$  were calculated following the Michaelis-Menten equation.

**3.3.3.3. Experiment II: *In vitro* experiments with dab liver**

*In vitro* metabolization of TNT was investigated by incubating pooled post mitochondrial fractions (PMF), S9, of dab liver (originating from 10 individuals) with aqueous dissolved TNT. The total protein content in each reaction mixture was 3.5 mg/mL and the PMF was diluted with Na<sup>+</sup>/K<sup>+</sup>-phosphate buffer (0.1 M, pH 7.5). The reaction mixture contained 1 mM NADPH, 1 mM uridine 5'-diphosphoglucuronic acid (UDPGA) or 0.1 mM acetyl-coenzyme A (AcCoA). The final volume of the reaction mixture was 500  $\mu$ L and the reaction was initiated by the addition of 15  $\mu$ L TNT (100 mg/L stock solution). The samples were incubated at 28 °C and 700 rpm for < 1 min (hereinafter designated as initial), 30 min or 120 min. The reaction was terminated by adding 800  $\mu$ L ice-cold acetonitrile and vortexing to ensure rapid precipitation of proteins. A total amount of 20 ng 1,4-dinitrobenzene (1,4-DNB) was added as an internal standard. The incubation tubes were centrifuged at 10 000 rpm and 4 °C for 10 min and the supernatant was filtered (0.2  $\mu$ m) for HPLC-MS analysis.

A blank contained Na<sup>+</sup>/K<sup>+</sup>-phosphate buffer instead of TNT and three different controls were prepared and treated analogously to the other samples. The TNT control was prepared without liver enzymes to investigate non-catalytic reactions of TNT. NADPH control and a cofactor control were prepared in order to find out if TNT metabolization only depends on the presence of NADPH. Detailed compositions of blanks, controls and samples are shown in Table 3.1. All controls and the blank were incubated for 120 min. With the exception of the blank, all of the controls and samples were prepared in duplicate.

**Table 3.1.:** Composition of the samples for *in vitro* metabolism experiments using post-mitochondrial fraction from dab liver. “X” present in sample, “-” not present in sample.

Content	Blank	TNT Control	NADPH Control	Cofactor Control	NADPH <sup>a</sup> < 1, 30, 120 min	UDPGA <sup>a</sup> < 1, 30, 120 min	AcCoA <sup>a</sup> < 1, 30, 120 min
Liver enzymes	X	-	X	X	X	X	X
TNT	-	X	X	X	X	X	X
NADPH	X	X	-	-	X	X	X
UDPGA	X	X	X	-	-	X	-
AcCoA	X	X	X	-	-	-	X
Buffer	X	X	X	X	X	X	X

<sup>a</sup>In each case all of the NADPH, UDPGA, AcCoA samples were terminated after < 1, 30, 120 min in duplicate.

### 3.3.4. HPLC-MS analysis

Prior to analysis, metabolites and explosives in the supernatant were concentrated under a stream of nitrogen to a final volume of about 50  $\mu$ L. From this extract, 5  $\mu$ L were injected onto the column in an Agilent 1290 Infinity High-Performance-Liquid-Chromatograph (HPLC) coupled with an AB Sciex QTrap 5500 Triple Quadrupole/Ion-Trap Mass Spectrometer (QQQ-MS). Gradient separation was carried out with a reverse phase Acclaim Explosives E2 Column (Thermo Fisher Scientific) at 22 °C, with eluents A: H<sub>2</sub>O (10 mM NH<sub>4</sub>Ac + 2.7 mL acetic acid, pH 4) and B: MeOH (10 mM NH<sub>4</sub>Ac + 2.7 mL acetic acid) starting at 45% B increasing to 95% B in several steps over 45 min keeping a constant flow rate of 0.3 mL/min.

Atmospheric pressure chemical ionization (APCI) was used in negative mode. Explosives and selected metabolites were detected via multiple reaction monitoring (MRM) mode based on characteristic MS/MS transitions, previously optimized with commercially available standard substances. The characterization of unknown compounds was accomplished in scan-mode of the linear ion trap by selecting nitroaromatic compounds using a neutral loss scan targeting loss of nitro groups ( $m/z$  46), followed by an enhanced product ion scan, producing a sensitive mass spectrum of the nitro-containing molecule.

The abundances of explosives and metabolites were quantified using the internal standard 1,4-dinitrobenzene and corrected for response factors of commercially available standards. Response factors were obtained using an external calibration covering the expected concentration range. Detection limits (LOD) of the compounds analysed ranged between 0.2 ng/mL - 1.6 ng/mL with an injection volume of 5  $\mu$ L.

### 3.3.5. Statistical analysis

Statistical analyses were carried out using R Version 3.3.1 (R Core Team, 2019). Enzyme activity data were calculated as mean relative activity and standard deviation. A Kruskal-Wallis rank sum test was used to test the differences in the activities of EROD and MROD between the control and treatments for dab, flounder and plaice. To determine which treatments showed significant differences in enzyme activity from the control, a pairwise multiple comparison according to Dunn using the “PMCMR-package” (Pohlert, 2014) was applied. P-values were adjusted according to Benjamini and Hochberg (1995). The half maximal inhibitory concentration (IC<sub>50</sub>) of EROD and MROD for each species was derived by applying a two-parameter log-logistic model,

with fixed lower and upper limits, to the enzyme activity data using the “drc-package” (Ritz and Streibig, 2005).



## 3.4. Results

### 3.4.1. *In vitro* effects of TNT on CYP1A enzymes

CYP1A activities were considerably influenced in the presence of TNT. A dose-dependent decrease of EROD as well as MROD activity was observed for all three fish species (Table 3.2). The lowest tested concentration of 0.15 mg/L TNT led to 9% inhibition of EROD activity in flounder livers. The extent of inhibition of EROD and MROD activity within one species was comparable. The greatest inequalities between the two enzymes were present in flounder liver, in which MROD was inhibited to a higher extent (20%) compared to EROD. The highest inhibition of CYP1A activities was observed in flounder samples, where the inhibition of EROD and MROD was significant even at 0.6 mg/L TNT compared to the control. Almost half of the initial EROD activity (52%) and 65% of MROD activity were inhibited at 7 mg/L of TNT (Table 3.2), providing an  $IC_{50}$  of 28.1  $\mu$ M TNT (EROD) respectively 37.7  $\mu$ M TNT (MROD) (Table 3.3). Since few samples did not reach the detection limit the calculated  $IC_{50}$  for MROD inhibition in flounder samples is subject to a corresponding uncertainty.  $IC_{50}$  values for CYP1A activity in dab and plaice were higher compared to flounder samples (Table 3.3). The slightest inhibition of EROD activity by TNT occurred in dab.

Dab, plaice and flounder liver samples contained different amount of protein as shown in Table 3.3. On average, dab samples contained the least protein (1.5 mg/mL) whereas plaice contained the most (2 mg/mL).

**Table 3.2.:** Relative activity of CYP1A enzymes (EROD, MROD measured in pmol/min/mg protein) in dab, flounder and plaice liver microsomes after *in vitro* exposure to TNT. Activity is expressed as the mean relative activity (standard deviation) compared to the control activity (0 mg/L TNT) in percentages (%). Number of individuals per species, n = 8. Significant differences between treatments compared to the control are marked with asterisk: \* p < 0.05 according to Dunn's pairwise multiple comparisons.

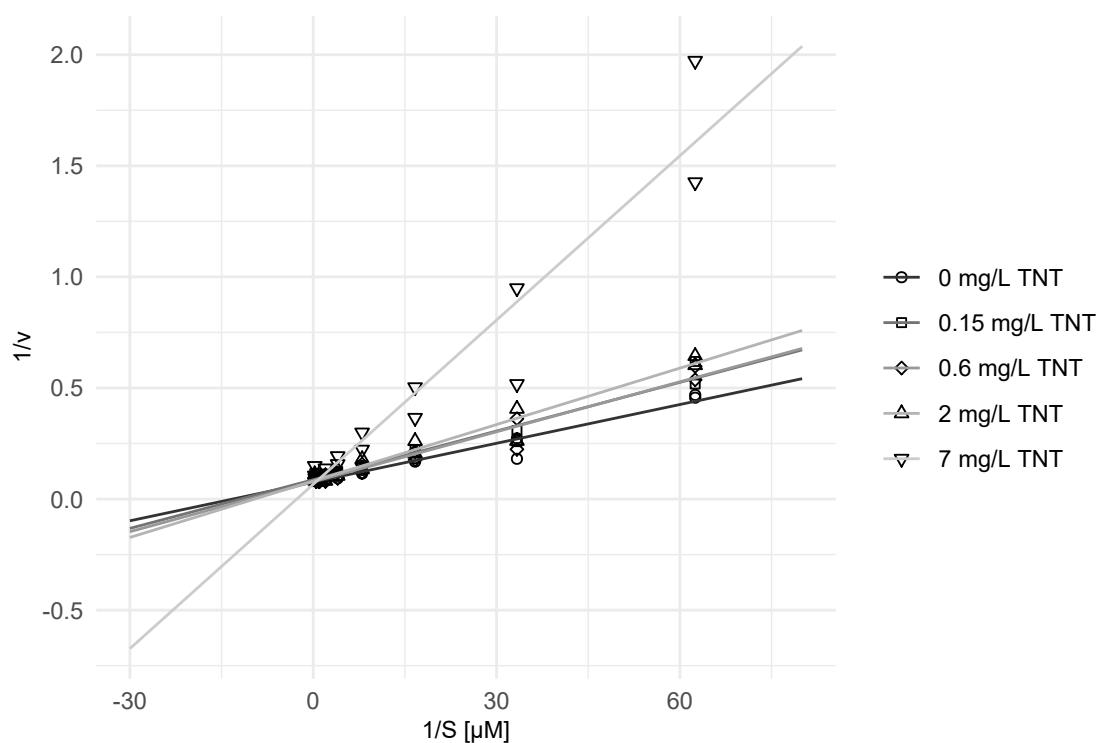
TNT concentration [mg/L]	0.15	0.3	0.6	2	7	15
<b>dab</b>						
EROD	93.7 (7.0)	90.7 (5.9)	88.6 (9.7)	81.7 (5.2)*	64.8 (3.6)*	47.1 (6.3)*
MROD	93.8 (6.0)	93.0 (12.8)	91.7 (12.0)	83.2 (13.3)*	58.5 (7.5)*	35.9 (6.2)*
<b>flounder</b>						
EROD	90.5 (5.7)	86.7 (5.0)	82.3 (10.1)*	72.3 (7.3)*	52.3 (4.7)*	30.9 (6.1)*
MROD	93.0 (12.1)	85.0 (17.8)	78.3 (17.0)*	55.1 (36.0)*	34.7 (29.3)*	8.4 (15.8)*
<b>plaice</b>						
EROD	93.7 (7.0)	89.8 (9.5)	86.5 (11.6)	77.8 (11.0)*	60.7 (10.7)*	39.8 (4.9)*
MROD	95.0 (7.4)	90.3 (7.1)	88.0 (9.4)	83.0 (12.9)*	56.5 (7.7)*	40.8 (7.9)*

**Table 3.3.:** Half maximal inhibitory concentration (IC<sub>50</sub>) of EROD and MROD in dab, plaice and flounder liver microsomes after *in vitro* exposure to TNT. IC<sub>50</sub> is expressed in 0 μM TNT (mg/L) and was calculated by running a log-logistic model. Protein content of liver microsomes is expressed in mg/mL (standard deviation). Number of individuals per species, n = 8.

Species	IC <sub>50</sub> EROD [μM TNT]	IC <sub>50</sub> MROD [μM TNT]	protein content [mg/mL]
dab	65.2 (14.8)	40.3 (9.2)	1.5 (0.4)
plaice	45.6 (10.4)	41.5 (9.4)	2.0 (0.2)
flounder	28.1 (6.4)	37.7 (8.6)	1.9 (0.3)

### 3.4.2. Kinetics of enzyme inhibition

The characteristic of EROD inhibition was investigated by adding different amounts of substrate (7-ethoxyresorufin) to the reaction mixture. The kinetic parameters were derived by Michaelis-Menten equation and confirmed the mechanism of competitive inhibition of EROD by TNT.  $V_{\max}$  did not change at different TNT concentrations in contrast to Michaelis constant  $K_m$ , which increased with rising TNT concentration. Competitive inhibition of EROD in dab liver microsomes is expressed in Lineweaver-Burk plot in Figure 3.1. As illustrated in the double reciprocal plot, the y-intercept is the same for all treatments and clearly shows that  $V_{\max}$  is not affected by TNT exposure. However, the x-intercept is different between the control and the TNT treatment, demonstrating that  $K_m$  is altered by different TNT concentrations.



**Figure 3.1.:** Lineweaver-Burk plot of EROD kinetics in dab liver microsomes after *in vitro* exposure to different concentrations of TNT (0 mg/L, 0.15 mg/L, 0.6 mg/L, 2 mg/L, 7 mg/L).

### 3.4.3. *In vitro* metabolization of TNT

*In vitro* incubation of TNT with PMF from dab liver led to a rapid formation of metabolites. Different compositions of TNT metabolites in the different treatments of

*in vitro* experiments are shown in Table 3.4. Combinations of three different types of cofactors (NADPH, UDPGA, AcCoA) were tested to artificially simulate the metabolic processes in dab liver. 2-amino-4,6-dinitrotoluene (2-ADNT) as well as 4-amino-2,6-dinitrotoluene (4-ADNT) were found in all samples and also in two of the controls (Table 3.4). When comparing the 120 min samples with the corresponding short-term incubation samples (initial), 2-ADNT and 4-ADNT was found to a greater extent after 120 min. After 120 min, approximately 12% to 30% more 4-ADNT than 2-ADNT was present in all samples.

Small amounts of 1,3,5-trinitrobenzene (TNB) were detected in the TNT control, which did not contain enzymes, and in comparable concentrations in the initial samples treated with the different cofactors. The TNB content was less in the cofactor control, the NADPH control as well as the treatments incubated for 30 min (Table 3.4). No TNB was found in the samples that were incubated for 120 min. In one sample 2,2,6,6-tetranitro-4,4-azoxytoluene (TNAzoxy) was detected at very low concentration (< 1 ng/mL). Compared to the samples only treated with NADPH as cofactor, no other compounds were detected in the samples treated additionally with UDPGA or AcCoA.

The TNT content in the samples decreased steadily over time in presence of the PMF (Table 3.4). After an incubation period of 120 min, the TNT content in the samples decreased by about 30% to 50%. The TNT content of the TNT control, also incubated for 120 min without enzyme addition, was comparable to the initial value of the enzyme-containing samples. At the mass transition from 211  $m/z$  to 167  $m/z$ , which was chosen for 3,5-dinitrobenzoic acid (3,5-DNBA), signals became larger with increasing incubation time, but showed a different retention time (5.56 min) compared to the 3,5-DNBA standard (5.99 min). The signal was found in each sample, especially in all samples incubated for 120 min and, with the exception of the blank, also in all controls.

**Table 3.4.:** Mean concentrations [ng/mL] of detected nitroaromatic compounds after *in vitro* metabolism experiments with TNT and dab liver PMF. The mean concentrations were calculated from duplicate samples. Blank and controls were incubated for 120 min. Initial samples were incubated for less than 1 min. Abbreviations: 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitrobenzene (TNB), 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,2,6,6-tetranitro-4,4-azoxytoluene (TNAzoxy). See Table 3.1 for the composition of the samples.

<b>Samples</b>	<b>TNT</b>	<b>TNB</b>	<b>2-ADNT</b>	<b>4-ADNT</b>	<b>TNAzoxy</b>
Blank	< LOD	< LOD	< LOD	< LOD	< LOD
TNT control	3316	5.5	< LOD	< LOD	< LOD
Cofactor control	2960	2.0	41.5	55.0	< LOD
NADPH control	2782	1.3	25.1	51.5	< LOD
NADPH initial	3135	4.8	16.5	6.3	< LOD
NADPH 30 min	3428	0.9	58.4	71.7	< LOD
NADPH 120 min	2237	< LOD	77.6	108.1	0.2
UDPGA initial	4104	5.4	9.5	10.3	< LOD
UDPGA 30 min	2773	0.7	18.0	46.7	< LOD
UDPGA 120 min	1996	< LOD	32.1	45.5	< LOD
AcCoA initial	3297	4.7	22.2	7.8	< LOD
AcCoA 30 min	2639	0.7	54.4	57.7	< LOD
AcCoA 120 min	2241	< LOD	105.4	118.7	< LOD

## 3.5. Discussion

### 3.5.1. Inhibition of CYP1A activities and species selection for *in vitro* metabolism experiments

In the marine environment fish close to ammunition dumpsites are potentially exposed to relatively low doses of TNT at a ng/L level over time (Gledhill et al., 2019). However, in close proximity to the munition shells concentration at the mg/L range were measured (Beck et al., 2018). Due to rapid biotransformation and excretion the biological half-life of TNT was estimated to be only 0.05 h by Ownby et al. (2005). Therefore, the concentrations we tested in this study are rather unlikely to be assumed as exposure concentrations for fish over longer periods of time, but are in principle conceivable for a short period of time in the field.

In the present study, experiments with flatfish microsomes for analysing *in vitro* inhibition of CYP1A enzyme activity revealed a clear decline of enzyme activity with rising TNT concentrations. In detail, EROD activity in dab liver was less inhibited compared to the other flatfish liver samples (Table 3.2). 2 mg/L of TNT caused around 10% less inhibition of EROD in dab than in flounder livers. In addition, the dab livers showed the highest mean enzyme activity for EROD and MROD (appendix B, Table B.1, Table B.2, Table B.3), in comparison to the flounder and plaice livers. Relatively high CYP1A activities in dab liver samples compared to other flatfish species were also shown in other studies and turned out to be dependent on sex, season, maturity and contaminants (Kirby et al., 1999; Westernhagen et al., 1999).

Although EROD and MROD are not directly involved in the metabolism of TNT, the activity of nitroreductases, which are responsible for the reduction of the nitro groups (Zbaida, 2002), is associated with various enzymes, including cytochrome P450s such as CYP1A or CYP3A (Belisario et al., 1990, 1991; Berson et al., 1993). More specifically, *in vitro* experiments with mammalian liver enzymes revealed that the reduction of nitro groups of TNT is mediated by NADPH-cytochrome P450 reductase (Shinkai et al., 2016). Therefore, the results on CYP1A activity shown in the present study served as a representative for the enzyme activity in TNT degradation reactions in the liver. In combination with a high enzyme activity and a weak enzyme inhibition by TNT, dab livers were the most promising flatfish samples to investigate *in vitro* degradation of TNT.

Since potential competitive inhibition of EROD after exposure to TNT has previously been described in European eel (Della Torre et al., 2008) and rainbow trout (Ek et al.,

2003), the mechanism of CYP1A inhibition was studied considering different potential types of chemical pathway disruption. Therein, TNT acted as a competitive inhibitor in the experiment.

The inhibition of biotransformation enzymes could impede the general enzymatic reaction of fish to xenobiotics. Therefore, inhibition of CYP1A enzymes by TNT may limit the ability of fish to interact with other pollutants present at the same time, such as polycyclic aromatic hydrocarbons (PAHs) which are commonly found in Baltic flatfish (Kammann, 2007; Vuorinen et al., 2006). In this context, flounder is more affected than other flatfish species. However, *in vivo* studies with flatfish may be useful to demonstrate the influence of CYP1A gene expression on the inhibition of enzyme activity, experiments with European eel showed that TNT does not alter CYP1A1 gene expression (Della Torre et al., 2008).

### **3.5.2. *In vitro* metabolization of TNT**

In the presence of liver enzymes and essential cofactors such as NADPH, TNT was metabolized within short time as shown in the experiments. In the absence of enzymes only a small amount of TNB was detected in the control after 120 min. As this concentration was comparable to the TNB content in the enzyme-containing initial samples, TNB was probably already present in the TNT stock solution. TNT is known to be photosensitive and TNB was found in photolysis experiments with TNT in seawater and estuary water by Luning Prak et al. (2017). The photolysis of TNT also takes place in pure water, but the conversion rates are lower compared to seawater (Burlinson et al., 1979). Moreover, TNB itself is not photosensitive (Luning Prak et al., 2017) and may have accumulated in the stock solution before the *in vitro* experiments were carried out. Despite the fact that the experiments were carried out in the dark, the stock solution was briefly exposed to sunlight during several handling steps and photolysis of TNT may have occurred.

Interestingly, the experiment confirmed that the existing TNB is probably metabolized by the PMF since no TNB was found after 120 min. Therefore, the persistence of TNB in the environment (Reddy et al., 1997) is limited when it is taken up by organisms. Reddy et al. (1996) investigated the metabolism of TNB in rat liver microsomes *in vitro* and TNB was completely metabolized after 5 min. The results suggest that possible TNB contamination in fish from dumpsites cannot be related to TNT exposure, but rather to direct uptake.

Formation of 2-ADNT and 4-ADNT in *in vitro* experiments with TNT and liver enzymes is well known and described in different studies (Leung et al., 1995; Rickert, 1987). In line with the known preferred reduction of TNT to 4-ADNT compared to 2-ADNT (McCormick et al., 1976), more 4-ADNT than 2-ADNT was found in all treatments after 120 min. The same observation was made in fish exposed to TNT in *in vivo* experiments (Mariussen et al., 2018). Low concentration of TNazoxy was detected in one sample. However, an enzymatically catalysed formation of this compound cannot exclusively be determined as its primary source, since it can be formed nonenzymatically by coupling of reactive intermediate forms such as 4-nitroso-dinitrotoluene under aerobic conditions (Haïdour and Ramos, 1996; McCormick et al., 1976; Shinkai et al., 2016).

A compound with the same mass transition as the 3,5-DNBA standard was detected in the samples and exhibited an enhanced signal with increasing incubation time in enzyme-containing samples, especially with the addition of NADPH. Since the retention time differed from the 3,5-DNBA standard, it cannot be proven that this is the latter compound. Not least due to the positioning of the nitro groups, 3,5-DNBA seems unlikely to be a degradation product of TNT in the metabolism of vertebrates. The mass signal could originate from another dinitrobenzoic acid isomer, such as 2,4-dinitrobenzoic acid (2,4-DNBA) and 2,6-dinitrobenzoic acid (2,6-DNBA), which have nitro groups at the same positions as TNT, 2-ADNT or 4-ADNT. 2,4-DNBA and 2,6-DNBA revealed similar fragmentation pattern at  $m/z$  167 in other studies, suggesting comparability of the fragmentation between different dinitrobenzoic acid isomers (Ma et al., 2007; Schmidt et al., 2006). 2,4-DNBA was previously identified as a degradation product of 2,4-dinitrotoluene (2,4-DNT) in metabolism experiments with rats (Rickert and Long, 1981), and 2,4-DNT is commonly found as an impurity in “military grade” TNT (Basch and Kraus, 1979; Murrmann, 1971), which was used in the present study and may explain this signal.

Shinkai et al. (2016) and Leung et al. (1995) showed that the reduction of TNT in rats is catalysed by a cytochrome P450 reductase (P450R) and requires NADPH. The reduction of TNT in fish also depends strongly on the presence of NADPH, as the present study shows. No differences among enzymatic degradation products were found between experiments with the cofactors UDPGA and AcCoA. Della Torre et al. (2008) showed that the activity of UDP-glucuronosyltransferase (UDPGT) increased in TNT-exposed eel. UDPGT is known for its involvement in phase II metabolism of environmental contaminants and requires UDPGA as a cofactor (Mulder, 1992). Results from TNT exposed rainbow trout suggest that TNT-derived glucuronides are present in fish bile (Ek et al., 2005). Such a conjugation with glucuronic acid has also been observed in the



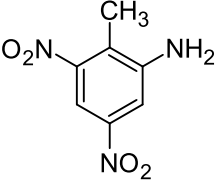
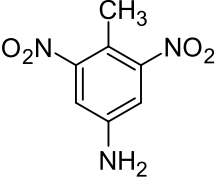
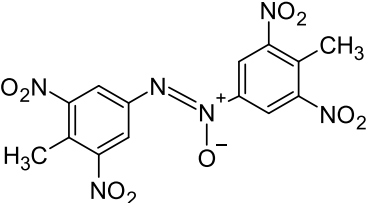
metabolism of dinitrotoluenes in rats (Rickert and Long, 1981). From this perspective, the smaller amounts of 2-ADNT and 4-ADNT in the samples additionally treated with UDPGA could be explained by the formation of glucuronides. Acetylation reactions with TNT should also be considered as a source of TNT-related conjugates and the importance of O-acetyltransferase with AcCoA as cofactor for TNT mutagenicity in bacteria has been demonstrated (Einistö, 1991).

### **3.5.3. On the way to a future marine monitoring of TNT exposure**

To understand the fate of TNT in the marine environment, in particular its impact on fish, the results contribute to implement a potential marine monitoring of TNT as a representative for dumped marine munitions. The compounds detected in *in vitro* metabolism studies such as the present one can be used for target analysis in field samples from munition dumpsites in the same species. They are also important for assessing the risk for fish exposed to ammunition. The performed experiments show, that different degradation products of TNT are produced in the liver within a short period of time and each of them has its own toxic potential. Toxicity studies with juvenile sheepshead minnows and nitroaromatic compounds showed that TNT ( $LC_{50}$  1.7 mg/L) is more toxic than 2-ADNT ( $LC_{50}$  8.6 mg/L), whereas TNB ( $LC_{50}$  1.2 mg/L) exposure revealed a higher toxicity than TNT (Lotufo et al., 2010b). Recently, TNT, 2-ADNT and 4-ADNT have been found to cause DNA damage in fish embryos, demonstrating the extensive toxicity of munition compounds beyond acute toxicity (Koske et al., 2019).

For monitoring purposes, target compounds must be stable and their fate in the organism clarified. Since the experiments revealed a time-dependent increase of the concentrations of 2-ADNT and 4-ADNT by enzymatic degradation, we recommend concentrating on these compounds in the search for TNT contamination in fish (Table 3.5). In addition, TNazoxy could serve as a non-enzymatically formed target compound for TNT contamination as it might accumulate over time. The parent compound TNT, on the other hand, appears to be unsuitable for monitoring purposes, as demonstrated by its rapid degradation in the environment and in fish.

**Table 3.5.:** Possible target compounds suitable for the detection of TNT contamination in fish.

Name	Structure	previously detected in fish
2-amino-4,6-dinitrotoluene (2-ADNT)		(Ek et al., 2005; Lotufo et al., 2010a; Mariussen et al., 2018; Ownby et al., 2005; Rosen and Lotufo, 2010)
4-amino-2,6-dinitrotoluene (4-ADNT)		(Ek et al., 2005; Lotufo et al., 2010a; Mariussen et al., 2018; Ownby et al., 2005; Rosen and Lotufo, 2010)
2,2,6,6-tetranitro-4,4-azoxytoluene (TNAzoxy)		This study (possibly formed non-enzymatically)

## 3.6. Conclusion

Due to their benthic lifestyle, flatfish are particularly vulnerable for exposure to explosives which are deposited in large numbers on the sea floor of the Baltic Sea. We showed that TNT competitively inhibits CYP1A enzymes of flatfish and that flounder was the most susceptible species. This could have a negative impact on their ability to deal with other pollutants present in the Baltic Sea.

*In vitro* exposure of dab liver PMF led to rapid degradation of TNT and primary degradation products such as ADNTs were formed. The results show that *in vitro* metabolism experiments with fish liver can serve well as a preparatory study for the analysis of field samples from ammunition dumpsites. Gathering knowledge about the expected toxic residues of explosives in fish is crucial as they may enter the marine food chain and can pose a risk also to the human consumer.

## 3.7. Acknowledgements

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## 4. Studying the metabolism of toxic chemical warfare agent-related phenylarsenic chemicals *in vitro* in cod liver

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## 4.1. Abstract

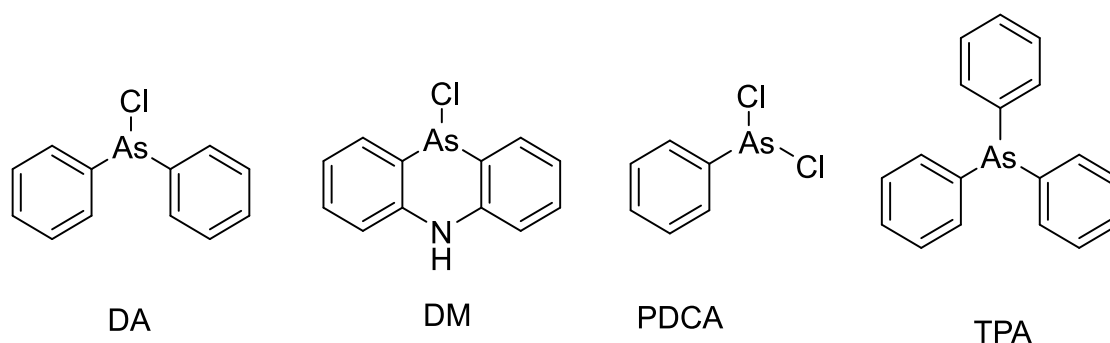
Large quantities of chemical warfare agents (CWAs), such as phenylarsenic chemicals, were disposed by sea-dumping after World War II. Nowadays, the release of these toxic chemicals from munitions poses a potential threat to living organisms. This study investigates the fate of these chemicals in fish by exposing selected CWA-related phenylarsenic chemicals and their oxidation products to cod (*Gadus morhua*) liver S9 fraction *in vitro*. Clark I (DA), Adamsite (DM) and their corresponding oxidation products as well as triphenylarsine oxide (TPA[ox]) and phenylarsonic acid (PDCA[ox]) were used as chemicals in *in vitro* experiments. Glutathione (GSH) conjugates of DA, DM and PDCA-related chemicals were found to be the most dominant metabolites, and methylated metabolites were detected as well, suggesting that these compounds are metabolised in the presence of cod liver enzymes. TPA[ox] was the only compound tested that did not form a GSH conjugate or methylated metabolite, indicating a different biotransformation pathway for this compound. Furthermore, hydroxylated metabolites were detected for each tested chemical. Due to their reactive nature, GSH conjugates may be difficult to detect in fish samples from CWA dumpsites. In contrast, both methylated and hydroxylated metabolites of phenylarsenic chemicals are promising target chemicals for the detection of CWA-related contamination in fish.

**Abbreviations:** CWA, chemical warfare agent; CW, chemical weapon; DA, Clark I; DM, Adamsite; PDCA, phenyldichloroarsine; TPA, triphenylarsine; PDCA[ox], phenylarsonic acid; DM[ox], 10-hydroxy-5*H*-phenarsazinine-10-oxide; DPA[ox], diphenylarsinic acid; TPA[ox], triphenylarsine oxide; GSH, glutathione; iAs, inorganic arsenic; SAM, *S*-adenosyl methionine; NMR, nuclear magnetic resonance spectroscopy; NADPH, nicotinamide adenine dinucleotide phosphate; MPAA, methylphenylarsinic acid; 10-M-5*H*-PA-10-O, 10-methyl-5*H*-phenarsazinine 10-oxide; MDPAO, methyldiphenylarsine oxide; HESI, heated electrospray ionization; HCD, higher-energy collisional dissociation; GST, glutathione-*S*-transferase; EIC, Extracted ion chromatogram.

## 4.2. Introduction

In order to understand the mechanism and pathway of arsenic induced toxicity in organisms, biotransformation of arsenicals is a crucial point. Extensive research on toxicity and biotransformation of inorganic arsenic (iAs) has been done and the toxicity of iAs with different oxidation states (iAs<sup>III</sup>, iAs<sup>V</sup>) is well known. Several studies have demonstrated that trivalent iAs<sup>III</sup> is responsible for the toxic properties of arsenic (Gomez-Camirero et al., 2010; Thomas et al., 2001). In humans, as in most mammalian species, the metabolism of inorganic arsenic can be distinguished in two routes: firstly, the reduction of the pentavalent state (iAs<sup>V</sup>) to the trivalent state (iAs<sup>III</sup>) (Naranmandura et al., 2006; Watanabe and Hirano, 2013; Zakharyan and Aposhian, 1999) and secondly, the methylation of trivalent forms of arsenic with the methyl donor S-adenosyl methionine (SAM). The latter reaction essentially needs glutathione (GSH) as a cofactor and reducing agent (Gomez-Camirero et al., 2010). The methylated forms of arsenic can finally be excreted by the organism in the urine (Kumagai and Sumi, 2006). In contrast to iAs species, the biotransformation reactions of phenylarsenic chemical warfare agents (CWAs) and their primary degradation products have not yet been investigated.

Phenylarsenic CWAs such as Clark I (diphenylchloroarsine, DA) and Adamsite (10-Chloro-5,10-dihydrophenarsazine, DM) were produced in large quantities during World War I (Bełdowski et al., 2017), mainly in Germany. Triphenylarsine (TPA) and Pfiffigus (phenyldichloroarsine, PDCA) are both components of technical Clark I, so-called arsine oil, which were used together with sulfur mustard to lower its freezing point (Missiaen et al., 2010). Structures of these arsenic containing CWAs are shown in Figure 4.1.



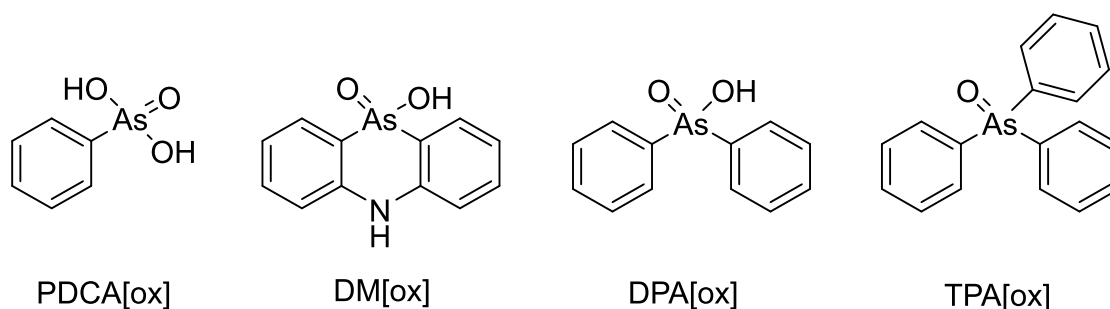
**Figure 4.1.:** Structures of CWA-related phenylarsenic chemicals.

After World War II, German chemical weapon (CW) stockpiles were captured by the Allies and disposed mainly by dumping in the Baltic Sea and the Skagerrak Strait (Szarejko and Namieśnik, 2009). Based on official records, at least 170 000 t of CWs were dumped in the Skagerrak and 50 000 t in the Baltic Sea (HELCOM, 2013).

Not only environmental issues but also the growing pressure on offshore investments and constructions using the sea floor have increased the compulsion to investigate the state of munitions. Several studies have been proven that shells filled with CWAs are often corroded allowing these chemicals to leak into the marine environment (Bełdowski et al., 2016a; Paka and Spiridonov, 2002; Szarejko and Namieśnik, 2009). For instance, in the 2010s during the Nord Stream pipeline project, several bombs containing CWAs with heavily corroded shells were observed in close vicinity to the pipeline route (HELCOM, 2013). Sediment analyses from samples taken near different CWA dumping areas have revealed high concentrations of phenylarsenic CWAs, which pose an unknown threat to the entire marine ecosystem (Bełdowski et al., 2017; Missiaen et al., 2010; Tørnes et al., 2006). This site-specific contamination is also reflected in marine organisms; diphenylarsinic acid (DPA[ox]), which is a primary degradation product of Clark I/II, has been detected in marine biota samples (Niemikoski et al., 2017), providing more convincing evidence on the environmental threat caused by continuously leaking munitions. The relevance of the existing exposure of marine organisms to these compounds is additionally emphasized by the evidence of bioaccumulation of CWA-related phenylarsenic chemicals in blue mussels (*Mytilus trossulus*) (Höher et al., 2019).

The uptake of CWA-related phenylarsenic chemicals raises the question of how they undergo biotransformation processes in marine organisms. In addition to the intact CWAs (Figure 4.1), the oxidation products of CWAs, which are considered as primary degradation products formed via hydrolysis and oxidation reactions in the water environment (Haas et al., 1998; Missiaen et al., 2010), are great of interest. Chemical structures of oxidation products of CWAs used in this study are presented in Figure 4.2. In order to illustrate the oxidation state of the specific chemical in this paper, the oxidation products of PDCA, DM, DA and TPA are represented by the abbreviations PDCA[ox], DM[ox], DPA[ox] and TPA[ox], respectively.





**Figure 4.2.:** Structures of oxidation products of phenylarsenic chemicals used in this study.

As mentioned previously, the available information on the metabolism of CWA-related phenylarsenic chemicals in any living organism is very limited and there are no published data concerning the metabolism of these chemicals in any fish or other marine biota. So far, research has only focused on the metabolism and toxicity of DPA[ox] in the context of the drinking water incident in Kamisu, Japan, where people suffered serious health impairments after drinking well water contaminated with DPA[ox] (Ishii et al., 2004). Further investigation showed that groundwater in this area was contaminated with DPA[ox] and other phenylarsenic chemicals which were originated from dumped CWs (Ishizaki et al., 2005). The toxicity of DPA[ox] is strongly influenced by the presence of GSH and the glutathione conjugate forming with DPA[ox] (Ochi et al., 2006). In an *in vitro* study with human HepG2-cells, a 1000-times higher toxicity of DPA-SG, the GSH adduct of DPA[ox], was demonstrated than for DPA[ox] itself (Kinoshita et al., 2006; Ochi et al., 2006) and its cellular uptake rate is directly linked to the presence of GSH.

As the seabed is heavily contaminated in some areas where dumping operations took place (Bełdowski et al., 2016a; Paka and Spiridonov, 2002; Szarejko and Namieśnik, 2009) and it has already been demonstrated that phenylarsenic CWAs enter the food web (Niemikoski et al., 2017), information on the biotransformation of these compounds is important both for the environmental risk assessment and for evaluating the risk for human sea food consumers. Existing studies have focused on detecting only primary degradation products of phenylarsenic CWAs, which do not necessarily provide an overall picture of the contamination levels of these toxic chemicals. In order to study the biotransformation of CWA-related chemicals, *in vivo* experiments in the field or in the laboratory are usually associated with major logistical precautions, especially in CWA dumpsites. In opposite to this, *in vitro* experiments with liver homogenates are much easier to handle under stable and reproducible conditions and can also serve

as study platform for metabolism studies of CWA-related chemicals (Halme et al., 2015).

In this study we exposed selected CWA-related phenylarsenic chemicals (Figure 4.1) and their primary oxidation products (Figure 4.2) *in vitro* to cod liver S9 fraction to pursue the following goals; 1) to investigate the fate of these chemicals in fish liver homogenates, 2) to identify formed metabolites which are not yet monitored and might be used as target chemicals when evaluating the total exposure status of CWA-related phenylarsenic chemicals in marine biota. Therefore, this study contributes to one of the recommendations of HELCOM from 2013 to develop and improve CWA-related chemical analytical methods (HELCOM, 2013), also with respect to ecotoxicology and physicochemical properties of CWAs.

## 4.3. Materials and methods

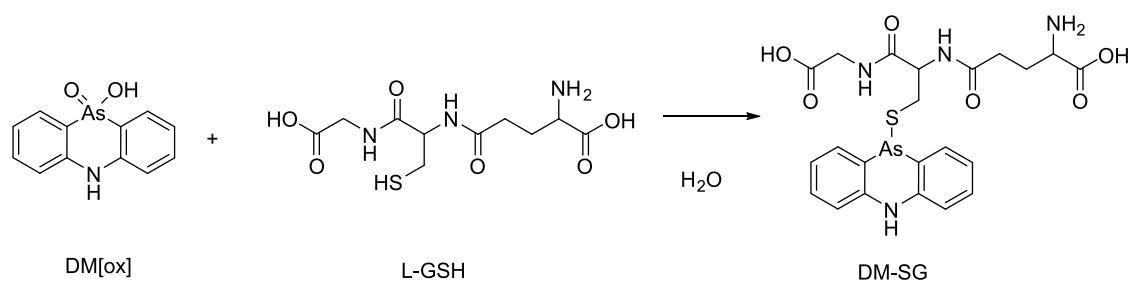
### 4.3.1. Chemicals and reagents

DA, DM, and reference chemicals for identification of formed metabolites were synthesized at the Finnish Institute for Verification of the Chemical Weapons Convention, VERIFIN. DM[ox] and DPA[ox] were obtained from Envilytix GmbH (Germany). PDCA[ox] and TPA[ox] were obtained from Acros Organics (Belgium) and Sigma Aldrich (Germany), respectively. Concentrations of phenylarsenic chemical stock solutions were determined by  $^1\text{H}$  nuclear magnetic resonance spectroscopy (NMR). 3-(Trimethylsilyl)propionic-2,2,3,3- $d_4$  acid sodium salt (TSP- $d_4$ ) used as reference chemical in NMR analysis was obtained from Sigma Aldrich (Germany). Methanol (MeOH), acetonitrile (ACN) and formic acid (99%) were obtained from Fisher Scientific (United Kingdom), Fluka (Germany) and Sigma Aldrich (Germany), respectively. Water was purified using a Direct-Q3 UV system (Millipore, Germany). Chemicals for Lowry protein assay and *in vitro* metabolism assay were purchased from Merck (Germany), except dithiothreitol (DTT) and ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, Germany). Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Biomol GmbH (Germany), L-GSH and SAM were obtained from Sigma-Aldrich (Germany).

### 4.3.2. Synthesis of reference chemicals

Glutathione complexes of PDCA[ox], DM and DPA[ox] were synthesized according to previously published procedures with minor modifications (Nakayama et al., 2005). Characterization of these synthesis products were done by ultrahigh performance liquid chromatography-high-resolution mass spectrometry (UHPLC-HRMS) and  $^1\text{H}$  NMR. The synthesis route for the GSH complex of DM[ox] is presented in Figure 4.3. GSH complexes for other phenylarsenic compounds were synthesized in a similar manner.

In this paper, GSH-conjugates of PDCA[ox], DM and DPA[ox] are presented as PA-SG2, DM-SG and DPA-SG, respectively.



**Figure 4.3.:** Synthesis of glutathione complex of 10-hydroxy-5*H*-phenarsazinine 10-oxide.

### 4.3.3. Sampling and isolation of liver enzymes

For performing *in vitro* metabolism experiments, cod (*Gadus morhua*) livers were taken in the area of Gdansk Bay (55.06 N - 55.26 N, 18.15 E - 18.58 E) onboard of RV Walther Herwig III in December 2016. After trawling, cod were put in water tanks filled with precooled seawater and further processing took place individually. The fish were sacrificed before sampling, after which the liver was removed and weighed. Afterwards, parts of the liver were put in cryovials and stored in liquid nitrogen at -80 °C.

The liver enzymes were prepared according to the method of Ronisz and Förlin (1998). Liver samples were homogenized in ice cold Na<sup>+</sup>/K<sup>+</sup>-phosphate buffer (0.1 M) with 0.15 M KCl, 1 mM EDTA, 1 mM DTT at a ratio of 1:10 (w/v) in a Potter-Elvehjem glass-Teflon homogenizer (Melsungen, Germany). Homogenates were put in a Beckman Coulter Optima MAX-XP ultracentrifuge (Brea, USA) and centrifuged (10 000 g for 20 min, 4 °C) to obtain the S9 fraction. For the purpose of the *in vitro* metabolism study, 8 liver samples were pooled to provide a sufficient amount of material for all experiments.

### 4.3.4. Protein content measurement

The protein content of the pooled liver sample was measured according to the method of Lowry et al. (1951). The measurement was carried out with a FLOUstar Optima microplate reader from BMG Labtech (Offenburg, Germany) and bovine serum albumin was used as standard.

### 4.3.5. *In vitro* metabolism experiments

*In vitro* metabolism was studied by incubating S9 liver fractions with different CWA-related phenylarsenic chemicals. Liver homogenates were diluted with Na<sup>+</sup>/K<sup>+</sup>-phosphate buffer (0.1 M, pH 7.5) and the protein concentration of the final reaction mixture was adjusted to 3.5 mg/L. The reaction was carried out in an overall volume of 500  $\mu$ L and depending on the composition it consisted of Na<sup>+</sup>/K<sup>+</sup>-phosphate buffer, 1 mM NADPH, 5 mM L-Glutathione (GSH) and 1 mM SAM. The reaction was initiated with the addition of 10  $\mu$ g/mL of the phenylarsenic compound (5  $\mu$ L). Compositions of the reaction mixtures are shown in Table 4.1. The reaction tubes were incubated and shaken (700 rpm) at 28 °C and the reaction was stopped after 0 min, 30 min or 120 min by addition of 800  $\mu$ L of ice-cold ACN. The reaction tubes were centrifuged (10 000 rpm, 4 °C, 10 min) and the supernatant was stored at -80 °C for further analysis. All samples were done in duplicate and the controls including the blanks were incubated for 120 min.

**Table 4.1.:** Composition of the samples for *in vitro* metabolism experiments with CWA-related phenylarsenic compounds using cod liver S9 fractions. “X” present in sample, “-” not present in sample.

Content	Blank	Substrate Control	NADPH Control	GSH Control	NADPH <sup>a</sup> 0, 30, 120 min	GSH <sup>a</sup> 0, 30, 120 min
Liver enzymes	X	-	X	X	X	X
CWA compound	-	X	X	X	X	X
NADPH	X	X	-	X	X	X
GSH	X	X	X	-	-	X
SAM	X	X	X	X	-	X
Buffer	X	X	X	X	X	X

<sup>a</sup>In each case, all the NADPH and GSH samples were terminated after 0, 30, 120 min in duplicate.

### 4.3.6. UHPLC-HRMS analysis

The UHPLC-HRMS analysis were performed with Thermo Scientific Orbitrap Fusion mass spectrometer (San Jose, USA) connected to a Thermo Scientific Dionex Ultimate 3000 ultrahigh performance liquid chromatography (Germering, Germany). UHPLC separations were done using Waters XBridge BEH C18 column (2.1  $\times$  50 mm, 1.7  $\mu$ m) at 40 °C using a linear gradient of two mobile phases: 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B). The gradient was run from 5% B at 0 min to 100% B at 5 min. After this the B eluent was kept at 100% for 1 min and at 5% for 2 min. The flow rate was 0.5 mL/min, and the injection volume was 5  $\mu$ L.

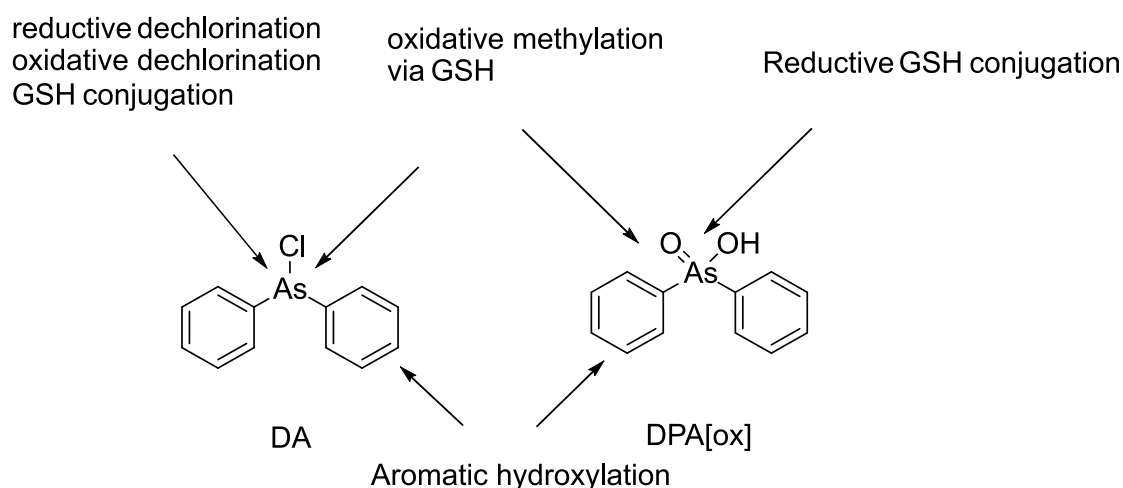
The ionization was done using heated electrospray ionization (HESI) in the positive ion mode. The instrumental parameters were set as follows: spray voltage 3000 V, source temperature 300 °C, ion transfer tube temperature 350 °C, sheath gas 30, auxiliary gas 10 and sweep gas 0. Mass measurements (HRMS) were done in the mass range of  $m/z$  70-800 using RF lens at 60% and quadrupole isolation ( $m/z$  70-800) at resolution of 120 000. Mass accuracy of the instrument using internal calibration was specified to be  $\leq 2.5$  ppm. For identification of formed metabolites, MS/HRMS technique was applied. Higher-energy collisional dissociation (HCD) was used in fragmentation of protonated molecules. Collision energies for different detected metabolites are presented in appendix C, Table C.1.

#### 4.3.7. $^1\text{H}$ NMR analysis

All  $^1\text{H}$  NMR measurements were carried out at 290 K using a Bruker Avance III 500 NMR spectrometer equipped with a 5 mm TXI probe head (Rheinstetten, Germany) operating at a proton frequency of 500 MHz. The NMR samples were prepared by transferring 500  $\mu\text{L}$  of the synthesis products into a 5 mm NMR tube (Wilmad, USA) and adding 100  $\mu\text{L}$  of TSP- $d_4$  solution (0.7 mg/mL) in  $\text{D}_2\text{O}$  as a reference chemical ( $\delta_{1\text{H}}$  0.00 ppm).

## 4.4. Results and discussion

*In vitro* metabolism of two CWA chemicals, DM and DA, their corresponding oxidation products DM[ox] and DPA[ox] as well as oxidation products of two components of arsine oil, PDCA[ox] and TPA[ox] were studied using the S9 fraction isolated from cod liver homogenates. Based on existing knowledge on the metabolism of inorganic arsenicals, the focus of this study was to investigate GSH mediated reactions and methylation. A non-targeted screening method was used for detecting metabolites in *in vitro* samples. The hypotheses pursued in this study on possible enzyme-catalysed metabolic reactions using DA and DPA[ox] as examples are shown in Figure 4.4.



**Figure 4.4.:** Hypothetical metabolic transformations for Clark I-related chemicals.

### 4.4.1. GSH-metabolites

Arsenic is a metalloid which acts as an electrophile in chemical reactions (Hughes, 2002; Kretzschmar et al., 2014). It is prone to react with nucleophiles, such as free thiol groups (Kretzschmar et al., 2014; Nakayama et al., 2005). Based on this, it was assumable that the studied CWA-related chemicals form GSH conjugates despite the oxidation state of arsenic. Accordingly, PDCA[ox], DM, DA, DM[ox] and DPA[ox] formed glutathione conjugates *in vitro*, and these were also formed non-enzymatically in the substrate control samples for PDCA[ox], DM, DA, DM[ox] and DPA[ox], respectively. As expected, intact chemicals (DA and DM) and their corresponding oxidation products (DPA[ox] and DM[ox]) formed the same GSH conjugates, namely DM-SG and DPA-SG (see Table 4.2), which were seen at  $m/z$  536 and at  $m/z$  549 in the mass

spectra, respectively. PAA formed di-glutathione conjugate, PA-SG2 (Table 4.2) which was seen at  $m/z$  765.

The identification of the detected metabolites was predominantly carried out using reference chemicals synthesized in this study. The identification criteria were based on the guidelines of the Organisation for the Prohibition of Chemical Weapons (OPCW) for the analysis of biomedical samples (Organisation for the Prohibition of Chemical Weapons (OPCW), 2018). The characterization of the synthesized reference chemicals was done by  $^1\text{H}$  NMR and HRMS techniques. In  $^1\text{H}$  NMR spectra, the most distinct difference in chemical shifts of formed GSH complexes compared to free GSH were chemical shifts of cysteine  $\alpha$  and  $\beta$  protons. When a covalent bond between arsenic and sulfur is formed, the  $^1\text{H}$  resonance signals of cysteine protons are shifted to higher frequency (lower ppm) compared to free GSH. This is clearly seen in the  $^1\text{H}$  NMR spectra of each synthesized GSH complex. The masses corresponding to the masses of protonated molecules of synthesized reference chemicals were detected by HRMS. Based on two spectrometric analysis techniques, it is justified to say that synthesis of reference chemicals was successful. NMR and HRMS data for synthesized reference chemicals for GSH metabolites are presented in appendix C (Table C.2).

Proposed elemental compositions for protonated molecules and formed fragment ions, their corresponding measured masses, mass differences ( $\Delta$  ppm) and retention times ( $R_t$ ) for reference chemicals and *in vitro* samples are presented in Table 4.2. Proposed fragmentation pathways for all three GSH metabolites are presented in appendix C (Figure C.1, Figure C.2, Figure C.3).

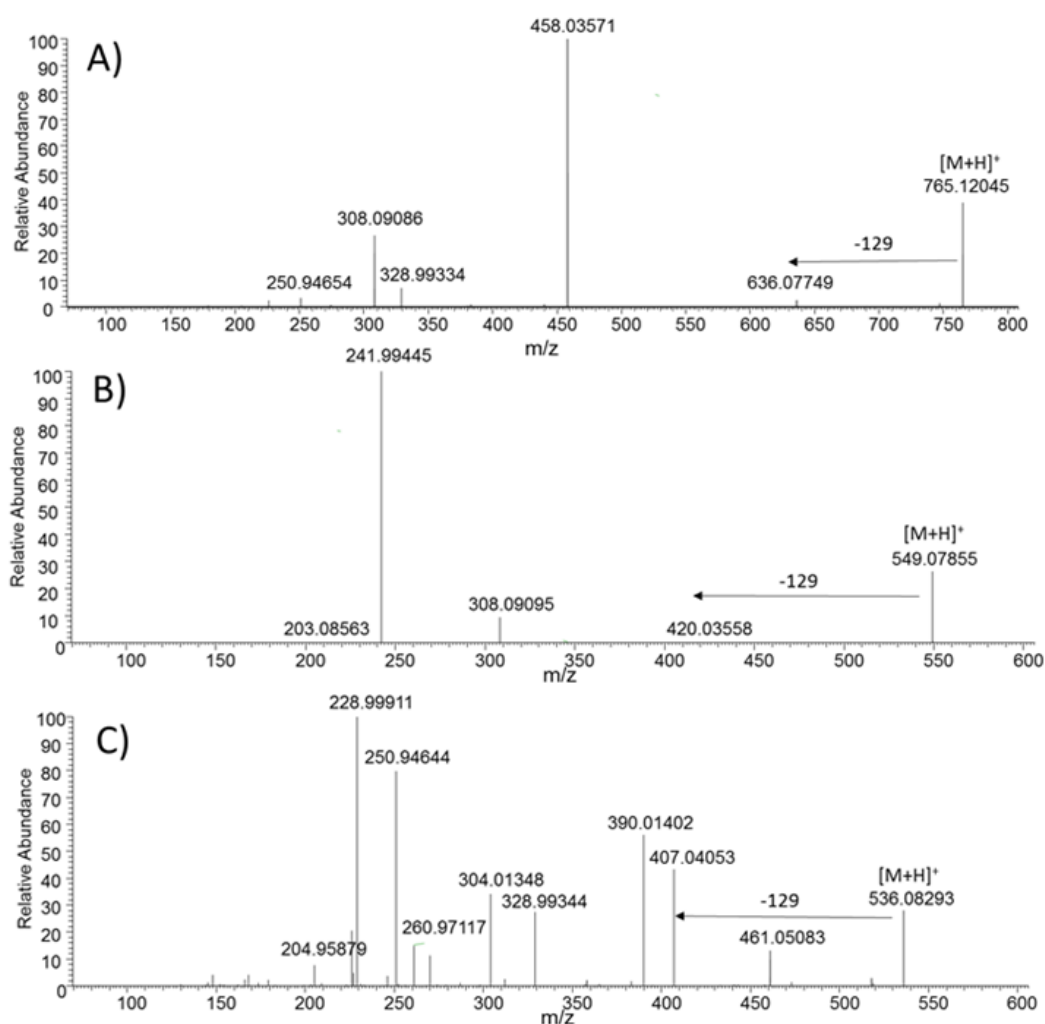
The elemental composition of the detected protonated molecules and fragment ions in *in vitro* samples were consistent with the corresponding elemental composition of synthesized reference chemicals. The spectra of detected GSH-metabolites for PDCA[ox], DM and DPA[ox] in *in vitro* samples are presented in Figure 4.5. The characteristic fragmentation pathway for all three GSH metabolites is the neutral loss of pyroglutamic acid (129.042 59 Da) which can be seen at  $m/z$  636.077 49 (A), 420.035 58 (B) and 407.040 53 (C) in the spectra in Figure 4.5.



**Table 4.2.:** Proposed elemental compositions for formed fragments in *in vitro* samples, measured masses and mass differences of detected GSH metabolites of phenylarsenic chemicals.

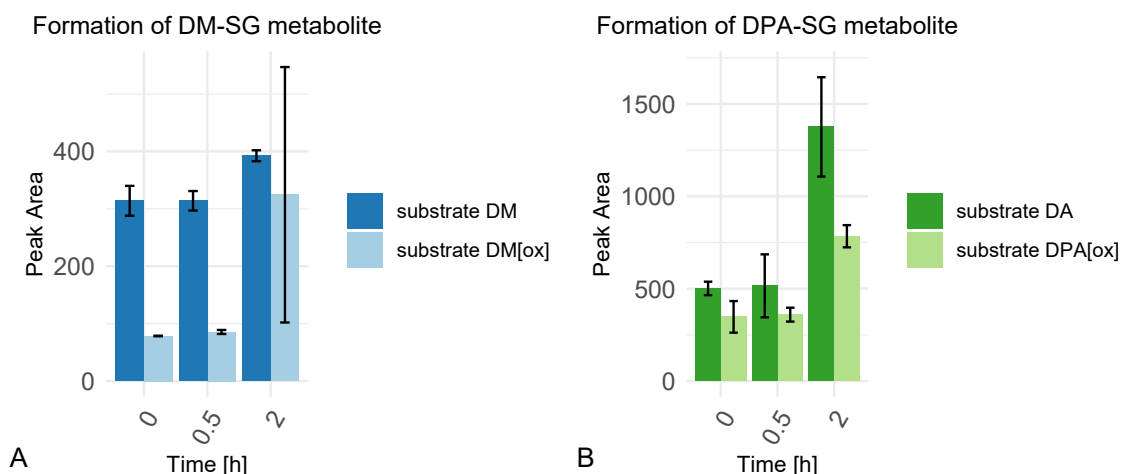
Compound	Theoretical elemental composition	Reference chemical			<i>In vitro</i> sample		
		Measured mass $m/z$	$\Delta$ ppm <sup>a</sup>	$R_t$ [min]	Measured mass $m/z$	$\Delta$ ppm <sup>a</sup>	$R_t$ [min]
PA-SG <sub>2</sub>	[M+H] <sup>+</sup>	765.12045 (32.8%)	0.64042		765.12036 (36.0%)	0.52280	
	C <sub>26</sub> H <sub>38</sub> AsN <sub>6</sub> O <sub>12</sub> S <sub>2</sub> <sup>+</sup>						
	C <sub>21</sub> H <sub>23</sub> AsN <sub>9</sub> O <sub>10</sub> S <sup>+</sup>	636.07749 (2.1%)	0.04064		636.07733 (2.5%)	0.33269	
	C <sub>8</sub> H <sub>17</sub> AsN <sub>9</sub> O <sub>9</sub> S <sup>+</sup>	458.03577 (100%)	0.96062	1.25	458.03577 (100%)	0.82963	1.24
	C <sub>5</sub> H <sub>8</sub> AsN <sub>2</sub> O <sub>3</sub> S <sup>+</sup>	250.94652 (3.4%)	0.27894		250.94653 (3.3%)	0.31879	
DM-SG	[M+H] <sup>+</sup>	549.07855 (50.7%)	0.36425		549.07886 (38.5%)	0.92883	
	C <sub>22</sub> H <sub>26</sub> AsN <sub>4</sub> O <sub>6</sub> S <sup>+</sup>						
	C <sub>22</sub> H <sub>19</sub> AsN <sub>3</sub> O <sub>3</sub> S <sup>+</sup>	420.03558 (0.2%)	0.42854		420.03564 (0.2%)	0.28570	
	C <sub>10</sub> H <sub>18</sub> N <sub>3</sub> O <sub>6</sub> S <sup>+</sup>	308.08095 (9.2%)	0.42195	2.35	308.09109 (9.1%)	0.03246	2.35
	C <sub>12</sub> H <sub>9</sub> AsN <sup>+</sup>	241.99445 (100%)	0.41323		241.99454 (100%)	0.04132	
DPA-SG	[M+H] <sup>+</sup>	536.08293 (16.5%)	0.31711		536.08295 (17.8%)	0.27980	
	C <sub>22</sub> H <sub>27</sub> AsN <sub>3</sub> O <sub>6</sub> S <sup>+</sup>						
	C <sub>17</sub> H <sub>20</sub> O <sub>3</sub> N <sub>2</sub> AsS <sup>+</sup>	407.04053 (28.8%)	0.04913		407.04044 (31.6%)	0.17197	
	C <sub>17</sub> H <sub>17</sub> O <sub>3</sub> NAsS <sup>+</sup>	390.01402 (37.4%)	0.15384	2.64	390.01389 (41.3%)	0.17948	2.64
	C <sub>12</sub> H <sub>10</sub> AsS <sup>+</sup>	260.97117 (14.2%)	0.76636		260.97116 (14.3%)	0.80468	
	C <sub>12</sub> H <sub>10</sub> As <sup>+</sup>	228.99911 (100%)	0.82969		228.99911 (100%)	0.82969	

<sup>a</sup> Mass difference between measured and theoretical mass (acceptable difference is 2.5 ppm (Organisation for the Prohibition of Chemical Weapons (OPCW), 2018)).



**Figure 4.5.:** MS/HRMS spectra for A) PA-SG2, B) DM-SG and C) DPA-SG detected in *in vitro* samples.

The formation of DPA-SG and DM-SG metabolites increased over time. As shown in Figure 4.6, DPA-SG and DM-SG conjugates were formed in larger scale when DA and DM were used as substrates compared to the oxidation products (DPA[ox] and DM[ox]). This can be explained by the reaction mechanism: Reduction of arsenic from oxidation state V to III is the rate limiting step in conjugation reactions of phenylarsenic (V) compounds (Kretzschmar et al., 2014). Therefore, DPA-SG and DM-SG are possibly formed from DA and DM by reductive dechlorination, while GSH is directly attached to arsenic instead of oxidative dechlorination when arsenic is first oxidized prior the conjugation with GSH.



**Figure 4.6.:** Time-dependent formation of DM-SG metabolite using DM and DM[ox] as a substrate (A) and formation of DPA-SG metabolite using DA and DPA[ox] as a substrate (B) in *in vitro* experiments with cod liver. Bars represent the mean value of the peak areas from two individual measurements. The error bars represent the lower and upper limits.

Also the formation of PA-SG<sub>2</sub> increased over the time. The data is shown in appendix C (Figure C.4). Although GSH can react non-enzymatically with electrophiles, such as arsenic, also described in herbicides (Jablonkai and Hatzios, 1993; Leavitt and Penner, 1979), this reaction is usually catalyzed by the glutathione-*S*-transferase (GST) superfamily (Ketterer, 1982). The results suggest that GSH conjugation reactions in fish liver are mainly enzyme-catalysed because the formation of GSH metabolites is increasing over time in samples containing enzymes. Furthermore, the results showed that the concentrations of non-enzymatically formed GSH-metabolites were lower compared to samples containing enzymes.

Arsenic-glutathione complexes have been previously detected in rat bile samples after exposure to iAs (Cui et al., 2004; Kala et al., 2000; Suzuki et al., 2001), and one study describes the existence of DPA-SG in rat bile after oral administration of DPA[ox] (Kobayashi and Hirano, 2013), suggesting that phenylarsenic chemicals can be excreted into the bile after conjugation with GSH. The biliary excretion of arsenic-GSH species depends on the multidrug resistance-associated protein 2 (MRP2/cMOAT) which is known to be responsible for the transport of GSH and GSH conjugates (Kala et al., 2000). In general, the conjugation reaction between GSH and xenobiotics is considered to be a detoxification step in metabolism, but in some cases the conjugation with GSH can produce more reactive and therefore more toxic metabolites (van Bladeren, 1988). According to previous studies, toxic properties are dramatically enhanced when DPA[ox] is conjugated with GSH (Ochi et al., 2006). Moreover, DPA-SG turned out to

be unstable in culture medium (Kinoshita et al., 2006), suggesting that the detection of DPA-SG in contaminated fish is challenging. To our knowledge, no studies describe the formation of DM-SG or PA-SG<sub>2</sub> in biota so far and their stability *e.g.* in bile is unclear. TPA[ox] was the only chemical in this study which did not form GSH conjugates, so the excretion pathway of TPA[ox] in fish might differ from those chemicals which formed GSH-conjugates. As TPA[ox] is the most lipophilic compound in this study, it might be more prone to accumulate in fish muscles and the liver compared to the other studied phenylarsenic chemicals.

#### 4.4.2. Methylated metabolites

In this study we were able to demonstrate that all CWA-related chemicals except TPA[ox] formed methylated products in *in vitro* samples which contained SAM as a cofactor. Several studies on methylation reactions of iAs species have shown that these reactions are catalyzed by the methyl-*S*-transferase family which utilizes SAM as a methyl group donor. In general, metabolism of inorganic arsenic has been considered to occur via sequential methylation and reduction reactions using SAM as a methyl group donor and GSH as a reducing agent (Gomez-Caminero et al., 2010). Therefore, it is assumable that methylated metabolites of CWA-related chemicals detected in this study are formed via GSH conjugation.

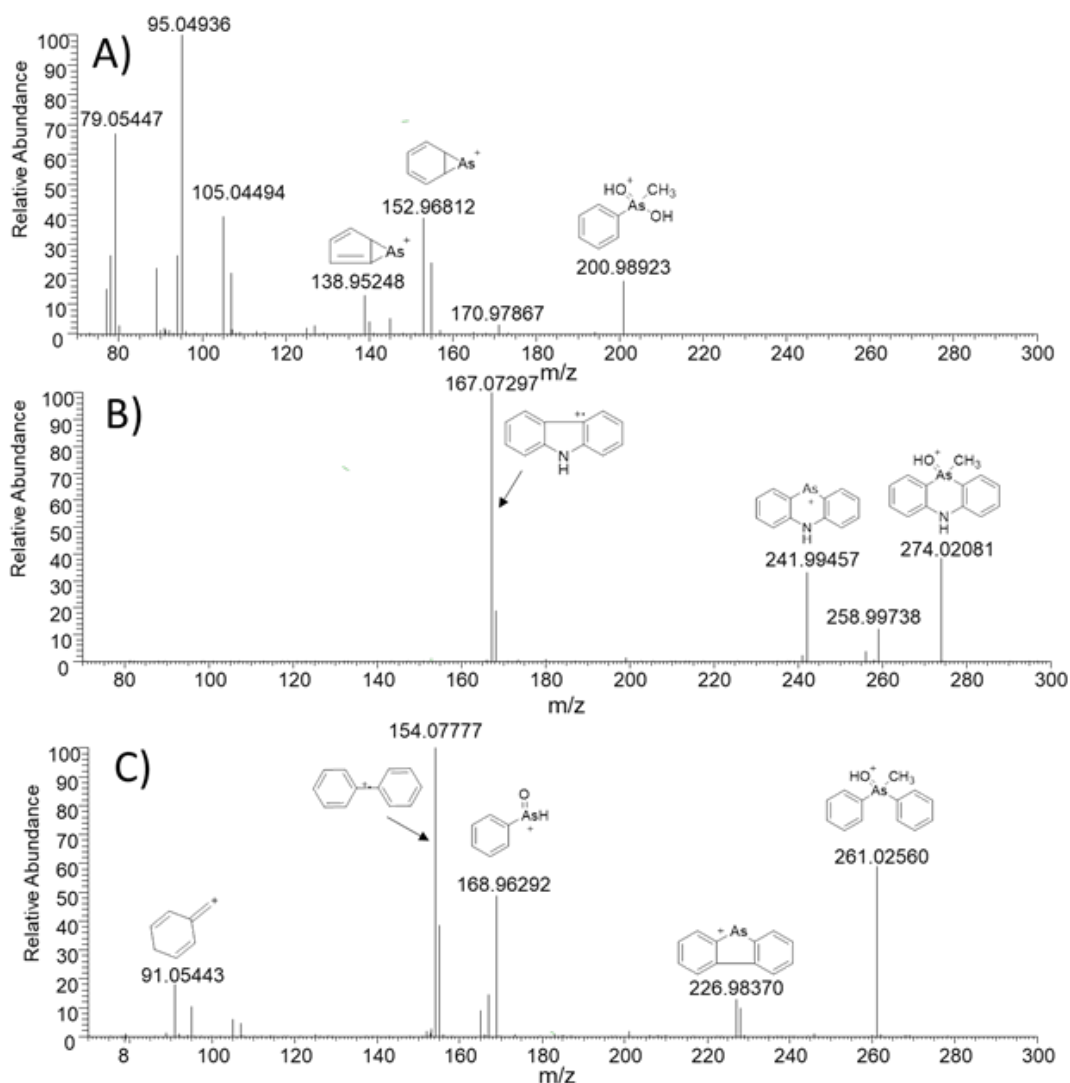
Of the compounds tested, DM and DM[ox] formed the same methylated metabolite, 10-methyl-5*H*-phenarsazinine (10-M-5*H*-PA-10-O), and DA and DPA[ox] formed the same methylated metabolite, methyldiphenylarsine oxide (MDPAO), and PDCA[ox] formed methylphenylarsinic acid (MPAA). The depletion of GSH as cofactor did not affect the formation of methylated metabolites, which strongly favors the assumption that the S9 fraction contains a certain amount of GSH or other reducing agents, such as free thiol groups. The elemental composition of the detected protonated molecules and fragment ions in *in vitro* samples was consistent with the corresponding elemental composition of the synthesized reference chemicals. The proposed elemental composition for the protonated molecules and fragment ions, their corresponding measured masses, mass differences and retention times of reference chemicals and *in vitro* samples are presented in Table 4.3.

**Table 4.3.:** Proposed elemental compositions for protonated molecule ion and formed fragments in *in vitro* samples, measured masses and mass differences of detected methylated metabolites of phenylarsenic chemicals.

Compound	Proposed elemental composition	Reference chemical			<i>In vitro</i> sample		
		Measured mass $m/z$	$\Delta$ ppm <sup>a</sup>	$R_t$ [min]	Measured mass $m/z$	$\Delta$ ppm <sup>a</sup>	$R_t$ [min]
MPAA	$[M+H]^+$ $C_7H_{10}AsO_2^+$	200.98915 (15.7%)	0.11693		200.98920 (16.2%)	0.34468	
	$C_6H_6As^+$	152.96808 (37.5%)	0.52582	0.82	152.9680 (38.3%)	0.33269	0.84
	$C_5H_4As^+$						
10-M-5 <i>H</i> -PA-10-O	$[M+H]^+$	274.02084 (36.1%)	0.29748		274.02081 (36.9%)	0.18611	
	$C_{13}H_{13}AsNO^+$						
	$C_{12}H_9AsN^+$	241.99460 (30.7%)	0.21124	1.44	241.99457 (35.7%)	0.08513	1.44
	$C_{12}H_9N^+$	167.07301 (100%)	0.37370		167.07297 (100%)	0.09971	
MDPAO	$[M+H]^+$ $C_{13}H_{14}AsO^+$	261.02557 (54.2%)	0.23258		261.02560 (56.1%)	0.03071	
	$C_{12}H_8As^+$	226.98367 (11.1%)	0.10950		226.98370 (12.6%)	0.012457	
	$C_8H_6AsO^+$	168.96286 (48.3%)	0.31174	1.82	168.96292 (46.2%)	0.23266	1.84
	$C_{12}H_{10}^+$	154.07777 (100%)	0.46826		154.07777 (100%)	0.78456	

<sup>a</sup>Mass difference between measured and theoretical mass (acceptable difference is 2.5 ppm (Organisation for the Prohibition of Chemical Weapons (OPCW), 2018)).

The spectra of MPAA, 10-M-5*H*-PA-10-O and MDPAO from *in vitro* samples are presented in Figure 4.7. As can be seen in Figure 4.7, the protonated molecule ions at  $m/z$  200.989 23 (A), at  $m/z$  274.020 81 (B) and at  $m/z$  261.025 60 (C) are the corresponding masses for the protonated molecules of MPAA, 10-M-5*H*-PA-10-O and MDPAO, respectively. Based on the measured mass of MPAA (Figure 4.7A), it appears that methylation took place only once at the arsenic atom and mono-methylated metabolites were formed. No di-methylated metabolites were detected.



**Figure 4.7.:** MS/HRMS spectra for MPAA (A), 10-M-5*H*-PA-10-O (B), and MDPAO (C) detected in *in vitro* samples.

Previous studies showed that in the urine of mice exposed to DPA[ox] or PDCA[ox], methylation occurred only for PDCA[ox] and small amounts of MPAA were detected, while no methylation of DPA[ox] was observed (Kinoshita et al., 2008). Despite the fact that only MPAA was found in mice urine, the *in vitro* experiments with cod liver homogenates prove that the methylation products MPAA, 10-M-5*H*-PA-10-O, MDPAO are formed and are potentially suitable to be found in bile or urine of contaminated fish. Furthermore, these findings suggest that PDCA[ox], DA and DM undergo the same metabolic reactions, although several metabolic pathways are conceivable. Methylated metabolites may also pose a higher risk to fish, as studies on the methylation of inorganic arsenic have shown higher toxicity and reactivity of these compounds than  $iAs^{III}$  itself (Kumagai and Sumi, 2006; Petrick et al., 2000; Thomas et al., 2004).

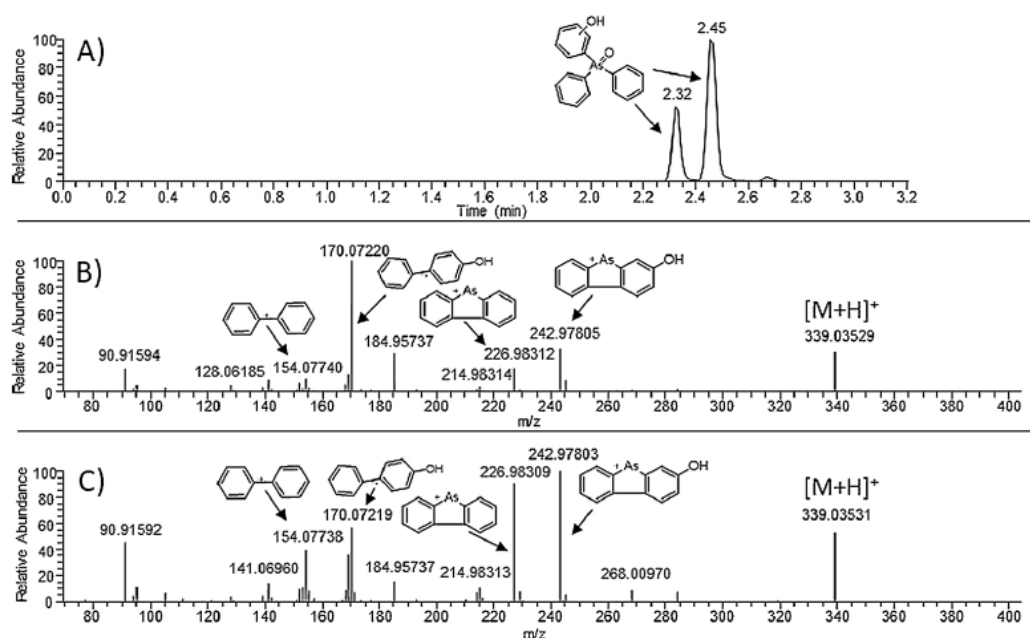
### 4.4.3. Hydroxylated metabolites

All studied CWA-related phenylarsenic chemicals formed metabolites in which hydroxylation took place on the aromatic ring. Hydroxylated metabolites were detected in the samples which contained NADPH as a cofactor, suggesting that these reactions are NADPH-dependent. Aromatic hydroxylation reactions are mediated by the cytochrome P450 monooxygenase family (Bernhardt, 2006), and these types of metabolites are already known, for example for polycyclic aromatic hydrocarbons in fish (Schlenk et al., 2008). The addition of OH-groups to the aromatic ring makes the substrate more hydrophilic (Schlenk et al., 2008), and further reactions to these aromatic OH-groups are possible as well.

There were no reference chemicals available for hydroxylated metabolites of the investigated chemicals, so the identification was based on accurate mass measurements of protonated molecules and formed fragment ions and their corresponding elemental composition. Based on the mass spectrometric measurements, it is not possible to say at which position hydroxylation took place in the aromatic ring. Due to lack of knowledge at which position the aromatic hydroxylation occurred, the hydroxylated metabolites of PDCA[ox], DM[ox], DPA[ox] and TPA[ox] are abbreviated as PDCA[ox]-OH, DM[ox]-OH, DPA[ox]-OH and TPA[ox]-OH, respectively.

Extracted ion chromatogram (EIC) and spectra for detected hydroxylated metabolites of TPA[ox] are presented in Figure 4.8. Two peaks with retention times of 2.32 min and 2.45 min and the same protonated molecule ions at  $m/z$  339.035 29 and 339.035 31 were detected, suggesting that two isomers with aromatic hydroxylation occurred in two different aromatic rings of TPA[ox]. The protonated molecules were isolated using

quadrupole isolation and collided using HCD 60%. The extracted ion chromatogram for mass at  $m/z$  339 and mass spectra of the corresponding peaks are presented in Figure 4.8 with proposed structures for fragment ions. The peaks at  $m/z$  154.07738 and 226.98309 are considered to be specific ions for TPA[ox] and the peaks at  $m/z$  170.07221 and  $m/z$  242.97803 were most probably generated from TPA[ox]-OH. The detection of protonated molecules at  $m/z$  218.96334, at  $m/z$  291.99507, and at  $m/z$  278.99948 suggests the formation of hydroxylated metabolites for PDCA[ox], DM[ox] and DPA[ox], respectively. Mass spectra and proposed structures of fragments ions for PDCA[ox]-OH, DM[ox]-OH and DPA[ox]-OH are presented in appendix C (Figure C.5).



**Figure 4.8.:** EIC for hydroxylated triphenylarsine oxide (TPA[ox]-OH) (A) and MS/HRMS spectra for two isomers of TPA[ox]-OH (B) and (C).



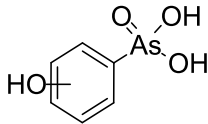
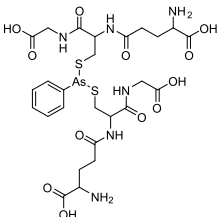
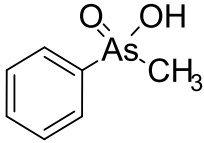
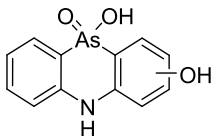
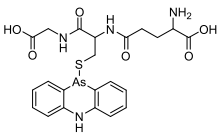
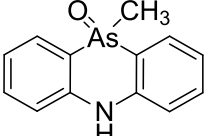
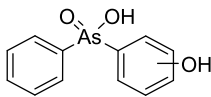
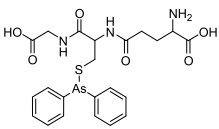
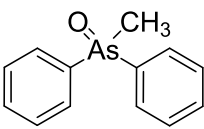
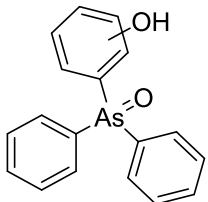
#### 4.4.4. Relevance of CWA-related phenylarsenic chemicals in fish

GSH-conjugates of the studied phenylarsenic CWAs were the major metabolites formed in *in vitro* experiments with cod liver samples. A previously published study performed with human hepatocytes demonstrated that the GSH-conjugate of DPA[ox] is highly toxic compared to DPA[ox] itself, suggesting that these major metabolites pose an increased risk to fish in the vicinity of dumpsites. Therefore, the question of whether fish are facing enhanced toxicity during biotransformation of CWAs and whether the compounds detected in this study pose a threat to fish in the vicinity of dumpsites should be addressed in further scientific studies. Based on the chemical similarities of the different GSH metabolites detected in this study, it is assumable to say that also these GSH metabolites have comparable toxic properties.

By this far, the evaluation of the ecological risks related to sea-dumped CWAs is limited to model-based assessments focusing on intact CWAs and their known primary degradation products, leaving out the biotransformation products which most likely have toxic properties. The results presented in this study strongly improve the knowledge on how CWA-related phenylarsenic chemicals are metabolised in fish and therefore improving the analytical capabilities with regard to the recommendations of the HELCOM report from 2013 (HELCOM, 2013) on addressing the problem of dumped chemical munitions in the Baltic Sea. Furthermore, the study proved that *in vitro* experiments with fish liver S9 fractions in combination with sophisticated chemical analysis technique can be utilized for the investigation of other CWA-related chemicals.

The results obtained in this study would also contribute to a future site-specific risk assessment of dumpsites requiring data from periodical surveys. Measured concentrations of primary degradation products and metabolites of CWA-related phenylarsenic chemicals in fish can also be evaluated in combination with the health status of fish in dumpsites, for example by using the Fish Disease Index for cod (Lang et al., 2018). In contrast to the hydroxylated and methylated metabolites, GSH-conjugates are not suitable for screening purposes in marine biota samples due to their reactive nature. The novel metabolites detected in *in vitro* samples and their structures are shown in Table 4.4.

**Table 4.4.:** Detected metabolites in cod liver *in vitro* samples exposed to CWA-related phenylarsenic chemicals.

Detected metabolites		
Phase I	Phase II	
<b>PDCA-related</b>		
 <p>PDCA[ox]-OH</p>	 <p>PA-SG<sub>2</sub></p>	 <p>MPAA</p>
<b>Adamsite-related</b>		
 <p>DM[ox]-OH</p>	 <p>DM-SG</p>	 <p>10-M-5H-PA-10-O</p>
<b>Clark-related</b>		
 <p>DPA[ox]-OH</p>	 <p>DPA-SG</p>	 <p>MDPAO</p>
<b>TPA-related</b>		
 <p>TPA[ox]-OH</p>	<p>not detected</p>	

## 4.5. Conclusion

Our results show that all studied CWA-related phenylarsenic chemicals undergo biotransformation reactions initiated by cod liver enzymes. With the exception of TPA[ox], all substrates formed GSH-conjugates and methylated metabolites which are described here for the first time.

We assume that the metabolism reactions shown here also occur in wild fish when they are exposed to these chemicals. For the environmental risk assessment and for the evaluation of the risk to the human seafood consumer, information on the total burden of phenylarsenic CWAs, including their metabolites, in fish and other marine biota species is essential. For this purpose, methylated and hydroxylated biotransformation products identified for the first time in this study should be used as target chemicals. Even though phenylarsenic CWAs and their metabolites are found in marine biota, their behavior, fate and toxicological impact on aquatic organisms are largely unknown, so this work ultimately improves the knowledge about the behavior of these chemicals in fish.

## 4.6. Acknowledgements

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## **5. First evidence of explosives and their degradation products in dab (*Limanda limanda* L.) from a munition dumpsite in the Baltic Sea**

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## 5.1. Abstract

Corrosion and disintegration of munition shells from the World Wars increase the risk that explosives are released into the marine environment, exposing a variety of organisms. Only few studies investigated contamination of fish with explosives in the field under environmental conditions. Here we present a comprehensive study on the contamination status of dab (*Limanda limanda*) from a munition dumpsite and from reference sites in the Baltic Sea. Bile of 236 dab from four different study sites, including a dumpsite for conventional munitions, was investigated and explosive compounds were detected by high performance liquid chromatography-mass spectrometry. Five explosive compounds were identified, including 2,4,6-trinitrotoluene, 4-amino-2,6-dinitrobenzene, and hexahydro-1,3,5-trinitro-1,3,5-triazine. 48% of the samples from the dumpsite contained at least one explosive compound. The results prove that toxic explosive compounds from a dumpsite in the Baltic Sea are accumulated by flatfish and may therefore pose a risk to fish health and human food safety.

**Abbreviations:** TNT, 2,4,6-trinitrotoluene; 2-ADNT, 2-amino-4,6-dinitrobenzene; 4-ADNT, 4-amino-2,6-dinitrobenzene; 1,3-DNB, 1,3-dinitrobenzene; TNB, 1,3,5-trinitrobenzene; HMX, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; 2,4-DNT, 2,4-dinitrotoluene; 2,5-DNT, 2,5-dinitrotoluene; 1,4-DNB, 1,4-dinitrobenzene.

## 5.2. Introduction

Recent evidence of explosive compounds (explosives including their degradation products) in blue mussels (*Mytilus edulis*) in the Baltic Sea (Strehse et al., 2017) raised the awareness of a possible contamination of marine biota living in the vicinity of dumped munitions. The compounds found in the mussels were the explosive trinitrotoluene (TNT) itself as well as 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT), known to be degradation products of TNT in different aquatic organisms including fish (Lotufo et al., 2010b; Talmage et al., 1999; Yoo et al., 2006).

Large parts of the munitions in the seas, including the Baltic Sea, originate from dumping in the aftermath of World Wars I and II (Beck et al., 2018; Beddington et al., 2005), but also all kinds of military operations during and outside wartime contributed to the current contamination (Böttcher et al., 2011). Huge quantities of munitions in the Baltic Sea are located in designated dumpsites, often in coastal areas (Beck et al., 2018), where they were deliberately dumped as a common practice applied until the 1970s (Carton and Jagusiewicz, 2009).

While being exposed to seawater over the years, the munition shells are corroding and the probability of leakage is increasing (Voie and Mariussen, 2017). However, corrosion rates of the shells are depending on various factors such as temperature, salinity, oxygen level and currents (Jurczak and Fabisiak, 2017), leading to huge differences in the corrosion status of munition shells at various locations in the Baltic Sea. Where shells are broken, the explosive compounds leak into the marine environment and may be taken up by marine organisms including fish. Predominant conventional explosives are nitroaromatics, including TNT and 1,3-dinitrobenzene (DNB), as well as nitramines like hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Gledhill et al., 2019).

The toxicity of TNT to fish has been demonstrated in several studies and also the degradation products 2-ADNT and 4-ADNT are toxic, although slightly less compared to TNT itself (Koske et al., 2019; Lotufo et al., 2010b; Talmage et al., 1999). Experiments on the bioaccumulation of TNT in fish showed a rapid uptake from the water column, but low bioconcentration factors, probably caused by the low octanol/water partition coefficient of 1.6 (Monteil-Rivera et al., 2009) in combination with the fast biotransformation of TNT in fish. However, the bioaccumulation of TNT including all its degradation products is much greater than the bioaccumulation of TNT itself (Belden et al., 2005; Ownby et al., 2005).

Despite the fact that explosive compounds have already been found in different marine biota (Gledhill et al., 2019; Strehse et al., 2017), these substances have not yet been identified in fish sampled from or near dumpsites. In order to provide information on the contamination status of the entire marine food chain with explosive compounds, fish are essential organisms and of particular interest for the human consumer. First approaches with experimentally exposed rainbow trout (*Oncorhynchus mykiss*) revealed that bile and blood plasma are convenient matrices to detect explosive compounds (Ek et al., 2003, 2005). Due to their benthic lifestyle (Hylland et al., 2017), flatfish such as dab (*Limanda limanda*) are particularly exposed to dumped munitions and, thus, represent a promising organism for analysing explosive compounds in fish from the Baltic Sea. This flatfish is a geographically widespread species and considered to be a relatively stationary species sensitive towards environmental stressors. It has been used as a bioindicator species in many studies on the prevalence of diseases, heavy metals (Lang et al., 2017) as well as PAH metabolites in bile fluids (Kammann, 2007) in the Baltic Sea and North Sea. Furthermore, it has been recommended and utilized as monitoring species by different working-groups of the International Council for Exploration of the Seas (ICES, 2012; Lang, 2002; Vethaak and Ap Rheinallt, 1992).

For the first time, we here investigated the contamination status of Baltic flatfish from the close vicinity of a dumpsite for conventional munition. For this purpose, dab from four different study sites in the western Baltic Sea, including a contaminated site, were caught and their bile was analysed for the presence of explosive compounds in order to improve our knowledge about the degree and spatial distribution of fish contamination originating from such dumpsites.



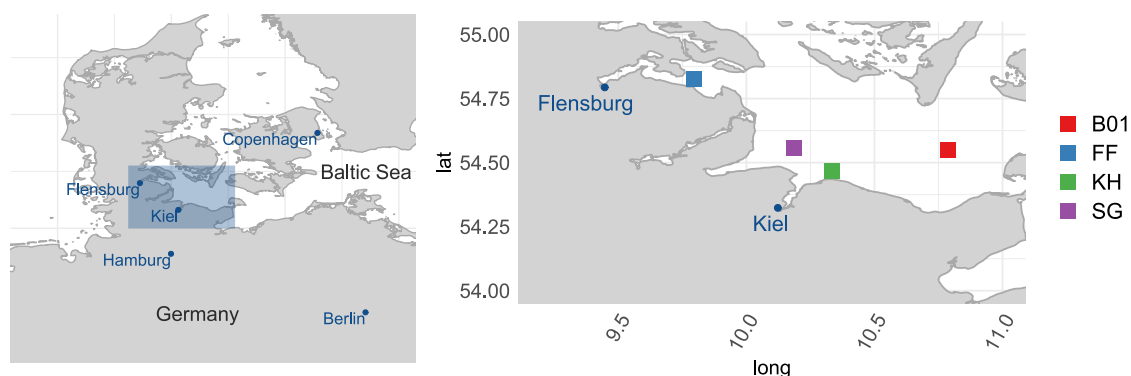
## 5.3. Material and methods

### 5.3.1. Chemicals

Dimethyl sulfoxide (DMSO, 99.9%), acetonitrile (99.9%), water (15 M $\Omega$  cm, HPLC grade) and methanol (MeOH, 99.9%) were obtained from Th. Geyer (Germany). Acetic acid (99%) was purchased from Merck (Germany) and ammonium acetate was obtained from Sigma Aldrich (Germany). Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 2,4,6-trinitrotoluene (TNT), 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) were obtained as reference standards from AccuStandard (USA). 2,4-dinitrotoluene (2,4-DNT) and 2,5-dinitrotoluene (2,5-DNT) were purchased from Neochema (Germany). 1,4-dinitrobenzene (1,4-DNB) was obtained from Sigma-Aldrich (Germany).

### 5.3.2. Study sites

All study sites were located in the western Baltic Sea. Kolberger Heide (KH) is a 1260 ha restricted munition dumpsite, approximately 30 000 t of conventional munitions were dumped in this area (Böttcher et al., 2016; Gledhill et al., 2019). Three reference sites were used for comparison: Stoller Ground (SG) located 10 km west of KH, B01 about 25 km northeast of KH and Flensburg Firth (FF). According to the AMUCAD database (EGEOS GmbH, 2019), no actual munition contamination is documented at SG. B01 is located close to the Fehmarn Belt which was contaminated by munition as hundreds of ground mines were dropped there during the war, but attempts were made to clear this important shipping route. FF is declared as a suspicious area for munitions (Böttcher et al., 2011). The locations of the sampling sites are shown in Figure 5.1 geographical coordinates are given in Table 5.2.



**Figure 5.1.:** Sampling sites of dab in the western Baltic Sea. Samples were taken at the Kolberger Heide dumpsite (KH) and at three different reference sites, B01, Flensburg Firth (FF) and Stoller Ground (SG) close to the German coastline.

### 5.3.3. Sampling

Dab were collected during cruises No. 301 (February 2016, KH, FF), No. 311 (February 2017, KH), No. 314 (August 2017, KH, SG) and No. 326 (August 2018, KH, SG) onboard RV Clupea by gillnet fishery at the edges of KH and in FF (fishing for 5 - 14 h) and by bottom trawling in SG (TV-300 bottom trawl, 15 - 20 min towing time at 3 - 4 knots towing speed). Additionally, dab were collected during cruise No. 408 (September 2018, B01) onboard RV Walther Herwig III by bottom trawling (140 feet bottom trawl, 60 min towing time at 3 - 4 knots towing speed) in B01.

Only alive dab were sorted from the catches and transferred to tanks containing running seawater of ambient temperature to keep them alive before examination. Fish were weighed, the total length measured and anesthetized by a blow on the head, followed by decapitation prior to the subsequent dissection. The bile was collected by puncture of the gall bladder with disposable needles (0.15 mm × 35 mm) into disposable syringes (1 mL) and transferring it into a glass vial (Agilent, Germany). The biometric data were used to determine the condition factor ( $CF = \text{weight [g]} \times 100 / \text{length [cm]}^3$ ) as an indicator of the general fish health status. Otoliths were removed for subsequent age determination according to Maier (1906) and Bohl (1957).

#### 5.3.4. Bile preparation

For the extraction of explosive compounds from bile samples, 25  $\mu\text{L}$  of each bile was transferred into reaction tubes filled with 1 mL of ice-cold acetonitrile. Subsequently, 5  $\mu\text{L}$  of the internal standard 1,4-dinitrobenzene (1,4-DNB, 10 ng/ $\mu\text{L}$ ) was added to the dilution, and the samples were thoroughly mixed. The reaction tubes were centrifuged at 6000 rpm (4  $^{\circ}\text{C}$ , 10 min) and the supernatant was in each case transferred into a 1.5 mL amber vial. The supernatant was reduced under a stream of nitrogen to a volume of 150  $\mu\text{L}$ . The remaining volume was transferred into a new insert and amber vial and further reduced to a final volume of 50  $\mu\text{L}$ , which was used for HPLC-MS analysis. The samples were stored at -20  $^{\circ}\text{C}$  until analysis. The entire extraction procedure was carried out under minimized light to reduce the influence of photodegradation.

#### 5.3.5. HPLC-MS

5  $\mu\text{L}$  of the final bile extract were injected on a column in an Agilent 1290 Infinity High-Performance-Liquid-Chromatograph coupled to an AB Sciex QTrap 5500 Triple Quadrupole/Ion-Trap Mass Spectrometer (HPLC-MS). Gradient separation was achieved using an Acclaim Explosives E2 Column (Thermo Fisher Scientific) kept at 22  $^{\circ}\text{C}$ .

Ionization was conducted in negative atmospheric pressure chemical ionization (APCI) mode. Explosives and selected metabolites were detected via a multiple reaction monitoring (MRM) mode based on characteristic MS/MS transitions, previously optimized using commercially available standard substances. Characterisation of unknown compounds was achieved in scan-mode of the linear ion trap by selecting nitroaromatic compounds using a neutral loss scan targeting the loss of nitro groups ( $m/z$  46), followed by an enhanced product ion scan, producing a sensitive mass spectrum of the nitro-containing molecule.

Abundances of explosive compounds were quantified via the internal standard 1,4-DNB and corrected for response factors. Response factors were determined using an external calibration covering the expected range of concentrations. The method used is described in detail by Koske et al. (2019). Limits of detection (LOD) and limits of quantitation (LOQ) for explosive compounds were calculated according to DIN 32645 (2008) and are listed in Table 5.1.

**Table 5.1.:** Detection limits (LOD) and quantification limits (LOQ) obtained for different explosive compounds, calculated according to DIN 32645 (2008). Abbreviations: HMX, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; TNT, 2,4,6-trinitrotoluene; 2,4-DNT, 2,4-dinitrotoluene; 2,5-DNT, 2,5-dinitrotoluene; 2-ADNT, 2-amino-4,6-dinitroloeuene; 4-ADNT, 4-amino-2,6-dinitroloeuene.

Substance	LOD [ng/mL bile fluid]	LOQ [ng/mL bile fluid]
HMX	0.4	2.4
RDX	1.2	4.4
TNT	2	7.6
2,4-DNT	13.2	46.4
2,5-DNT	9.2	33.2
2-ADNT	3.2	12.4
4-ADNT	2.8	10.8

### 5.3.6. Statistical analysis

Statistical analyses were carried out using R Version 3.6.2 (R Core Team, 2019). The correlation between concentration of 4-ADNT in bile and the age of fish from Kolberger Heide was tested using Pearson's product moment correlation coefficient.

## 5.4. Results

A total of 236 dab were examined in this study (biometric data in Table 5.2) and individual bile samples were taken for chemical analysis. On average, the dab from the munition dumpsite KH were the largest and oldest fish (mean values 30.2 cm and 5.4 years, resp.), while fish from the reference site SG were the smallest and youngest (24.8 cm; 3.2 years). At all study sites, more female than male dab were caught. Dab from FF showed comparable biometric characteristics (29.1 cm; 5.1 years) as dab from KH. Dab from B01 (25.7 cm; 3.6 years) were comparable with those of SG. The mean values of the condition factor (CF) at all four study sites were in the range of 1.0 to 1.2.

**Table 5.2.:** Biometric data of dab from study sites B01, Stoller Ground (SG), Kolberger Heide (KH) and Flensburg Firth (FF). Geographical coordinates of the sampling stations are given as latitude and longitude for a point or rectangle. Total length, condition factor (CF) and age are expressed as mean values  $\pm$  standard deviation.

Sampling site	Coordinates	Sex m/f	Total length [cm]	CF [g/cm <sup>3</sup> ]	Age [years]
B01 (n = 20)	54°33,04 N 10°47,31 E	5/15	25.7 $\pm$ 3.1	1.01 $\pm$ 0.09	3.60 $\pm$ 1.20
SG (n = 94)	54°32,87 N - 54°33,52 N 10°09,94 E - 10°11,23 E	26/68	24.8 $\pm$ 2.9	1.03 $\pm$ 0.14	3.20 $\pm$ 1.09
KH (n = 115)	54°27,28 N - 54°27,97 N 10°19,28 E - 10°20,56 E	12/103	30.2 $\pm$ 3.2	1.10 $\pm$ 0.15	5.37 $\pm$ 1.36
FF (n = 7)	54°49,47 N 09°47,81 E	0/7	29.1 $\pm$ 2.3	1.16 $\pm$ 0.11	5.14 $\pm$ 0.83

Bile samples from three different reference sites in the vicinity of Kiel Bight (B01, FF, SG) and samples taken from the edges of the Kolberger Heide munition dumpsite (KH) were analysed for explosive compounds. No explosive compounds were detected in 94 samples from SG (Table 5.3). Seven samples from FF were analysed and one sample contained 1.24 ng/mL HMX. Of B01, 20 samples were analysed, three of them

containing either 4-ADNT or HMX. 9.35 ng/mL HMX were measured in one sample, resulting in an extrapolated mean of 0.47 ng/mL per sample with a correspondingly great range, as shown in Table 5.3. In total, 115 bile samples were taken at KH, and HMX, RDX, TNT, 2-ADNT as well as 4-ADNT were detected (Table 5.3). The mean concentration of 4-ADNT (17.06 ng/mL) was considerably higher than that of 2-ADNT (1.6 ng/mL). The maximum concentration overall measured in all samples from KH was 141 ng/mL 4-ADNT. The highest single 2-ADNT concentration measured was 32.5 ng/mL bile.

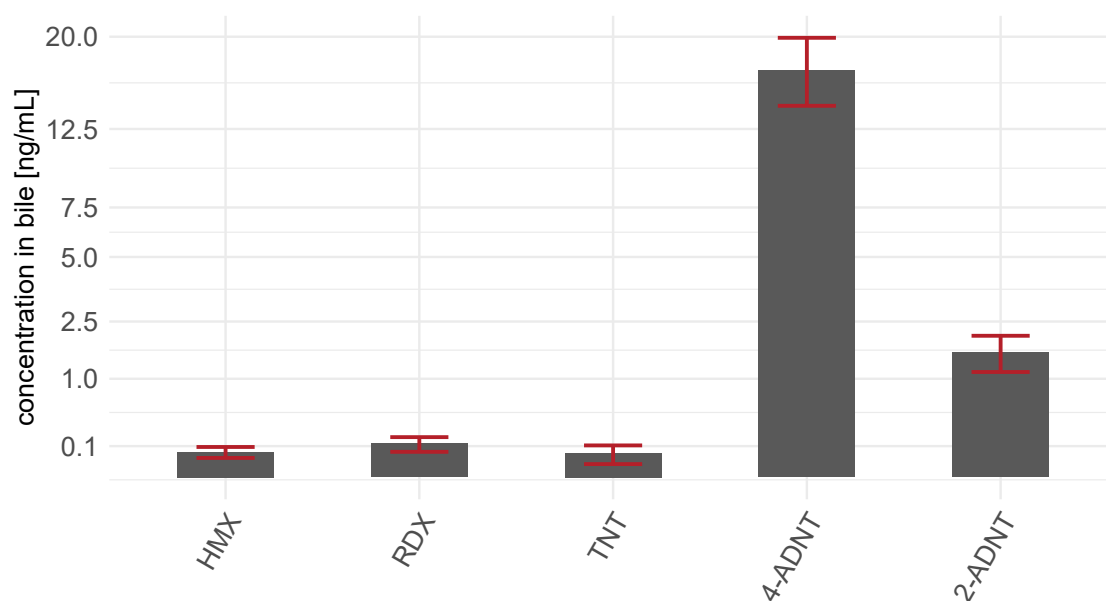
When comparing the proportions of bile contamination in the two study sites with the biggest sample sizes, a clear difference between the reference site SG and the munition dumpsite KH becomes obvious, as shown in Table 5.3. No explosive compounds were found in bile samples from SG, or rather their concentrations were below the detection limit. In contrast, 2-ADNT was detected in 12% and 4-ADNT in 45% of the dab bile samples from KH. 2% of the samples from KH contained TNT. In summary, 55 individual samples of a total of 115 samples from KH contained at least one explosive compound, which corresponds to a contamination rate of 48%.

To test for a possible correlation between the concentration of explosive compounds in bile and the age of fish from Kolberger Heide, 4-ADNT was used as example since this compound was the most predominant found. The correlation between 4-ADNT concentration and fish age ( $r = 0.06$ ) was poor and not significant.

A detailed look at the concentrations of the explosive compounds measured in bile from KH, shown in Figure 5.2, reveals a much more pronounced contamination with 4-ADNT in contrast to 2-ADNT. Not only more samples contained 4-ADNT, but also the concentrations were considerably higher compared to 2-ADNT. In 11 samples from KH, 2-ADNT as well as 4-ADNT was detected. The TNT concentrations measured in two samples were lower than all measured concentrations of 2-ADNT and 4-ADNT.

**Table 5.3.:** Mean concentrations (ng/mL) of different explosive compounds measured in dab bile from four study sites in the vicinity of Kiel Bight: Reference sites (B01, FF, SG) and munition dumpsite (KH). The range (minimum and maximum value) is given in brackets. For LOD (limit of detection) see Table 5.1. Percentage of bile contaminated with explosive compounds is calculated based on the sample size per area.

Area	Sample size	HMX	HMX	RDX	RDX	TNT	TNT	4-ADNT	4-ADNT	2-ADNT	2-ADNT
		[ng/mL]	contami- nation per area	[ng/mL]	contami- nation per area	[ng/mL]	contami- nation per area	[ng/mL]	contami- nation per area	[ng/mL]	contami- nation per area
B01	20	0.47 (0 - 9.35)	5%	< LOD	0%	< LOD	0%	0.59 (0 - 6.92)	10%	< LOD	0%
FF	7	0.18 (0 - 1.24)	14%	< LOD	0%	< LOD	0%	< LOD	0%	< LOD	0%
SG	94	< LOD	0%	< LOD	0%	< LOD	0%	< LOD	0%	< LOD	0%
KH	115	0.07 (0 - 1.99)	5%	0.12 (0 - 3.73)	5%	0.06 (0 - 3.87)	2%	17.06 (0 - 141)	45%	1.60 (0 - 32.5)	12%



**Figure 5.2.:** Concentrations of explosive compounds (ng/mL) found in dab bile from the Kolberger Heide dumpsite (KH). Bars represent the mean concentration for each compound. Error bars (red) represent the standard error of the mean (SEM), sample size  $n = 115$ .



## 5.5. Discussion

The aim of the present study was to investigate the degree of contamination with explosive compounds in fish caught near a dumpsite for conventional munition and to improve the knowledge about the spatial distribution of this contamination. Different reference sites in the surrounding area of the dumpsite should be investigated to identify a possible spread of the contamination in fish, caused, for example, by the distribution of the compounds via the water phase or by migration of the fish themselves.

In total, 55 out of 115 bile samples (48%) from dab caught at the Kolberger Heide dumpsite contained one or more explosive compounds. This high degree of contamination is in striking contrast to the low contamination level of bile samples from the three reference sites. The uptake of explosive compounds, like TNT, 2-ADNT and 4-ADNT, by marine biota at this dumpsite has already been shown in an earlier study that used blue mussels as biomonitoring system (Strehse et al., 2017). Since mussels are usually attached to solid surfaces and are filter-feeding organisms, their body burden strongly depends on the water concentrations of explosive compounds in the surrounding area. In comparison, fish caught at the edges of the dumpsite probably passed through a larger area and, therefore, integrate the munition contamination present at various points in the vicinity — this could be both inside and outside the dumpsite.

Degradation products of TNT accounted for most of the contamination in bile samples from the Kolberger Heide, as 12% 2-ADNT and 45% 4-ADNT but only 2% TNT were found in the samples. This finding raises the question of whether the degradation products were only formed in the fish or already in the water, in the sediment or in the prey before uptake. TNT degradation usually implies the reduction of the nitro groups and can take place via abiotic or biotic processes (Beck et al., 2018). Therefore, it is likely that both TNT and TNT degradation products such as 2-ADNT or 4-ADNT are present in the water column and can be taken up by fish. This was confirmed by the analysis of water samples taken from Kolberger Heide near a pile of sea mines containing several TNT degradation products, including 2-ADNT and 4-ADNT (Gledhill et al., 2019). Recently, an in situ study determined the dissolution flux of TNT at Kolberger Heide with 0.0047 - 0.277 mg/cm<sup>2</sup>/day (Beck et al., 2019), proving that explosive compounds are released into the water column and are, thus, also available for fish. These findings suggest that the dab from Kolberger Heide caught in this study were temporarily exposed directly to TNT, 2-ADNT and 4-ADNT via the surrounding water. Furthermore, the possibility of ingestion of contaminated sediment by dab should not be excluded as an additional source of exposure.

In addition, Gledhill et al. (2019) have demonstrated that macroalgae, sea stars and ascidians take up TNT and its metabolites in the KH dumpsite. The concentrations measured in these benthic organisms ranged up to 24 µg/g dry weight. Since sea stars, molluscs and crustaceans are among the most important food resources of dab (De Clerck and Torreale, 1988), it is very likely that fish will also ingest these substances via the food web. However, due to the low bioconcentration potential of TNT in fish (Ownby et al., 2005), the accumulation of explosive compounds via the food chain appears to be less decisive than direct uptake through water or sediment (Lotufo et al., 2017).

Obviously, at the reference site SG no fish was found to contain explosive compounds despite the fact that, for instance, the distance between SG and KH is only about 10 km. Usually dab tend to stay in their territory which can be demonstrated by specific fish disease prevalence patterns (Diamant and McVicar, 1990; McVicar et al., 1988). However, it is also possible that dab swim further distances (Damm et al., 1991; Temming, 1989). Therefore, it cannot be ruled out that dab leave the Kolberger Heide and this could also explain why two dab caught in the reference area B01 contained 4-ADNT. In addition, the presence of munitions from military operations in the areas B01 and FF (see section study sites) may also explain the explosive compounds detected in the respective samples. In summary, it is likely that dab contaminated with explosive compounds are spread in the larger vicinity of Kolberger Heide, however, the compounds can no longer be detected in the bile after a short time due to the rapid biotransformation (Belden et al., 2005).

The evidence that a prolonged exposure of fish in the direct proximity of munitions leads to a higher concentration of explosive compounds in bile has also been demonstrated in a cage experiment with dab in the Kolberger Heide (Straumer et al., unpublished data). Analysis of bile samples after three weeks of exposure of the dab in the cages in direct vicinity of dumped munitions revealed that concentrations of 2-ADNT (range 20.9 ng/mL - 358.8 ng/mL), 4-ADNT (range 148.9 ng/mL - 1291.6 ng/mL) and TNT (range 0 ng/mL - 6.8 ng/mL) in the bile were considerably increased compared to concentrations detected in the wild dab from KH addressed in the present study (Straumer et al., unpublished data).

Since biotransformation reactions of xenobiotics in fish are mainly catalysed by enzymes of the cytochrome P450 (CYPs) superfamily (Schlenk et al., 2008), important transformation reactions of explosives are also taking place in the liver. In this respect, the degradation of TNT in fish has been demonstrated in *in vivo* exposure studies (Lotufo et al., 2010b; Mariussen et al., 2018), and *in vitro* experiments with liver samples also revealed the metabolization of TNT specifically in dab (Koske et al., 2020a).

In addition to biotransformation in fish themselves, photolysis also plays a decisive role for the degradation of TNT in seawater (Luning Prak et al., 2017). This means that the presence of TNT degradation products cannot be narrowed down to just one source.

The accumulation of explosive compounds in fish was also investigated by Ek et al. (2006) using European flounder (*Platichthys flesus*) exposed to cleaved artillery shells for 8 weeks. Neither TNT nor its degradation products were detected in bile and blood plasma of these fish. Extensive surveys for detecting explosive compounds in biota were conducted at Vieques Island in Puerto Rico, a former naval training area, and high concentrations of TNT, 1,3-DNB, TNB and RDX in the mg/kg range were detected in tube worms, corals and sea urchins (Porter et al., 2011). Not every explosive compound was detected in each sample, but in addition TNB was detected in dusky damselfish (*Stegastes adustus*), sampled at the same site. Other fish sampled in the same area, including groupers, snappers and parrotfish, did not show detectable concentrations of explosive compounds (Porter et al., 2011). Other studies investigated contamination in various biota samples with explosive compounds in areas affected by munitions in the USA, Hawaii and Norway. Up to 10 µg/kg HMX were detected in mussels and snails (Rosslund et al., 2010), high concentrations of TNB (7800 µg/kg) were detected in bent-nosed clam (*Macoma nasuta*), and 3-nitrotoluene (up to 460 µg/kg) and nitrobenzene (up to 320 µg/kg) were measured in starry flounder (*Platichthys stellatus*) at Jackson Park Housing Complex, USA (Lotufo et al., 2017). Few deep-sea shrimp (*Heterocarpus ensifer*) sampled at a sea-disposal site on Hawaii (HI-05) contained 4-ADNT (up to 45 µg/kg) (Koide et al., 2016). Goatfish samples taken from “Ordnance Reef” (HI-06), Hawaii, contained explosive compounds including TNB, 2,4-DNT, HMX and high concentrations of RDX (1600 µg/kg) (Lotufo et al., 2017; University of Hawaii, 2014). These results show that marine organisms living in the vicinity of munition dumpsites accumulate explosive compounds, making the degree of contamination shown here appear realistic. However, the variations in the identified explosive compounds in biota samples between the different studies result not least from the different analytical methods (Beck et al., 2018) and are therefore difficult to compare.

An explanation for the difference in biometric data between sampling sites are the different fishing methods. Due to the selectivity of the gillnet for larger fish, which was used in KH and FF, larger and, thus, mainly older fish were caught there. As female become larger than male dab (Rijnsdorp et al., 1992), proportionally more female fish were caught in KH and FF compared to SG and B01. According to the CF, dab from KH were fitter than fish from SG and B01. However, the mean CF values from all four sampling sites were similar to the CF of dab from the North and Baltic Seas described

in other studies (Htun-Han, 1978; Lang et al., 2017) and are subject to seasonal changes (Ortega-Salas, 1980; Saborowski and Buchholz, 1996). We, therefore, assume that the dab used in this study had a common CF at each study site. A correlation between the amount of explosive compounds in the bile and the age of the fish from Kolberger Heide could not be determined in the present study using the example of 4-ADNT.

The detection of explosive compounds in fish from the Baltic Sea shown here is in line with the findings of the same chemicals in mussels and other marine biota (Gledhill et al., 2019; Strehse et al., 2017) at the same site. This raises concerns about contamination of the marine food chain up to larger organisms such as fish. Furthermore, the genotoxicity of TNT and its degradation products in fish (Koske et al., 2019) poses a potential risk for long-term effects in fish living near munition dumpsites. In order to determine the amount of explosive compounds that fish may accumulate over time, measurements of liver or filet samples are recommended, as bile is excreted periodically.

## 5.6. Conclusion

We have shown the degree of contamination with explosive compounds in bile samples from wild dab caught in the close vicinity of the Kolberger Heide munition dumpsite. In comparison, bile samples from a nearby reference site, where no munitions were directly disposed, did not show any contamination associated with munitions. Therefore, we have proven that chemicals released from dumped munition are taken up by fish in the Baltic Sea. To our knowledge, this is the first study showing a broad contamination of fish caused by dumped conventional munition in the Baltic Sea. The rapid biotransformation of explosive compounds in fish leads to a close local correlation of the results. The determination of explosive compounds in the bile of dab opens the possibility to detect the same compounds in potentially contaminated other fish species important to the human consumer. The same analytical method may also be applied to fish muscle samples after the extraction method has been adapted. Further studies on the detection of explosive compounds in fish will help to obtain an overall picture of the contamination status of fish exposed to dumped munitions and to achieve a targeted management of munition dumpsites.

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## 6. General Discussion

The intention of the work was to gain new insights into the effect and fate of munition compounds in fish in the context of unsolved future questions concerning the management of dumped munitions. The results are important to enable an appropriate risk assessment of explosives in the marine environment and to develop a possible monitoring concept for dumped munitions. In accordance with the three topics of this thesis, the toxicity (1), the metabolism (2) and the detection (3) of compounds originating from dumped munitions, the objectives of this work are briefly summarized here.

*Objective 1: Do 2,4,6-trinitrotoluene and its primary degradation products cause genotoxic effects in fish and are there differences in the level of effect?*

TNT as well as 2-ANT and 4-ADNT caused significant genotoxic effects in zebrafish embryos. The maximum genotoxic induction caused by the concentrations tested in this thesis was about three to four times higher for TNT than for 2-ADNT and 4-ADNT, showing a weaker genotoxic effect induced by the degradation products. These results, derived from an *in vivo* exposure scenario, increase the biological relevance of genotoxic effects in exposed fish caused by nitroaromatic compounds from dumped munitions. Together with existing studies demonstrating the mutagenicity and carcinogenicity of nitroaromatic compounds, there is considerable evidence that long-term health effects must be expected in fish exposed to dumped conventional munitions.

*Objective 2: Which biotransformation products of 2,4,6-trinitrotoluene and chemical warfare agent-related phenylarsenic chemicals can be found in fish from the Baltic Sea, using *Limanda limanda* and *Gadus morhua* as common species, and could be used for monitoring purposes?*

A rapid time-dependent enzymatic degradation of TNT took place in dab liver and the concentrations of 2-ADNT and 4-ADNT increased over time. A preferential formation of 4-ADNT was observed, which is also known from laboratory exposure studies

with other fish species. Based on these results, it is highly promising to use aminodinitrotoluenes as target compounds for monitoring contamination in flatfish exposed to conventional munitions.

For three phenylarsenic chemicals, Clark I, Adamsite and phenylarsonic acid, and their oxidation products, glutathione conjugates and methylated metabolites were identified in cod liver. Hydroxylated metabolites were also identified for these three chemicals and additionally for triphenylarsine oxide. Due to the reactivity of glutathione conjugates, some of which have already been shown to be clearly toxic in toxicity tests, it is recommended that the hydroxylated and methylated metabolites of CWA-related phenylarsenic chemicals should be used as target compounds for a monitoring in fish.

*Objective 3: What is the actual level of contamination with explosive compounds in fish from a munition dumpsite in the Baltic Sea?*

48% of 115 dab specimen analyzed from Kolberger Heide dumpsite contained at least one explosive compound. In contrast, 94 samples analyzed from the reference site did not contain any explosive compound. HMX, RDX, TNT, 2-ADNT and 4-ADNT were identified in bile samples from Kolberger Heide. In accordance with the *in vitro* metabolism experiments carried out in this thesis, 2-ADNT and 4-ADNT were the most frequently detected compounds.

The results demonstrate that a considerable proportion of flatfish caught in the vicinity of a munition dumpsite in the Baltic Sea are contaminated with explosive compounds, some of which are known to be genotoxic, mutagenic and carcinogenic. In this context, adverse health effects for exposed fish have to be expected and the same compounds could be found in other fish species important for human consumers. As some dab sampled at other sites in the western Baltic Sea also contained small amounts of explosive compounds, it is likely that several areas in this part of the Baltic Sea contain dumped munitions. This is also evident in countless historical documents, and therefore fish may be contaminated even further away from dedicated munition dumpsites.



In the following sections, the results of the present thesis will be comprehensively and critically discussed in the context of ecotoxicological relevance, the application in future monitoring programmes and in the light of climate change.

## 6.1. Genotoxicity of nitroaromatics and its implications

Previous research on genotoxic effects of TNT and its degradation products using vertebrate systems provided partly ambiguous results and did not cover effects in fish (Ashby et al., 1985; Furedi et al., 1984a; Homma-Takeda et al., 2002; Lachance et al., 1999). This makes it difficult to predict the genotoxic effects of dumped conventional munitions on fish. However, various ecotoxicological *in vitro* assays demonstrated the mutagenic and genotoxic potential of nitroaromatic compounds, including TNT, (George et al., 2001; Kennel et al., 2000; Liao et al., 2017; Neuwoehner et al., 2007; Spangford et al., 1982) suggesting that the adverse effects of TNT are depending on the organism, the cell type and particularly the metabolism. In this context, genotoxins are chemicals that damage the DNA, which can lead to lesions or mutations, while mutagens are chemicals that cause changes in the genetic material, resulting in mutations and even cancer. Thus, the DNA damage caused by nitroaromatic compounds in zebrafish embryos shown in chapter 2 demonstrates that the genotoxic effects can be caused in living fish by aqueous exposure. In order to evaluate the implications of these results, a closer look at the role of the *in vivo* comet assay in toxicity testing within the regulatory risk assessment of chemicals is useful.

The *in vivo* comet assay for regulatory purposes is used as an indicator test to detect DNA damage in organisms that may lead to mutations. It aims at identifying the risk for mutations and cancer development within a dose-response relationship. In this way, the results of an *in vivo* comet assay contribute to the risk assessment for a certain substance, ultimately for humans (Brendler-Schwaab et al., 2005). The DNA damage detected by increased DNA migration in the *in vivo* comet assay includes DNA strand breaks and alkali-labile sites. However, the degree of DNA damage can be influenced by DNA excision repair which generally reduces DNA fragmentation. During repair, DNA fragmentation may also increase due to incision-induced DNA strand breaks, so the timing of the analysis may influence the result. Finally, the DNA damage caused by a genotoxin may be correctly repaired without introducing any genetic alterations,

which has to be taken into account when discussing the results (Brendler-Schwaab et al., 2005).

The positive genotoxicity of TNT and its primary degradation products 2-ADNT and 4-ADNT in zebrafish embryos indicated by the comet assay demonstrates the genotoxic potential of these explosive compounds. It was also shown that the maximum genotoxic induction of TNT is three to four times higher than that of 2-ADNT and 4-ADNT. This result is of particular biological relevance when considering the mutagenicity of TNT, which has already been demonstrated *in vitro* (Ahlborg et al., 1988; Lachance et al., 1999; Neuwoehner et al., 2007; Spanggard et al., 1982). Scientific studies on the carcinogenicity of TNT are limited and are based on two rodent experiments using rats and mice with an exposure period of 2 years (Furedi et al., 1984a,b). Carcinoma of the urinary bladder were found in rats fed 50 mg TNT per kg/d (Furedi et al., 1984a), and malignant lymphoma in combination with lymphocytic leukemia in the spleen increased significantly in mice administered 1.5 mg TNT per kg/d (Furedi et al., 1984b). Taking these findings into account, TNT must be considered genotoxic, mutagenic and carcinogenic in vertebrate species, including the *in vivo* genotoxicity in fish shown here. It is therefore necessary to recognize that serious health effects in fish, including carcinogenicity, are likely to occur in marine ecosystems that are exposed to explosive compounds originating from dumped munitions.

Generally, toxicological effects in organisms are linked to a certain dose, and the relationship can be described by dose-response models (Borzelleca, 2000). For example, significant genotoxic effects occurred in zebrafish embryos at 0.1 mg/L TNT, meaning that this concentration in the water will cause such effects in fish. However, the effects caused by carcinogenic chemicals do not necessarily follow traditional dose-response models and therefore do not always have a toxicological threshold. Depending on the mechanism of action of a carcinogenic chemical, the carcinogenic effect may occur in an organism as soon as the concentration is greater than zero, a so-called non-threshold carcinogen (Bevan and Harrison, 2017; Bolt et al., 2004).

For cancer risk assessment of non-threshold carcinogens, linear dose-response models are often used to extrapolate from higher doses used in animal experiments to lower doses necessary for the risk assessment. These linear dose-response models as well as non-threshold mechanisms are controversially discussed and probably do not always reflect the mechanism of action of the respective chemicals (Bolt et al., 2004; Calabrese, 2019; Henderson et al., 2000; Kobets and Williams, 2019; Waddell, 2005). It must be explicitly mentioned that the mechanism of action of genotoxic carcinogens is the crucial aspect that needs to be clarified in order to allow proper risk assessment and

discussion on thresholds or non-thresholds of the chemical. This also applies to the carcinogenicity of TNT in fish mediated by genotoxic mechanisms.

As the mechanism of carcinogenicity caused by TNT in organisms is still unknown, the question of whether these effects occur with or without a threshold cannot be answered either. However, since the comet assay has shown the genotoxicity of TNT in fish *in vivo*, direct and indirect mechanisms can be assumed that damage the DNA. On the one hand, direct mechanisms include reactions with the DNA in target cells leading to DNA damage, the misreplication of damaged DNA and mutations in critical genes (Greim and Albertini, 2015), all of which can lead to a positive results in the comet assay. These effects can be expected to be caused by TNT or its biodegradation products, including reactive intermediates (Bolt et al., 2006). On the other hand, indirect mechanisms as the inhibition of excision repair may have contributed to the observed DNA damage as well. In this context, the production of reactive oxygen species during the biodegradation of TNT, which has been demonstrated in *in vitro* experiments (Shinkai et al., 2016), is also likely to contribute to genotoxicity. Both direct and indirect mechanisms can exhibit a non-linear dose response (Bevan and Harrison, 2017; Greim and Albertini, 2015), such as supra-linear or sub-linear (Nohmi and Fukushima, 2016), which would ultimately lead to a threshold genotoxicity of TNT. However, this has yet to be demonstrated. In order to further investigate these possible mechanisms, specially designed experiments that exclude, for example, the inhibition of DNA repair must be carried out.

The results on genotoxicity of TNT and its degradation products in fish in this thesis have strengthened the chain of arguments that carcinogenic effects are likely in marine organisms exposed to dumped conventional munitions. Using the *in vivo* approach, the biological relevance of genotoxic effects was demonstrated. In order to enable a proper risk assessment of TNT, understanding the genotoxic mechanism of action is of crucial importance and should therefore be further targeted. As long as this mechanism of action has not been clearly understood, adverse effects in exposed fish must be assumed, in the spirit of precaution, even at considerably lower concentrations of explosive compounds in the marine environment.

## **6.2. Metabolism of munition compounds in fish – how *in vitro* experiments can be used as pilot study**

Understanding the metabolism of compounds leaking from dumped munitions in fish plays a key role for the identification, risk assessment and management of munition

compounds in the marine environment. As shown in chapter 3, explosive compounds undergo rapid degradation reactions *in vitro* in the presence of fish liver enzymes. This corresponds to the general understanding of the biotransformation of xenobiotics in organisms (Parkinson and Ogilvie, 2008), which are constantly transforming and eliminating foreign chemicals. Furthermore, it is also in accordance with current knowledge that TNT in fish is initially degraded by nitro-reduction (Belden et al., 2005; Lotufo et al., 2010b; Mariussen et al., 2018). This first reduction step occurs rapidly and reduction to 4-ADNT is preferred over 2-ADNT. The increased reduction to 4-ADNT has been described as thermodynamically favourable (McCormick et al., 1976). A higher concentration of 4-ADNT compared to 2-ADNT was also observed in dab caught at the Kolberger Heide munition dumpsite (chapter 5), demonstrating that this preferred reaction occurs equally *in vivo* in exposed wild fish.

Another degradation product of TNT, 1,3,5-trinitrobenze (TNB) occurring by photodegradation (Luning Prak et al., 2017), which is also found in the water phase at munition dumpsites as Kolberger Heide (Gledhill et al., 2019), was completely degraded by dab liver enzymes *in vitro* within 2 hours. The *in vitro* metabolism of TNT studied in chapter 3 shows that even after a short exposure time, degradation products occur more frequently and the concentration of the parent compound decreases considerably. These degradation processes need to be seriously considered when trying to identify target compounds suitable for monitoring explosive compounds in fish that are potentially exposed to dumped munitions.

The insights into the *in vitro* metabolism of CWA-related phenylarsenic chemicals in fish shown in chapter 4 provide entirely new knowledge about hydroxylation, methylation and conjugation reactions of these chemicals in marine organisms. The existing knowledge on the metabolism of these chemicals in organisms is very limited and studies that focus on the metabolism in fish do not exist. Since phenylarsenic chemicals from dumped CW tend to oxidize as soon as they are in contact with water (Haas et al., 1998), mainly the oxidized forms of these chemicals can be expected to be metabolized inside marine organisms.

All four phenylarsenic chemicals investigated, Clark I (DA), Adamsite (DM), phenyl-dichloroarsine (PDCA) and triphenylarsine (TPA), were metabolized in a first step by cod liver enzymes and formed hydroxylated metabolites, possibly catalyzed with the same mechanism by the cytochrome P450 monooxygenase enzyme family. In addition, glutathione conjugates and methylated metabolites of DA, DM and PDCA were identified, which probably allow the fish to eliminate the chemicals, as glutathione conjugates of Clark I oxidation products were detected in the bile of exposed rats (Kobayashi and Hirano, 2013). As these conjugation products were found for DA, DM and PDCA, a

comparable degradation pathway can be assumed in CWA-exposed fish. Due to their reactive nature, glutathione conjugates of phenylarsenic chemicals are rather not recommended for monitoring purposes in fish and detection should focus on hydroxylated and methylated metabolites.

The *in vitro* metabolism experiments performed in this thesis with conventional munition compounds on one side and CWA-related phenylarsenic chemicals on the other side used fish liver homogenates and generally the same bioanalytical methodology. Using sophisticated chemical analytical methods, biodegradation products of TNT and phenylarsenic chemicals have been identified as suitable target compounds to monitor possible exposure of fish to such compounds in the marine environment. Thereby, the *in vitro* methodology proved to be a fast, cost-effective, robust and altogether efficient method to expand the knowledge on the metabolism of compounds related to dumped munitions in fish. The method can be applied using liver material from different fish species. As samples of exposed fish collected on scientific cruises are valuable and expensive, it is recommendable to carry out *in vitro* experiments with existing sample material as pilot study in order to establish the research questions or the analytical method. Also in the context of Russell et al. (1959) “3 R concept” (replacement, reduction and refinement) to reduce the number of animal experiments in scientific studies, *in vitro* methods are preferable to *in vivo* exposure of fish, especially in metabolism experiments, if they answer the same research question.

As shown in chapter 3 and chapter 4 of this thesis, the metabolism in fish is considerably influencing the fate of conventional munition compounds and CWA-related phenylarsenic chemicals. Both for the monitoring of these compounds in the marine environment and for the risk assessment of dumped munitions, the metabolism must be considered.

### **6.3. Towards a future monitoring of dumped munitions in the sea?**

Embedded in the Marine Strategy Framework Directive (MSFD) (European Commission, 2008), the ongoing objective in Europe is to protect and accordingly manage the marine environment. Structured in various descriptors, the good environmental status should be achieved by continuous monitoring and targeted measures, even if this is not the case for all pollutant classes by 2020, as shown by the examples of descriptors D8 and D9 (European Commission, 2018).

Detached from the MSFD, a successful monitoring programme must be able to react flexibly to changing circumstances in order to constantly reflect the current state and threats to the marine environment and to remain competitive for the future. This requires the integration of emerging pollutants or chemicals that have not been considered so far, such as dumped munitions. Since the environmental problem of dumped munitions in Europe has only received specific and growing attention in the last decade, it is plausible that the environmental burden of dumped munitions in the marine environment has not yet been monitored on a legal basis such as the MSFD. In Germany, a detailed report on the pollution of German waters with dumped munitions was published for the first time in 2011 (Böttcher et al., 2011). Since then, various national and international research projects addressed scientific questions in this context, all of which have contributed to the fact that in 2020 much more detailed knowledge exists on analytical methods, the contamination and the effects of dumped munitions.

The dimension of the underwater munition inventory, increasing maritime economic activities, leaking munition shells, the toxicity of the compounds contained, together with the growing knowledge about it, have led to a broader consensus among scientists that action should be taken (Ballentine et al., 2015; Beck et al., 2018; Bełdowski et al., 2017; Carton and Jagusiewicz, 2009; Della Torre et al., 2010; Kampmeier et al., 2020; Maser and Strehse, 2020; Missiaen and Henriët, 2002; Niemikoski et al., 2017; Voie and Mariussen, 2017). This opinion is also reflected in the decision of the Conference of German Environmental Ministers in 2019, which requested a screening for munition compounds in the German waters of the North Sea and Baltic Sea (Behörde für Umwelt und Energie der Freien und Hansestadt Hamburg, 2019). In addition, in 2016, Germany addressed the problem of dumped munitions in the marine environment as part of the national implementation process of the MSFD and provided a factsheet with appropriate measures (BLANO, 2016a,b).

However, since dumped munitions are a cross-border problem, the initiative for monitoring should be international for maximum success, even if the following measures would have to be taken by national authorities. This includes a comprehensive international policy process to agree on a consensus, as the MSFD has done for other pollutants. First steps have already been taken by acknowledging the problem, expanding scientific knowledge and discussing possible solutions, albeit partly at national level. International scientific projects are an important part to achieve this goal, because researchers can share the most recent scientific findings with national authorities and, conversely, the specific need for scientific studies can be communicated. Military institutions should also be involved in this process, as many results are already existing but not freely available.

When thinking about implementing a monitoring of compounds from dumped munitions either in Europe or at national level, several general elements need to be considered. First of all, the exploration of the seafloor in terms of dumped munitions varies considerably from area to area and monitoring sites need to be chosen seriously. In the Baltic Sea, certain munition dumpsites, such as the Kolberger Heide or the Bornholm Basin, have been extensively investigated and detailed information on the number and condition of munition objects is already existing (Bełdowski et al., 2016a; Kampmeier et al., 2020; Missiaen et al., 2010). Such information is not available for many other known or suspected contaminated sites in the Baltic Sea, and site-specific information is lacking, particularly in the North Sea. To explore areas that have not been investigated, hydroacoustic and optical approaches can be used, for example, but archives also provide valuable information. The “Ammunition Cadastre Sea” (AMUCAD) database (EGEOS GmbH, 2019) currently offers probably the most comprehensive dataset on the spatial distribution of dumped munitions worldwide and especially in the North Sea and Baltic Sea.

When defining monitoring sites, reference sites offering comparable site-specific parameters but most likely not contaminated with dumped munitions must be selected. As an example, the study design used in this thesis in chapter 5 for the investigation of dab from the Kolberger Heide dumpsite can be referred here. Approximately 100 bile samples each were taken at the edges of the dumpsite and a nearby reference site (10 km) and analyzed for explosive compounds. Since 48% of the samples from the dumpsite contained at least one explosive compound and no explosive compounds were identified in samples from the reference site, a close spatial correlation between contaminated fish and the munitions can be assumed. However, the exploration of munition objects on the seafloor is incomplete and objects are also located outside the designated dumpsites (Kampmeier et al., 2020). To account for this uncertainty in the monitoring, a sufficient number of samples should be taken at each site to enable the detection of differences in the contamination status.

Different environmental compartments, including water, sediment and biota samples, should be monitored at one site. Due to different physico-chemical properties in the compartments, different exposure conditions and the mobility of the water phase and biota, the concentrations of munition compounds will differ considerably. For this purpose, different analytical methods using various analytical instruments have been published (Appel et al., 2018; Craig et al., 2019; Darrach et al., 1998; Gledhill et al., 2019; Lotufo et al., 2017; Rodacy et al., 2001), including the method used in this thesis for fish bile. It should be mentioned that different analytical methods achieve differ-

ent sensitivities and a multi-compartment monitoring must take this into account by complying with analytical quality criteria, such as the use of ring tests.

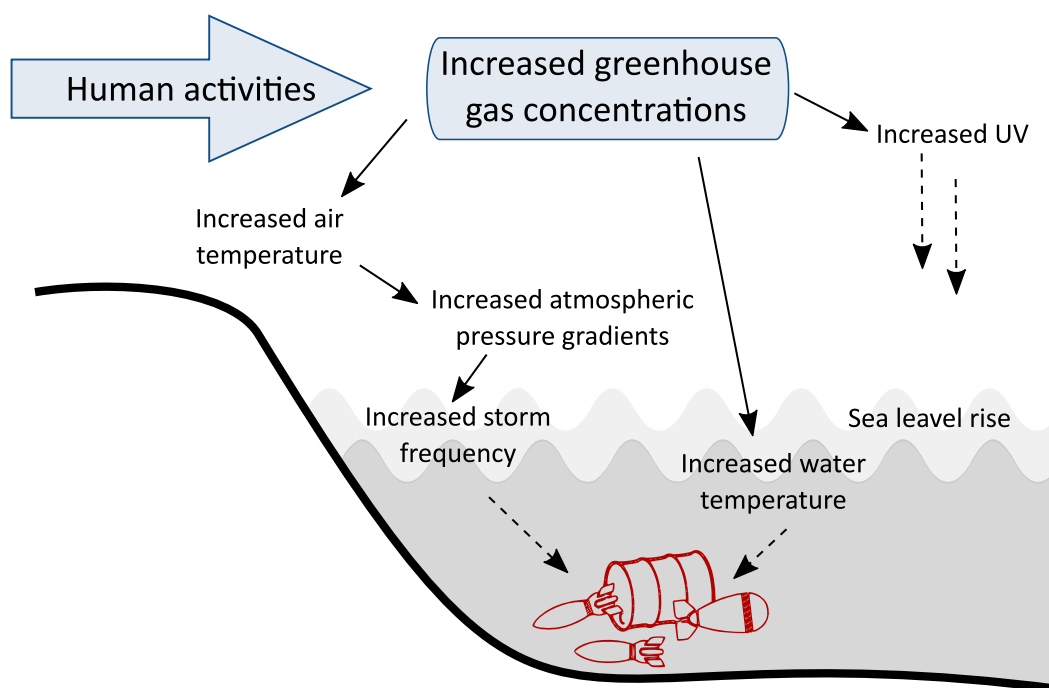
Following the results of this thesis and other studies in this field, monitoring of compounds originating from dumped munitions should focus on degradation products of the parent compounds. For the monitoring of conventional explosives in fish, 2-ADNT and 4-ADNT should be measured; in heavily polluted areas the detection of TNT may also be possible. The monitoring of CWA-related phenylarsenic compounds in fish should focus on the oxidation products of the parent compounds or the hydroxylated metabolites. The scope of monitoring should, on the one hand, allow an adequate assessment of the environmental status and, on the other hand, remain affordable for the responsible authorities. Particularly with regard to a current topic such as dumped munitions, it is useful to regularly review the monitoring concept on the basis of the latest scientific findings.

## **6.4. Munition dumpsites in a changing environment**

The mean atmospheric carbon dioxide (CO<sub>2</sub>) concentration is constantly increasing, from approximately 280 parts per million (ppm) in pre-industrial times (Siegenthaler and Sarmiento, 1993) to a recent concentration of 413 ppm in the beginning of the year 2020 (National Oceanic and Atmospheric Administration, 2020). While a rising atmospheric CO<sub>2</sub> level contributes to climate change more than any other greenhouse gas, its implications are for instance global warming, sea-level rise and ocean acidification (Coelho et al., 2013; Doney et al., 2011; Dore et al., 2009), which affect the entire marine environment and, conversely, humans.

Although the impact on marine pollution is less well understood than the direct consequences of climate change, it is likely that climate change will also affect the pollutants already present in the marine environment and their effects. As climate change alters abiotic conditions (Figure 6.1) such as salinity, pH, temperature, precipitation and circulation of the water column in the marine environment (Harley et al., 2006; Schiedek et al., 2007), the state, environmental fate and potential risk of the pollutants change as well. These changes also affect dumped munitions in marine environments around the world.





**Figure 6.1.:** Abiotic changes in the marine environment due to climate change according to Harley et al. (2006), which will also affect the condition of dumped munitions in the future.

Although the possible consequences of climate change for dumped munitions have not yet been specifically investigated, the general effects on the marine environment in the future in terms of climate change may help to discuss possible implications. The average global sea surface temperature has risen by 0.85 °C over the past 130 years and this trend is expected to accelerate in the future (Harley et al., 2006; IPCC, 2014). A higher water temperature is likely to increase the solubility of compounds from dumped munitions (Lynch et al., 2001). However, an in situ study by Beck et al. (2019), investigating the solubility of exposed conventional munition material in the Baltic Sea, found no substantial differences in the solubility during different seasons with changing water temperatures. The authors also consider the effect of salinity on the solubility to be relatively small, suggesting that changing ocean salinity, for example depending on precipitation in certain regions (IPCC, 2014), will not significantly change the solubility of munition compounds.

Although temperature and salinity changes, which strongly depend on site-specific factors, are probably not significantly affecting the solubility of munition compounds, they do change the exposure conditions for marine organisms. Different processes that alter toxicokinetics, such as partition coefficients, diffusion rates, ventilation and metabolic rates, or feeding activity, result in increased uptake of pollutants as water temper-

ature rises, leading to increased exposure and toxicity for the organism. In return, an increased elimination and detoxification rate of pollutants in the organism can be assumed due to increased metabolic activity, which counteracts the exposure of the organism (Buchwalter et al., 2003; Heugens et al., 2001; Maruya et al., 2005; Schiedek et al., 2007). However, different studies report increased toxicity at higher temperatures to aquatic organisms, with the assumption that the change in toxicity is based on the altered substance-specific bioactivation and detoxification (Capkin et al., 2006; Gaunt and Barker, 2000; Noyes et al., 2009; Ratushnyak et al., 2005). Since the biotransformation of munition compounds is crucial for their toxicity in fish, as highlighted in this thesis, a future rise in water temperature will also have consequences for exposed fish. Taking this into account, as a precautionary principle, higher toxicity to fish species exposed to munition compounds should be expected with rising temperatures.

Besides temperature and salinity, UV radiation is expected to change in the future by halogenated pollutants and rising greenhouse gas concentrations, which alter the spatial distribution of ozone that protects the earth's surface from excessive UV radiation (Coelho et al., 2013; Schiedek et al., 2007). Exposure of aquatic organisms in combination with UV radiation can enhance the toxicity of pollutants (Peachey, 2005), and there is also evidence of increased toxicity of explosive compounds through photoactivation and photolysis to other toxic compounds (Dave et al., 2000; Woodley and Downs, 2014). The photosensitivity of conventional munition compounds is sufficiently known (Luning Prak et al., 2017), so that increased UV radiation will lead to enhanced degradation processes and the formation of degradation products. However, the effect of UV radiation is particularly significant for munition objects lying in shallow water, since the radiation intensity decreases with water depth (Beck et al., 2018).

Another important factor that will affect the condition of dumped munitions is the sea level rise, as well as extreme winds, waves and storms, which are expected to intensify and become more frequent in the future (IPCC, 2014). It is probable that the dissolution of munition compounds in storm events will be enhanced by stronger water movements, for example in shallow areas of the North Sea and Baltic Sea. The deposition or burial of munition objects and distribution of particulate munition material by currents (Menzel et al., 2018) is also likely to increase during extreme weather events. This implies that the location of munition objects will change over time and encounters of objects along traffic routes or beaches will continue to occur.

As it is quite unrealistic, given the global amount of dumped munitions and the expected costs, to expect extensive clearance and remediation in the near future, this contamination problem needs serious attention and should be monitored regularly.

When clearing individual sites or areas, monitoring of the surrounding marine environment in different compartments and matrices should also be carried out in order to draw conclusions concerning the meaningfulness and risk of a potential large-scale clearance.



## 7. Outlook

Even if dumped munitions usually have a historical origin, the constant discoveries show that this is a current problem and its dimension and understanding still has some uncertainties. This fact teaches us that this way of disposing hazardous substances was perhaps the most obvious step at the time, but not the most sustainable. Scientific progress and increased research on dumped munitions in the marine environment in the past years have led to a better understanding and greater evidence of their effects. In this context, this thesis shows that the combination of laboratory experiments and field work provides a powerful and effective framework for studying the consequences of emerging or poorly investigated pollutants in the marine environment.

Together with the existing toxicological concerns and the known contamination of the marine ecosystem, the future prospects of a changing environment should further manifest the awareness of this problem among the broader public. After all, this is the most effective way to promote the process of developing appropriate management measures at the political level and to enable further research on dumped munitions in the future. Both contribute to ensure the sustainable use of marine regions, which will increase in the future. Future research should focus on areas, species or mechanisms that have not yet been studied and that are of importance for future management.



## References

- G. Ahlborg, P. Einisto, and M. Sorsa. Mutagenic activity and metabolites in the urine of workers exposed to trinitrotoluene (TNT). *British Journal of Industrial Medicine*, 45:353–358, 1988.
- J. Aker, B. Howard, and M. Reid. Risk Management For Unexploded Ordinance (UXO) In The Marine Environment. *Dalhousie Journal of Interdisciplinary Management*, 8 (2), 2012. ISSN 1923-6530.
- E. Amato, L. Alcaro, I. Corsi, C. Della Torre, C. Farchi, S. Focardi, G. Marino, and A. Tursi. An integrated ecotoxicological approach to assess the effects of pollutants released by unexploded chemical ordnance dumped in the southern Adriatic (Mediterranean Sea). *Marine Biology*, 149(1):17–23, 2006. ISSN 0025-3162.
- G. Ampleman, D. Baucher, S. Thiboutot, J. Hawari, and F. Monteil-Rivera. Evaluation of underwater contamination by explosives and metals at Point Amour Labrador and in the Halifax Harbour area. Technical report, 2004.
- D. Appel, J. S. Strehse, H. J. Martin, and E. Maser. Bioaccumulation of 2,4,6-trinitrotoluene (TNT) and its metabolites leaking from corroded munition in transplanted blue mussels (*M. edulis*). *Marine Pollution Bulletin*, 135(April):1072–1078, 2018. ISSN 18793363. doi: 10.1016/j.marpolbul.2018.08.028.
- J. Ashby, B. Burlinson, P. A. Lefevre, and J. Topham. Non-genotoxicity of 2,4,6-Trinitrotoluene (TNT) to the mouse bone marrow and the rat liver: Implications for its carcinogenicity. *Archives of Toxicology*, 58(1):14–19, 1985.
- H. C. Bailey and R. J. Spanggord. The Relationship Between the Toxicity and Structure of Nitroaromatic Chemicals. In *Aquatic Toxicology and Hazard Assessment: Sixth Symposium*. ASTM International, 1983.
- M. Ballentine, C. Tobias, P. Vlahos, R. Smith, and C. Cooper. Bioconcentration of TNT and RDX in Coastal Marine Biota. *Archives of Environmental Contamination and Toxicology*, 68(4):718–728, 2015. ISSN 0090-4341.
- A. Basch and S. Kraus. Analysis and Characterization of Military-Grade Trinitrotoluene by Gas Chromatography. *Journal of Forensic Science*, 24(4):870–874, 1979. ISSN 0022-1198.

- A. J. Beck, M. Gledhill, C. Schlosser, B. Stamer, C. Böttcher, J. Sternheim, J. Greinert, and E. P. Achterberg. Spread, Behavior, and Ecosystem Consequences of Conventional Munitions Compounds in Coastal Marine Waters. *Frontiers in Marine Science*, 5(April):1–26, 2018. ISSN 2296-7745. doi: 10.3389/fmars.2018.00141.
- A. J. Beck, E. M. van der Lee, A. Eggert, B. Stamer, M. Gledhill, C. Schlosser, and E. P. Achterberg. In Situ Measurements of Explosive Compound Dissolution Fluxes from Exposed Munition Material in the Baltic Sea. *Environmental Science & Technology*, 2019. ISSN 0013-936X.
- J. Beddington, A. J. Kinloch, A. J. Kinloch, and F. R. Eng. Munitions Dumped at Sea: A Literature Review. *Imperial College Consultants Ltd. London, UK*, 2005.
- Behörde für Umwelt und Energie der Freien und Hansestadt Hamburg. Endgültiges Ergebnisprotokoll, Umweltministerkonferenz Hamburg 2019. Technical report, Behörde für Umwelt und Energie der Freien und Hansestadt Hamburg, Hamburg, 2019.
- J. B. Belden, D. R. Ownby, G. R. Lotufo, and M. J. Lydy. Accumulation of trinitrotoluene (TNT) in aquatic organisms: Part 2 - Bioconcentration in aquatic invertebrates and potential for trophic transfer to channel catfish (*Ictalurus punctatus*). *Chemosphere*, 58(9):1161–1168, 2005. ISSN 00456535. doi: 10.1016/j.chemosphere.2004.09.058.
- J. Bełdowski, Z. Klusek, M. Szubska, R. Turja, A. I. Bulczak, D. Rak, M. Brenner, T. Lang, L. Kotwicki, K. Grzelak, J. Jakacki, N. Fricke, A. Östin, U. Olsson, J. Fabisiak, G. Garnaga, J. R. Nyholm, P. Majewski, K. Broeg, M. Söderström, P. Vanninen, S. Popiel, J. Nawała, K. Lehtonen, R. Berglind, and B. Schmidt. Chemical Munitions Search & Assessment - An evaluation of the dumped munitions problem in the Baltic Sea. *Deep Sea Research Part II: Topical Studies in Oceanography*, 128:85–95, 2016a. ISSN 0967-0645. doi: 10.1016/j.dsr2.2015.01.017.
- J. Bełdowski, M. Szubska, E. Emelyanov, G. Garnaga, A. Drzewińska, M. Bełdowska, P. Vanninen, A. Östin, and J. Fabisiak. Arsenic concentrations in Baltic Sea sediments close to chemical munitions dumpsites. *Deep Sea Research Part II: Topical Studies in Oceanography*, 128:114–122, 2016b. ISSN 0967-0645.
- J. Bełdowski, R. Been, and E. K. Turmus. *Towards the Monitoring of Dumped Munitions Threat (MODUM): A Study of Chemical Munitions Dumpsites in the Baltic Sea*. Springer, 2017. ISBN 9402411534.



- M. A. Belisario, R. Pecce, R. D. Morte, A. R. Arena, A. Cecinato, P. Ciccioli, and N. Staiano. Characterization of oxidative and reductive metabolism *in vitro* of nitrofluoranthenes by rat liver enzymes. *Carcinogenesis*, 11(2):213–218, 1990. ISSN 1460-2180.
- M. A. Belisario, A. R. Arena, R. Pecce, R. Borgia, N. Staiano, and F. De Lorenzo. Effect of enzyme inducers on metabolism of 1-nitropyrene in human hepatoma cell line HepG2. *Chemico-Biological Interactions*, 78(3):253–268, 1991. ISSN 0009-2797.
- Y. Benjamini and Y. Hochberg. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, 57(1):289–300, 1995.
- D. Bernet, A. Liedtke, D. Bittner, R. I. L. Eggen, S. Kipfer, C. Küng, C. R. Largiader, M. J.-F. Suter, T. Wahli, and H. Segner. Gonadal Malformations in Whitefish from Lake Thun: Defining the Case and Evaluating the Role of EDCs. *CHIMIA International Journal for Chemistry*, 62(5):383–388, 2008. ISSN 0009-4293.
- R. Bernhardt. Cytochromes P450 as versatile biocatalysts. *Journal of Biotechnology*, 124(1):128–145, 2006. ISSN 0168-1656.
- A. Berson, C. Wolf, C. Chachaty, C. Fisch, D. Fau, D. Eugene, J. Loeper, J.-C. Gauthier, P. Beaune, D. Pompon, P. Maurel, and D. Pessayre. Metabolic Activation of the Nitroaromatic Antiandrogen Flutamide by Rat and Human Cytochromes P-450, Including Forms Belonging to the 3A and 1A Subfamilies. *Journal of Pharmacology and Experimental Therapeutics*, 265(1):366–372, 1993. ISSN 0022-3565.
- R. J. Bevan and P. T. C. Harrison. Threshold and non-threshold chemical carcinogens: A survey of the present regulatory landscape. *Regulatory Toxicology and Pharmacology*, 88:291–302, 2017. ISSN 0273-2300.
- B. BLANO. MSRL-Maßnahmenprogramm zum Meeresschutz der deutschen Nord- und Ostsee - Anlage 1 Maßnahmenkennblätter. Technical report, BMUB, Bundesministerium für Umwelt, Naturschutz, Bau und Reaktorsicherheit, Bonn, 2016a.
- B. BLANO. MSRL-Maßnahmenprogramm zum Meeresschutz der deutschen Nord- und Ostsee. Technical report, BMUB, Bundesministerium für Umwelt, Naturschutz, Bau und Reaktorsicherheit, Bonn, 2016b.
- M. Boettcher, T. Kosmehl, and T. Braunbeck. Low-dose effects and biphasic effect profiles: Is trenbolone a genotoxicant? *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 723(2):152–157, 2011. ISSN 1383-5718.

- H. Bohl. *Die Biologie der Kliesche (Limanda Limanda L.) in der Nordsee: Mit 26 Zahlentaf. u. 27 Abb. im Text, auf Taf. 1-3 u. auf 4 Beil.* Schweizerbart, 1957.
- H. M. Bolt, H. Foth, J. G. Hengstler, and G. H. Degen. Carcinogenicity categorization of chemicals — new aspects to be considered in a European perspective. *Toxicology Letters*, 151(1):29–41, 2004. ISSN 0378-4274.
- H. M. Bolt, G. H. Degen, S. B. Dorn, S. Plöttner, and V. Harth. Genotoxicity and Potential Carcinogenicity of 2,4,6-Trinitrotoluene: Structural and Toxicological Considerations. *Reviews on Environmental Health*, 21(4):217–228, 2006. ISSN 2191-0308.
- T. Borchardt, S. Burchert, H. Hablitzel, L. Karbe, and R. Zeitner. Trace metal concentrations in mussels: Comparison between estuarine, coastal and offshore regions in the southeastern North Sea from 1983 to 1986. *Marine Ecology Progress Series*, 42(1):17–31, 1988. ISSN 0171-8630.
- A. Borja, S. B. Bricker, D. M. Dauer, N. T. Demetriades, J. G. Ferreira, A. T. Forbes, P. Hutchings, X. Jia, R. Kenchington, and J. C. Marques. Overview of integrative tools and methods in assessing ecological integrity in estuarine and coastal systems worldwide. *Marine Pollution Bulletin*, 56(9):1519–1537, 2008. ISSN 0025-326X.
- Á. Borja, M. Elliott, J. Carstensen, A.-S. Heiskanen, and W. van de Bund. Marine management - Towards an integrated implementation of the European Marine Strategy Framework and the Water Framework Directives. *Marine Pollution Bulletin*, 60(12):2175–2186, 2010. ISSN 0025-326X.
- J. F. Borzelleca. Paracelsus: Herald of Modern Toxicology. *Toxicological Sciences*, 53(1):2–4, 2000. ISSN 1096-0929.
- C. Böttcher, T. Knobloch, N.-P. Rühl, J. Sternheim, U. Wichert, and J. Wöhler. Munitionsbelastung der deutschen Meeresgewässer - Bestandsaufnahme und Empfehlungen. Technical report, Bundesamt für Seeschifffahrt und Hydrographie (BSH), 2011.
- C. Böttcher, T. Knobloch, J. Sternheim, I. Weinberg, U. Wichert, and J. Wöhler. Munitionsbelastung der deutschen Meeresgewässer - Entwicklungen und Fortschritt (Jahr 2015). *MELUR*, 2016.
- T. Braunbeck and E. Lammer. Fish Embryo Toxicity Assays. *German Federal Environment Agency*, pages 1–298, 2006.
- A. P. Breen and J. A. Murphy. Reactions of oxyl radicals with DNA. *Free Radical Biology & Medicine*, 18(6):1033–1077, 1995. ISSN 0891-5849.

- S. Brendler-Schwaab, A. Hartmann, S. Pfuhler, and G. Speit. The *in vivo* comet assay: use and status in genotoxicity testing. *Mutagenesis*, 20(4):245–254, 2005. ISSN 1464-3804.
- C. Briggs, S. M. Shjegstad, J. A. K. Silva, and M. H. Edwards. Distribution of chemical warfare agent, energetics, and metals in sediments at a deep-water discarded military munitions site. *Deep Sea Research Part II: Topical Studies in Oceanography*, 128: 63–69, 2016. ISSN 0967-0645.
- D. B. Buchwalter, J. J. Jenkins, and L. R. Curtis. Temperature Influences on Water Permeability and Chlorpyrifos Uptake in Aquatic Insects with Differing Respiratory Strategies. *Environmental Toxicology and Chemistry*, 22(11):2806–2812, 2003. ISSN 0730-7268.
- E. Bueding and N. Jolliffe. Metabolism of Trinitrotoluene (TNT) *in vitro*. *Journal of Pharmacology and Experimental Therapeutics*, 88(3):300–312, 1946.
- D. M. Burke and R. T. Mayer. Ethoxyresorufin: Direct Fluorimetric Assay of a Microsomal O-Dealkylation which is Preferentially Inducible by 3-Methylcholanthrene. *Drug Metabolism and Disposition*, 2(6):583–588, 1974. ISSN 0090-9556.
- N. E. Burlinson, M. E. Sitzmann, D. J. Glover, and L. A. Kaplan. Photochemistry of TNT and Related Nitroaromatics, Part III. *NSWC/WOL TR*, pages 78–198, 1979.
- E. J. Calabrese. The linear No-Threshold (LNT) dose response model: A comprehensive assessment of its historical and scientific foundations. *Chemico-Biological Interactions*, 2019. ISSN 0009-2797.
- E. Capkin, I. Altinok, and S. Karahan. Water quality and fish size affect toxicity of endosulfan, an organochlorine pesticide, to rainbow trout. *Chemosphere*, 64(10): 1793–1800, 2006. ISSN 0045-6535.
- G. Carton and A. Jagusiewicz. Historic Disposal of Munitions in U.S. and European Coastal Waters, How Historic Information Can be Used in Characterizing and Managing Risk. *Marine Technology Society Journal*, 43(4):16–32, 2009. ISSN 00253324. doi: 10.4031/MTSJ.43.4.1.
- Z. Ceylan, T. Şişman, Z. Yazıcı, and A. Ö. Altıkat. Embryotoxicity of nitrophenols to the early life stages of zebrafish (*Danio rerio*). *Toxicology and Industrial Health*, 32(8):1414–1422, 2016. ISSN 0748-2337. doi: 10.1177/0748233714562444.
- R. B. Clark. *Marine Pollution*. Clarendon Press, Oxford, 3 edition, 1992. ISBN 0-19-854685-8.

- F. J. R. C. Coelho, A. L. Santos, J. Coimbra, A. Almeida, Â. Cunha, D. F. R. Cleary, R. Calado, and N. C. M. Gomes. Interactive effects of global climate change and pollution on marine microbes: the way ahead. *Ecology and Evolution*, 3(6):1808–1818, 2013. ISSN 2045-7758.
- H. D. Craig and S. Taylor. Framework for Evaluating the Fate, Transport, and Risks From Conventional Munitions Compounds in Underwater Environments. *Marine Technology Society Journal*, 45(6):35–46, 2011.
- H. D. Craig, T. F. Jenkins, M. T. Johnson, D. M. Walker, D. E. Dobb, and B. V. Pepich. Method development and laboratory intercomparison of an RP-HPLC-UV method for energetic chemicals in marine tissues. *Talanta*, 198:284–294, 2019. ISSN 0039-9140.
- P. J. Crutzen. The “Anthropocene”. In *Earth System Science in the Anthropocene*, pages 13–18. Springer, 2006.
- X. Cui, Y. Kobayashi, T. Hayakawa, and S. Hirano. Arsenic Speciation in Bile and Urine Following Oral and Intravenous Exposure to Inorganic and Organic Arsenics in Rats. *Toxicological Sciences*, 82(2):478–487, 2004. ISSN 10966080. doi: 10.1093/toxsci/kfh265.
- U. Damm, T. Lang, and A. D. Rijnsdorp. *Movements of dab (Limanda limanda L.) in the German Bight and Southern Bight: Results of German and Dutch tagging experiments in 1988, 1989*. International Council for the Exploration of the Sea, Demersal Fish Committee, 1991.
- M. R. Darrach, A. Chutjian, and G. A. Plett. Trace Explosives Signatures from World War II Unexploded Undersea Ordnance. *Environmental Science and Technology*, 32(9):1354–1358, 1998. ISSN 0013936X. doi: 10.1021/es970992h.
- G. Dave, E. Nilsson, and A.-S. Wernersson. Sediment and water phase toxicity and UV-activation of six chemicals used in military explosives. *Aquatic Ecosystem Health & Management*, 3(3):291–299, 2000. ISSN 1463-4988.
- R. De Clerck and E. Torrele. Feeding habits of common dab (*Limanda limanda* L.) in the southern North Sea. *ICES CM1988/G*, 26, 1988.
- Á. M. de Miranda Cabral Gontijo, R. E. Barreto, G. Speit, V. A. Valenzuela Reyes, G. L. Volpato, and D. M. Favero Salvadori. Anesthesia of fish with benzocaine does not interfere with comet assay results. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 534(1):165–172, 2003. ISSN 1383-5718. doi: 10.1016/S1383-5718(02)00276-0.

- C. Della Torre, I. Corsi, A. Arukwe, M. Valoti, and S. Focardi. Interactions of 2,4,6-trinitrotoluene (TNT) with xenobiotic biotransformation system in European eel *Anguilla anguilla* (Linnaeus, 1758). *Ecotoxicology and Environmental Safety*, 71(3): 798–805, 2008. ISSN 01476513. doi: 10.1016/j.ecoenv.2008.03.003.
- C. Della Torre, T. Petochi, I. Corsi, M. M. Dinardo, D. Baroni, L. Alcaro, S. Focardi, A. Tursi, G. Marino, and A. Frigeri. DNA damage, severe organ lesions and high muscle levels of As and Hg in two benthic fish species from a chemical warfare agent dumping site in the Mediterranean Sea. *Science of the Total Environment*, 408(9): 2136–2145, 2010. ISSN 0048-9697.
- A. Diamant and A. H. McVicar. Distribution of X-cell disease in common dab, *Limanda limanda* L., in the North Sea, and ultrastructural observations of previously undescribed developmental stages. *Journal of Fish Diseases*, 13(1):25–37, 1990. ISSN 0140-7775.
- G. J. Dietrich, A. Szpyrka, M. Wojtczak, S. Dobosz, K. Goryczko, and A. Ciereszko. Effects of UV irradiation and hydrogen peroxide on DNA fragmentation, motility and fertilizing ability of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. *Theriogenology*, 64(8):1809–1822, 2005. ISSN 0093-691X.
- DIN 32645. Chemical analysis - Decision limit, detection limit and determination limit under repeatability conditions - Terms, methods, evaluation. 2008.
- S. C. Doney, M. Ruckelshaus, J. E. Duffy, J. P. Barry, F. Chan, C. A. English, H. M. Galindo, J. M. Grebmeier, A. B. Hollowed, and N. Knowlton. Climate Change Impacts on Marine Ecosystems. *Annual Review of Marine Science*, 2011. doi: 10.1146/annurev-marine-041911-111611.
- J. E. Dore, R. Lukas, D. W. Sadler, M. J. Church, and D. M. Karl. Physical and biogeochemical modulation of ocean acidification in the central North Pacific. *Proceedings of the National Academy of Sciences*, 106(30):12235–12240, 2009. ISSN 0027-8424.
- EGEOS GmbH. AMUCAD: Ammunition Cadastre Sea, 2019. URL [www.amucad.org](http://www.amucad.org).
- M. L. Eggens and F. Galgani. Ethoxyresorufin-*O*-deethylase (EROD) Activity in Flatfish : Fast Determination with a Fluorescence Plate-Reader. *Marine Environmental Research*, 33(3):213–221, 1992.
- L. Ehrenberg, E. Moustacchi, and S. Osterman-Golkar. Dosimetry of genotoxic agents and dose-response relationships of their effects. *Mutation Research/Reviews in Genetic Toxicology*, 123(2):121–182, 1983. ISSN 0165-1110.

- P. Einistö. Role of bacterial nitroreductase and *O*-acetyltransferase in urine mutagenicity assay of rats exposed to 2,4,6-trinitrotoluene (TNT). *Mutation Research Letters*, 262(3):167–169, 1991. ISSN 0165-7992. doi: 10.1016/0165-7992(91)90017-X.
- H. Ek, G. Dave, G. Birgersson, and L. Förlin. Acute effects of 2,4,6-trinitrotoluene (TNT) on haematology parameters and hepatic EROD-activity in rainbow trout (*Oncorhynchus mykiss*). *Aquatic Ecosystem Health & Management*, 6(4):415–421, 2003. ISSN 1463-4988. doi: 10.1080/14634980390255100.
- H. Ek, G. Dave, J. Sturve, B. C. Almroth, E. Stephensen, L. Förlin, and G. Birgersson. Tentative biomarkers for 2,4,6-trinitrotoluene (TNT) in fish (*Oncorhynchus mykiss*). *Aquatic Toxicology*, 72(3):221–230, 2005. ISSN 0166445X. doi: 10.1016/j.aquatox.2005.01.001.
- H. Ek, G. Dave, E. Nilsson, J. Sturve, and G. Birgersson. Fate and Effects of 2,4,6-Trinitrotoluene (TNT) from Dumped Ammunition in a Field Study with Fish and Invertebrates. *Archives of Environmental Contamination and Toxicology*, 51(2):244–252, 2006. ISSN 00904341. doi: 10.1007/s00244-005-0117-5.
- H. Ek, E. Nilsson, and G. Dave. Effects of TNT leakage from dumped ammunition on fish and invertebrates in static brackish water systems. *Ecotoxicology and Environmental Safety*, 69(1):104–111, 2008. ISSN 01476513. doi: 10.1016/j.ecoenv.2006.12.016.
- J. Eum, J. Kwak, H. J. Kim, S. Ki, K. Lee, A. A. Raslan, O. K. Park, M. A. U. Chowdhury, S. Her, Y. Kee, S. H. Kwon, and B. J. Hwang. 3D Visualization of Developmental Toxicity of 2,4,6-Trinitrotoluene in Zebrafish Embryogenesis Using Light-Sheet Microscopy. *International Journal of Molecular Sciences*, 17(11):1–14, 2016. ISSN 14220067. doi: 10.3390/ijms17111925.
- European Commission. Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008 establishing a framework for community action in the field of marine environmental policy (Marine Strategy Framework Directive). *Official Journal of the European Union*, 164:19–40, 2008.
- European Commission. Report from the Commission to the European Parliament and the Council assessing Member States’ programmes of measures under the Marine Strategy Framework Directive. Technical report, Brussels, 2018.
- European Commission. Coastal and marine policy, 2019. URL [https://ec.europa.eu/environment/marine/eu-coast-and-marine-policy/marine-strategy-framework-directive/index\\_en.htm](https://ec.europa.eu/environment/marine/eu-coast-and-marine-policy/marine-strategy-framework-directive/index_en.htm).

- T. Frische. Screening for Soil Toxicity and Mutagenicity Using Luminescent Bacteria - A Case Study of the Explosive 2,4,6-Trinitrotoluene (TNT). *Ecotoxicology and Environmental Safety*, 51:133–144, 2002. doi: 10.1006/eesa.2001.2124.
- E. M. Furedi, B. S. Levine, D. E. Gordon, V. S. Rac, and P. M. Lish. Determination of the Chronic Mammalian Toxicological Effects of TNT (Twenty-four Month Chronic Toxicity/Carcinogenicity Study of Trinitrotoluene (TNT) in the Fischer 344 Rat) Final Report Phase III Volume 1. Technical report, US Army Medical Research and Development Command, Fort Detrick, Frederick, 1984a.
- E. M. Furedi, B. S. Levine, J. W. Sagartz, V. S. Rac, and P. M. Lish. Determination of the Chronic Mammalian Toxicological Effects of TNT (Twenty-four Month Chronic Toxicity/Carcinogenicity Study of Trinitrotoluene (TNT) in the B6C3F1 Hybrid Mouse) Final Report Phase IV Volume 3. Technical report, US Army Medical Research and Development Command, Fort Detrick, Frederick, 1984b.
- P. Gaunt and S. A. Barker. Matrix solid phase dispersion extraction of triazines from catfish tissues; examination of the effects of temperature and dissolved oxygen on the toxicity of atrazine. *International Journal of Environment and Pollution*, 13(1-6): 284–312, 2000. ISSN 0957-4352.
- S. E. George, G. Huggins-Clark, and L. R. Brooks. Use of a *Salmonella* microsuspension bioassay to detect the mutagenicity of munitions compounds at low concentrations. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 490(1):45–56, 2001. ISSN 1383-5718.
- J. P. Giesy and K. Kannan. Global Distribution of Perfluorooctane Sulfonate in Wildlife. *Environmental Science & Technology*, 35(7):1339–1342, 2001. ISSN 0013-936X.
- M. Gledhill, A. J. Beck, B. Stamer, C. Schlosser, and E. P. Achterberg. Quantification of munition compounds in the marine environment by solid phase extraction - ultra high performance liquid chromatography with detection by electrospray ionisation - mass spectrometry. *Talanta*, 200(November 2018):366–372, 2019. ISSN 00399140. doi: 10.1016/j.talanta.2019.03.050.
- J. V. Goldstone, A. G. McArthur, A. Kubota, J. Zanette, T. Parente, M. E. Jönsson, D. R. Nelson, and J. J. Stegeman. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. *BMC Genomics*, 11(1): 643, 2010. ISSN 1471-2164.

- A. Gomez-Camirero, P. Howe, M. Hughes, and E. Kenyon. Environmental Health Criteria 224 Arsenic and Arsenic Compounds. 2010.
- H. Greim and R. J. Albertini. Cellular response to the genotoxic insult: the question of threshold for genotoxic carcinogens. *Toxicology Research*, 4(1):36–45, 2015. ISSN 2045-4538.
- T. Grummt, H. Wunderlich, A. Chakraborty, M. Kundi, B. Majer, F. Ferk, A. K. Nersesyan, W. Parzefall, and S. Knasmüller. Genotoxicity of Nitrosulfonic Acids, Nitrobenzoic Acids, and Nitrobenzylalcohols, Pollutants Commonly Found in Ground Water Near Ammunition Facilities. *Environmental and Molecular Mutagenesis*, 47(2):95–106, 2006. ISSN 0893-6692.
- R. Haas, T. C. Schmidt, K. Steinbach, and E. Von Löw. Chromatographic determination of phenylarsenic compounds. *Fresenius' Journal of Analytical Chemistry*, 361(3):313–318, 1998. ISSN 09370633. doi: 10.1007/s002160050892.
- A. Haïdour and J. L. Ramos. Identification of Products Resulting from the Biological Reduction of 2,4,6-Trinitrotoluene, 2,4-Dinitrotoluene, and 2,6-Dinitrotoluene by *Pseudomonas* sp. *Environmental Science & Technology*, 30(7):2365–2370, 1996. ISSN 0013-936X.
- M. Halme, M. Pesonen, U. Hakala, M. Pasanen, K. Vähäkangas, and P. Vanninen. Applying human and pig hepatic *in vitro* experiments for sulfur mustard study: Screening and identification of metabolites by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, 29(14):1279–1287, 2015. ISSN 10970231. doi: 10.1002/rcm.7218.
- B. S. Halpern, S. Walbridge, K. A. Selkoe, C. V. Kappel, F. Micheli, C. D'Agrosa, J. F. Bruno, K. S. Casey, C. Ebert, and H. E. Fox. A Global Map of Human Impact on Marine Ecosystems. *Science*, 319(5865):948–952, 2008. ISSN 0036-8075.
- C. D. G. Harley, A. Randall Hughes, K. M. Hultgren, B. G. Miner, C. J. B. Sorte, C. S. Thornber, L. F. Rodriguez, L. Tomanek, and S. L. Williams. The impacts of climate change in coastal marine systems. *Ecology Letters*, 9(2):228–241, 2006. ISSN 1461-023X.
- J. Hawari, A. Halasz, L. Paquet, E. Zhou, B. Spencer, G. Ampleman, and S. Thiboutot. Characterization of Metabolites in the Biotransformation of 2,4,6-Trinitrotoluene with Anaerobic Sludge: Role of Triaminotoluene. *Applied and Environmental Microbiology*, 64(6):2200–2206, 1998. ISSN 0099-2240.



- HELCOM. Final Report of the ad hoc Working Group on Dumped Chemical Munition (HELCOM CHEMU) to the 16th Meeting of the Helsinki Commission (March 1995). Technical report, 1995.
- HELCOM. Chemical Munitions Dumped in the Baltic Sea: Report of the ad hoc Expert Group to Update and Review the Existing Information on Dumped Chemical Munitions in the Baltic Sea (HELCOM MUNI). In *Baltic Sea Environment Proceedings*, 2013.
- L. Henderson, S. Albertini, and M. Aardema. Thresholds in genotoxicity responses. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 464(1):123–128, 2000. ISSN 1383-5718.
- E. H. W. Heugens, A. J. Hendriks, T. Dekker, N. M. van Straalen, and W. Admiraal. A Review of the Effects of Multiple Stressors on Aquatic Organisms and Analysis of Uncertainty Factors for Use in Risk Assessment. *Critical Reviews in Toxicology*, 31(3):247–284, 2001. ISSN 1040-8444.
- J. C. Hoffsommer and J. M. Rosen. Analysis of Explosives in Sea Water. *Bulletin of Environmental Contamination and Toxicology*, 7(2-3):177–181, 1972. ISSN 0007-4861.
- N. Höher, R. Turja, M. Brenner, J. R. Nyholm, A. Östin, P. Leffler, L. Butrimavičienė, J. Baršienė, M. Halme, M. Karjalainen, H. Niemikoski, P. Vanninen, K. Broeg, K. K. Lehtonen, and R. Berglind. Toxic effects of chemical warfare agent mixtures on the mussel *Mytilus trossulus* in the Baltic Sea: A laboratory exposure study. *Marine Environmental Research*, 145(February):112–122, 2019. ISSN 18790291. doi: 10.1016/j.marenvres.2019.02.001.
- S. Homma-Takeda, Y. Hiraku, Y. Ohkuma, S. Oikawa, M. Murata, K. Ogawa, T. Iwamuro, S. Li, G. F. Sun, Y. Kumagai, N. Shimojo, and S. Kawanishi. 2,4,6-Trinitrotoluene-induced Reproductive Toxicity via Oxidative DNA Damage by its Metabolite. *Free Radical Research*, 36(5):555–566, 2002. ISSN 10715762. doi: 10.1080/10715760290025933.
- M. E. Honeycutt, A. Jarvis, and V. A. McFarland. Cytotoxicity and Mutagenicity of 2,4,6-Trinitrotoluene and Its Metabolites. *Ecotoxicology and Environmental Safety*, 35:282–287, 1996.

- S. E. Hook and R. F. Lee. Genotoxicant induced DNA damage and repair in early and late developmental stages of the grass shrimp *Palaemonetes pugio* embryo as measured by the comet assay. *Aquatic Toxicology*, 66(1):1–14, 2004. ISSN 0166-445X.
- M. Htun-Han. The reproductive biology of the dab *Limanda limanda* (L.) in the North Sea: gonosomatic index, hepatosomatic index and condition factor. *Journal of Fish Biology*, 13(3):369–378, 1978. ISSN 0022-1112.
- M. F. Hughes. Arsenic toxicity and potential mechanisms of action. *Toxicology Letters*, 133(1):1–16, 2002. ISSN 0378-4274.
- T. H. Hutchinson, N. Scholz, and W. Guhl. Analysis of the ECETOC aquatic toxicity (EAT) database IV - Comparative toxicity of chemical substances to freshwater versus saltwater organisms. *Chemosphere*, 36(1):143–153, 1998. ISSN 0045-6535.
- K. Hylland, B. B. Skei, G. Brunborg, T. Lang, M. J. Gubbins, J. le Goff, and T. Burgeot. DNA damage in dab (*Limanda limanda*) and haddock (*Melanogrammus aeglefinus*) from European seas. *Marine Environmental Research*, 124:54–60, 2017. ISSN 18790291. doi: 10.1016/j.marenvres.2016.01.001.
- ICES. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO). Technical report, International Council for the Exploration of the Sea, 2012.
- L. Inouye, B. Lachance, and P. Gong. *Genotoxicity of Explosives*. Number June 2009. 2009. ISBN 9781420004342. doi: 10.1201/9781420004342.ch8.
- I. International Maritime Organization. Status of IMO Treaties: Comprehensive information on the status of multilateral Conventions and instruments in respect of which the International Maritime Organization or its Secretary-General performs depositary or other functions. Technical report, International Maritime Organization, United Nations, London, 2020.
- IPCC. Climate Change 2014 Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Technical report, IPCC, Geneva, 2014.
- K. Ishii, A. Tamaoka, F. Otsuka, N. Iwasaki, K. Shin, A. Matsui, G. Endo, Y. Kumagai, T. Ishii, S. Shoji, T. Ogata, M. Ishizaki, M. Doi, and N. Shimojo. Diphenylarsinic Acid Poisoning from Chemical Weapons in Kamisu, Japan. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, 56(5):741–745, 2004. ISSN 0364-5134.

- N. Ishii, M. Gi, M. Fujioka, S. Yamano, M. Okumura, A. Kakehashi, and H. Wanibuchi. Diphenylarsinic acid exerts promotion effects on hepatobiliary carcinogenesis in a rat medium-term multiorgan carcinogenicity bioassay. *Journal of Toxicologic Pathology*, 30(1):39–45, 2017. ISSN 0914-9198.
- M. Ishizaki, T. Yanaoka, M. Nakamura, T. Hakuta, S. Ueno, M. Komuro, M. Shibata, T. Kitamura, A. Honda, M. Doy, K. Ishii, A. Tamaoka, N. Shimojo, T. Ogata, E. Nagasawa, and S. Hanaoka. Detection of Bis(diphenylarsine)oxide, Diphenylarsinic Acid and Phenylarsonic Acid, Compounds Probably Derived from Chemical Warfare Agents, in Drinking Well Water. *Journal of Health Science*, 51(2):130–137, 2005.
- I. Jablonkai and K. K. Hatzios. *In Vitro* Conjugation of Chloroacetanilide Herbicides and Atrazine with Thiols and Contribution of Nonenzymatic Conjugation to Their Glutathione-Mediated Metabolism in Corn. *Journal of Agricultural and Food Chemistry*, 41(10):1736–1742, 1993. ISSN 0021-8561.
- T. F. Jenkins, S. R. Bigl, A. D. Hewitt, J. L. Clausen, H. D. Craig, M. E. Walsh, R. Martel, K. Nieman, S. Taylor, and M. R. Walsh. Site Characterization For Munitions Constituents, EPA Federal Facilities Forum Issue Paper. *Environmental Protection Agency (EPA-505-S-11-001), Washington, DC, US Environmental Protection Agency*, 2012.
- P. D. Jepson and R. J. Law. Persistent pollutants, persistent threats. *Science*, 352(6292):1388–1389, 2016. ISSN 0036-8075.
- L. R. Johnson, R. Davenport, H. Balbach, and D. J. Schaeffer. Phototoxicology: 3. Comparative Toxicity of Trinitrotoluene and Aminodinitrotoluenes to *Daphnia magna*, *Dugesia dorotocephala*, and Sheep Erythrocytes. *Ecotoxicology and Environmental Safety*, 27(1):34–49, 1994. ISSN 0147-6513.
- A. L. Juhasz and R. Naidu. Explosives: Fate, Dynamics, and Ecological Impact in Terrestrial and Marine Environments. In *Reviews of Environmental Contamination and Toxicology*, pages 163–215. Springer, 2007.
- W. Jurczak and J. Fabisiak. Corrosion of ammunition dumped in the Baltic Sea. *Journal of KONBiN*, 41(1):227–246, 2017. ISSN 2083-4608.
- S. V. Kala, M. W. Neely, G. Kala, C. I. Prater, D. W. Atwood, J. S. Rice, and M. W. Lieberman. The MRP2/cMOAT Transporter and Arsenic-Glutathione Complex Formation Are Required for Biliary Excretion of Arsenic. *Journal of Biological Chemistry*, 275(43):33404–33408, 2000. ISSN 0021-9258.

- U. Kammann. PAH Metabolites in Bile Fluids of Dab (*Limanda limanda*) and flounder (*Platichthys flesus*): Spatial Distribution and Seasonal Changes. *Environmental Science and Pollution Research-International*, 14(2):102–108, 2007. ISSN 0944-1344.
- U. Kammann, M. Vobach, and W. Wosniok. Toxic Effects of Brominated Indoles and Phenols on Zebrafish Embryos. *Archives of Environmental Contamination and Toxicology*, 51(1):97–102, 2006. ISSN 0090-4341.
- M. Kampmeier, E. M. van der Lee, U. Wichert, and J. Greinert. Exploration of the munition dumpsite Kolberger Heide in Kiel Bay, Germany: Example for a standardised hydroacoustic and optic monitoring approach. *Continental Shelf Research*, page 104108, 2020. ISSN 0278-4343.
- S. J. Kennel, L. J. Foote, M. Morris, A. A. Vass, and W. H. Griest. Mutation Analyses of a Series of TNT-related Compounds Using the CHO-hprt Assay. *Journal of Applied Toxicology*, 20:441–448, 2000.
- M. J. Kennish. *Practical Handbook of Estuarine and Marine Pollution*. CRC press, 1 edition, 1997. ISBN 0849384249.
- B. Ketterer. The Role of Nonenzymatic Reactions of Glutathione in Xenobiotic Metabolism. *Drug Metabolism Reviews*, 13(1):161–187, 1982. ISSN 0360-2532.
- K. Kinoshita, T. Ochi, T. Suzuki, K. Kita, and T. Kaise. Glutathione plays a role in regulating the formation of toxic reactive intermediates from diphenylarsinic acid. *Toxicology*, 225(2-3):142–149, 2006. ISSN 0300483X. doi: 10.1016/j.tox.2006.05.010.
- K. Kinoshita, A. Noguchi, K. Ishii, A. Tamaoka, T. Ochi, and T. Kaise. Urine analysis of patients exposed to phenylarsenic compounds via accidental pollution. *Journal of Chromatography B*, 867(2):179–188, 2008. ISSN 1570-0232. doi: 10.1016/j.jchromb.2008.03.033.
- M. F. Kirby, P. Neall, and T. Tylor. EROD Activity Measured in Flatfish From the Area of the *Sea Empress* Oil Spill. *Chemosphere*, 38(12):2929–2949, 1999.
- Y. Kobayashi and S. Hirano. The role of glutathione in the metabolism of diphenylarsinic acid in rats. *Metallomics*, 5(5):469–478, 2013.
- T. Kobets and G. M. Williams. Review of the evidence for thresholds for DNA-reactive and epigenetic experimental chemical carcinogens. *Chemico-Biological Interactions*, 2019. ISSN 0009-2797.

- S. Koide, J. A. K. Silva, V. Dupra, and M. Edwards. Bioaccumulation of chemical warfare agents, energetic materials, and metals in deep-sea shrimp from discarded military munitions sites off Pearl Harbor. *Deep Sea Research Part II: Topical Studies in Oceanography*, 128:53–62, 2016. ISSN 0967-0645.
- K. Końca, A. Lankoff, A. Banasik, H. Lisowska, T. Kuszewski, S. Gózdź, Z. Koza, and A. Wojcik. A cross-platform public domain PC image-analysis program for the comet assay. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, 534(1-2):15–20, 2003. ISSN 13835718. doi: 10.1016/S1383-5718(02)00251-6.
- D. Koske, N. I. Goldenstein, and U. Kammann. Nitroaromatic compounds damage the DNA of zebrafish embryos (*Danio rerio*). *Aquatic Toxicology*, 217:105345, 2019. ISSN 0166-445X. doi: 10.1016/j.aquatox.2019.105345.
- D. Koske, N. I. Goldenstein, T. Rosenberger, U. Machulik, R. Hanel, and U. Kammann. Dumped munitions: New insights into the metabolization of 2,4,6-trinitrotoluene in Baltic flatfish. *Marine Environmental Research*, 160:104992, 2020a. ISSN 0141-1136. doi: 10.1016/j.marenvres.2020.104992.
- D. Koske, K. Straumer, N. I. Goldenstein, R. Hanel, T. Lang, and U. Kammann. First evidence of explosives and their degradation products in dab (*Limanda limanda* L.) from a munition dumpsite in the Baltic Sea. *Marine Pollution Bulletin*, 155:111131, 2020b. doi: 10.1016/j.marpolbul.2020.111131.
- T. Kosmehl, A. V. Hallare, G. Reifferscheid, W. Manz, T. Braunbeck, and H. Hollert. A Novel Contact Assay for Testing Genotoxicity of Chemicals and Whole Sediments in Zebrafish Embryos. *Environmental Toxicology and Chemistry*, 25(8):2097–2106, aug 2006. ISSN 0730-7268. doi: 10.1897/05-460R.1.
- T. Kosmehl, A. V. Hallare, T. Braunbeck, and H. Hollert. DNA damage induced by genotoxicants in zebrafish (*Danio rerio*) embryos after contact exposure to freeze-dried sediment and sediment extracts from Laguna Lake (The Philippines) as measured by the comet assay. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 650(1):1–14, 2008. ISSN 1383-5718.
- J. Kretzschmar, E. Brendler, J. Wagler, and A. C. Schmidt. Kinetics and activation parameters of the reaction of organoarsenic(V) compounds with glutathione. *Journal of Hazardous Materials*, 280:734–740, 2014. ISSN 18733336. doi: 10.1016/j.jhazmat.2014.08.036.

- Y. Kumagai and D. Sumi. Arsenic: Signal Transduction, Transcription Factor, and Biotransformation Involved in Cellular Response and Toxicity. *Annual Review of Pharmacology and Toxicology*, 47(1):243–262, 2006. ISSN 0362-1642. doi: 10.1146/annurev.pharmtox.47.120505.105144.
- B. Lachance, P. Y. Robidoux, J. Hawari, G. Ampleman, S. Thiboutot, and G. I. Sunahara. Cytotoxic and genotoxic effects of energetic compounds on bacterial and mammalian cells in vitro. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 444(1):25–39, 1999. ISSN 1383-5718.
- R. E. Laflamme and R. A. Hites. The global distribution of polycyclic aromatic hydrocarbons in recent sediments. *Geochimica et Cosmochimica Acta*, 42(3):289–303, 1978. ISSN 0016-7037.
- T. Lang. Fish disease surveys in environmental monitoring: The role of ICES. In *ICES Marine Science Symposia*, volume 215, pages 202–212, 2002.
- T. Lang, R. Kruse, M. Haarich, and W. Wosniok. Mercury species in dab (*Limanda limanda*) from the North Sea, Baltic Sea and Icelandic waters in relation to host-specific variables. *Marine Environmental Research*, 124:32–40, 2017. ISSN 0141-1136.
- T. Lang, L. Kotwicki, M. Czub, K. Grzelak, L. Weirup, and K. Straumer. The Health Status of Fish and Benthos Communities in Chemical Munitions Dumpsites in the Baltic Sea. In *Towards the Monitoring of Dumped Munitions Threat (MODUM)*, pages 129–152. Springer, 2018.
- R. Law, G. Hanke, M. Angelidis, J. Batty, A. Bignert, J. Dachs, I. Davies, Y. Denga, A. Duffek, and B. Herut. Marine Strategy Framework Directive: Task Group 8 Report Contaminants and pollution effects. Technical report, European Commission, 2010.
- J. R. C. Leavitt and D. Penner. In Vitro Conjugation of Glutathione and Other Thiols with Acetanilide Herbicides and EPTC Sulfoxide and the Action of the Herbicide Antidote R-25788. *Journal of Agricultural and Food Chemistry*, 27(3):533–536, 1979. ISSN 0021-8561.
- P. Leffler, E. Brännäs, D. Ragnvaldsson, H. Wingfors, and R. Berglind. Toxicity and Accumulation of Trinitrotoluene (TNT) and its Metabolites in Atlantic Salmon Alevins Exposed to an Industrially Polluted Water. *Journal of Toxicology and Environmental Health, Part A*, 77(19):1183–1191, 2014. ISSN 1528-7394. doi: 10.1080/15287394.2014.920756.

- K. H. Leung, M. Yao, R. Stearns, and S.-H. L. Chiu. Mechanism of bioactivation and covalent binding of 2,4,6-trinitrotoluene. *Chemico-Biological Interactions*, 97:37–51, 1995.
- K. M. Y. Leung, D. Morritt, J. R. Wheeler, P. Whitehouse, N. Sorokin, R. Toy, M. Holt, and M. Crane. Can Saltwater Toxicity be Predicted from Freshwater Data? *Marine Pollution Bulletin*, 42(11):1007–1013, 2001. ISSN 0025-326X.
- J. Lewis, R. Martel, L. Trépanier, G. Ampleman, and S. Thiboutot. Quantifying the Transport of Energetic Materials in Unsaturated Sediments from Cracked Unexploded Ordnance. *Journal of Environmental Quality*, 38(6):2229–2236, 2009. ISSN 1537-2537.
- H.-Y. Liao, C.-M. Kao, C.-L. Yao, P.-W. Chiu, C.-C. Yao, and S.-C. Chen. 2,4,6-Trinitrotoluene Induces Apoptosis via ROS-Regulated Mitochondrial Dysfunction and Endoplasmic Reticulum Stress in HepG2 and Hep3B Cells. *Scientific Reports*, 7(1):1–11, 2017. ISSN 2045-2322.
- G. Liebezeit. Dumping and re-occurrence of ammunition on the German North Sea coast. In *Chemical Munition Dump Sites in Coastal Environments*, pages 13–26. Office for Scientific, Technical and Cultural Affairs (OSTC), Brussels, 2002.
- D. H. Liu, H. C. Bailey, and J. G. Pearson. Toxicity of a Complex Munitions Wastewater to Aquatic Organisms. In W. Bishop, R. Cardwell, and B. Heidolph, editors, *Aquatic Toxicology and Hazard Assessment: Sixth Symposium*, pages 135–150, 1983. doi: 10.1520/STP33503S.
- D. R. Livingstone. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 120(1):43–49, 1998. ISSN 1095-6433. doi: 10.1016/S1095-6433(98)10008-9.
- G. R. Lotufo. Toxicity and Bioaccumulation of Munitions Constituents in Aquatic and Terrestrial Organisms. In *Energetic Materials*, pages 445–479. Springer, 2017.
- G. R. Lotufo, M. Nipper, R. S. Carr, and J. M. Conder. Fate and Toxicity of Explosives in Sediments. In *Ecotoxicology of Explosives*, pages 117–134. 2009. doi: 10.1201/9781420004342.
- G. R. Lotufo, W. Blackburn, S. J. Marlborough, and J. W. Fleeger. Toxicity and bioaccumulation of TNT in marine fish in sediment exposures. *Ecotoxicology and Environmental Safety*, 73(7):1720–1727, 2010a. ISSN 01476513. doi: 10.1016/j.ecoenv.2010.02.009.

- G. R. Lotufo, W. M. Blackburn, and A. B. Gibson. Toxicity of trinitrotoluene to sheepshead minnows in water exposures. *Ecotoxicology and Environmental Safety*, 73(5):718–726, 2010b. ISSN 01476513. doi: 10.1016/j.ecoenv.2010.02.007.
- G. R. Lotufo, G. Rosen, W. Wild, and G. Carton. Summary Review of the Aquatic Toxicology of Munitions Constituents. Technical report, 2013.
- G. R. Lotufo, M. A. Chappell, C. L. Price, M. L. Ballentine, A. A. Fuentes, R. D. George, E. Glisch, and G. Carton. Review and Synthesis of Evidence Regarding Environmental Risks Posed by Munitions Constituents (MC) in Aquatic Systems. Technical report, 2017.
- O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein Measurement With the Folin Phenol Reagent. *Journal of Biological Chemistry*, 193(1):265–275, 1951.
- D. J. Luning Prak, J. E. Breuer, E. A. Rios, E. E. Jedlicka, and D. W. O’Sullivan. Photolysis of 2,4,6-trinitrotoluene in seawater and estuary water: Impact of pH, temperature, salinity, and dissolved organic matter. *Marine Pollution Bulletin*, 114(2):977–986, 2017. ISSN 18793363. doi: 10.1016/j.marpolbul.2016.10.073.
- J. C. Lynch, K. F. Myers, J. M. Brannon, and J. J. Delfino. Effects of pH and Temperature on the Aqueous Solubility and Dissolution Rate of 2,4,6-Trinitrotoluene (TNT), Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). *Journal of Chemical & Engineering Data*, 46(6):1549–1555, 2001. ISSN 0021-9568.
- W.-T. Ma, W. Chan, K. Steinbach, and Z. Cai. Determination of five nitrobenzoic acids in groundwater by solid-phase extraction and liquid chromatography-mass spectrometry. *Analytical and Bioanalytical Chemistry*, 387(6):2219–2225, 2007. ISSN 1618-2642.
- H. N. Maier. *Beiträge zur Altersbestimmung der Fische: Allgemeines; die Altersbestimmung nach den Otolithen bei Scholle und Kabeljau*. Littmann, 1906.
- T. M. Mallon, J. M. Ortiz, W. H. Candler, G. Rogers, and R. Hillburn. Investigation of an Outbreak of Anemia Cases at an Army Trinitrotoluene Munitions Production Plant From 2004 to 2005 and Subsequent Surveillance 2005-2013. *Military Medicine*, 179(11):1374–1383, 2014. ISSN 1930-613X.



- E. Mariussen, S. Stornes, K. Bøifot, B. Rosseland, B. Salbu, and L. Heier. Uptake and effects of 2,4,6-trinitrotoluene (TNT) in juvenile Atlantic salmon (*Salmo salar*). *Aquatic Toxicology*, 194(November 2017):176–184, 2018. ISSN 18791514. doi: 10.1016/j.aquatox.2017.11.016.
- J. Martins, L. O. Teles, and V. Vasconcelos. Assays with *Daphnia magna* and *Danio rerio* as alert systems in aquatic toxicology. *Environment International*, 33(3):414–425, 2007. ISSN 0160-4120.
- K. A. Maruya, K. L. Smalling, and W. Vetter. Temperature and Congener Structure Affect the Enantioselectivity of Toxaphene Elimination by Fish. *Environmental Science & Technology*, 39(11):3999–4004, 2005. ISSN 0013-936X.
- E. Maser and J. S. Strehse. “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. *Archives of Toxicology*, pages 1–13, apr 2020. ISSN 0340-5761. doi: 10.1007/s00204-020-02743-0.
- N. G. McCormick, F. E. Feeherry, and H. S. Levinson. Microbial Transformation of 2,4,6-Trinitrotoluene and Other Nitroaromatic Compounds. *Applied and Environmental Microbiology*, 31(6):949–958, 1976. ISSN 0099-2240.
- A. H. McVicar, D. W. Bruno, and C. O. Fraser. Fish Diseases in the North Sea in Relation to Sewage Sludge Dumping. *Marine Pollution Bulletin*, 19(4):169–173, 1988. ISSN 0025-326X.
- P. Menzel, C. Schütt, H. Wranik, M. Paschen, and A. Drews. Towards a general prediction-model for the current-induced mobilisation of objects on the sea floor. *Ocean Engineering*, 164:160–167, 2018. ISSN 0029-8018.
- J. C. Mirsalis and B. E. Butterworth. Induction of unscheduled DNA synthesis in rat hepatocytes following *in vivo* treatment with dinitrotoluene. *Carcinogenesis*, 3(3):241–245, 1982. ISSN 1460-2180.
- T. Missiaen and J.-P. Henriët. Chemical munition dump sites in coastal environments: a border-transgressing problem. In *Chemical Munition Dump Sites in Coastal Environments*, pages 1–12. Office for Scientific, Technical and Cultural Affairs (OSTC), Brussels, 2002.
- T. Missiaen, M. Söderström, I. Popescu, and P. Vanninen. Evaluation of a chemical munition dumpsite in the Baltic Sea based on geophysical and chemical investigations. *Science of the Total Environment*, 408(17):3536–3553, 2010. ISSN 00489697. doi: 10.1016/j.scitotenv.2010.04.056.

- F. Monteil-Rivera, A. Halasz, C. Groom, J.-S. Zhao, S. Thiboutot, G. Ampleman, and J. Hawari. Fate and Transport of Explosives in the Environment: A Chemist's View. In *Ecotoxicology of Explosives*, pages 26–54. CRC Press, 2009.
- G. J. Mulder. Glucuronidation and its role in regulation of biological activity of drugs. *Annual Review of Pharmacology and Toxicology*, 32(1):25–49, 1992. ISSN 0362-1642.
- R. P. Murrmann. Composition and Mass Spectra of Impurities in Military Grade TNT Vapor. Technical report, 1971.
- T. Nakayama, T. Isobe, K. Nakamiya, J. S. Edmonds, Y. Shibata, and M. Morita. Complexes of diphenylarsinic acid and phenylarsonic acid with thiols: a  $^1\text{H}$  and  $^{13}\text{C}$  NMR study. *Magnetic Resonance in Chemistry*, 43(7):543–550, 2005. ISSN 07491581. doi: 10.1002/mrc.1585.
- H. Naranmandura, N. Suzuki, and K. T. Suzuki. Trivalent Arsenicals Are Bound to Proteins During Reductive Methylation. *Chemical Research in Toxicology*, 19(8):1010–1018, 2006. ISSN 0893-228X.
- N. National Oceanic and Atmospheric Administration. Earth System Research Laboratory, 2020.
- J. Neuwoehner, A. Schofer, B. Erlenkaemper, K. Steinbach, K. Hund-Rinke, and A. Eisentraeger. Toxicological Characterization of 2,4,6-Trinitrotoluene, its Transformation Products, and Two Nitramine Explosives. *Environmental Toxicology and Chemistry*, 26(6):1090–1099, 2007. ISSN 0730-7268.
- M. C. Newman. *Fundamentals of Ecotoxicology: The Science of Pollution*. CRC press, 4 edition, 2014. ISBN 9781466582293.
- H. Niemikoski, M. Söderström, and P. Vanninen. Detection of Chemical Warfare Agent-Related Phenylarsenic Compounds in Marine Biota Samples by LC-HESI/MS/MS. *Analytical Chemistry*, 89(20), 2017. ISSN 15206882. doi: 10.1021/acs.analchem.7b03429.
- H. Niemikoski, D. Koske, U. Kammann, T. Lang, and P. Vanninen. Studying the metabolism of toxic chemical warfare agent-related phenylarsenic chemicals *in vitro* in cod liver. *Journal of Hazardous Materials*, 391:122221, 2020a. ISSN 0304-3894. doi: 10.1016/j.jhazmat.2020.122221.

- H. Niemikoski, M. Söderström, H. Kiljunen, A. Östin, and P. Vanninen. Identification of Degradation Products of Sea-Dumped Chemical Warfare Agent-Related Phenylarsenic Chemicals in Marine Sediment. *Analytical Chemistry*, 92(7):4891–4899, 2020b. ISSN 0003-2700.
- M. Nipper, R. S. Carr, J. M. Biedenbach, R. L. Hooten, K. Miller, and S. Saepoff. Development of Marine Toxicity Data for Ordnance Compounds. *Archives of Environmental Contamination and Toxicology*, 41(3):308–318, 2001. ISSN 0090-4341.
- T. Nohmi and S. Fukushima. *Thresholds of Genotoxic Carcinogens: From Mechanisms to Regulation*. Academic Press, 2016. ISBN 0128018038.
- P. D. Noyes, M. K. McElwee, H. D. Miller, B. W. Clark, L. A. Van Tiem, K. C. Walcott, K. N. Erwin, and E. D. Levin. The toxicology of climate change: Environmental contaminants in a warming world. *Environment International*, 35(6):971–986, 2009. ISSN 0160-4120.
- T. Ochi, K. Kinoshita, T. Suzuki, K. Miyazaki, A. Noguchi, and T. Kaise. The role of glutathione on the cytotoxic effects and cellular uptake of diphenylarsinic acid, a degradation product of chemical warfare agents. *Archives of Toxicology*, 80(8):486–491, 2006. ISSN 1432-0738. doi: 10.1007/s00204-006-0067-3.
- OECD. OECD Guideline for Testing of Chemicals: Fish, Acute Toxicity Test, No. 203. *Organization for Economic Co-operation and Development, Paris*, 1992.
- OECD. OECD Guidelines for the Testing of Chemicals: Fish Embryo Acute Toxicity (FET) Test, No. 236. *Organization for Economic Co-operation and Development, Paris*, 2013.
- Organisation for the Prohibition of Chemical Weapons (OPCW). Work instruction for the reporting of the results of the OPCW biomedical proficiency test. Technical report, 2018.
- A. A. Ortega-Salas. Seasonal changes in the common dab, *Limanda limanda* (L.) in Isle of Man Waters. *Journal of Fish Biology*, 16(1):75–82, 1980. ISSN 0022-1112.
- D. R. Ownby, J. B. Belden, G. R. Lotufo, and M. J. Lydy. Accumulation of trinitrotoluene (TNT) in aquatic organisms: Part 1 - Bioconcentration and distribution in channel catfish (*Ictalurus punctatus*). *Chemosphere*, 58(9):1153–1159, 2005. ISSN 00456535. doi: 10.1016/j.chemosphere.2004.09.059.
- M. Paetzel. Deep marine munition dump sites: example from Arendal, Norway. In *Chemical munition dump sites in coastal environments*, page 133. 2002.

- V. Paka and M. Spiridonov. Research of dumped chemical weapons made by R/V “Professor Shtokman” in the Gotland, Bornholm & Skagerrak dump sites. In *Chemical munition dump sites in coastal environments*, pages 27–42. Citeseer, 2002.
- A. Parkinson. Biotransformation of Xenobiotics. In C. Klaassen, editor, *Casarett and Doull’s Toxicology: The Basic Science of Poisons*, pages 133–224. New York, 2001.
- A. Parkinson and B. W. Ogilvie. Biotransformation of Xenobiotics. In *Casarett and Doull’s Toxicology: The Basic Science of Poisons*, volume 7, pages 161–304. McGraw-Hill Inc New York, USA, 2008.
- R. B. J. Peachey. The synergism between hydrocarbon pollutants and UV radiation: a potential link between coastal pollution and larval mortality. *Journal of Experimental Marine Biology and Ecology*, 315(1):103–114, 2005. ISSN 0022-0981.
- J. C. Pennington, C. A. Hayes, S. Yost, T. A. Crutcher, T. E. Berry, J. U. Clarke, and M. J. Bishop. Explosive Residues from Blow-in-Place Detonations of Artillery Munitions. *Soil & Sediment Contamination*, 17(2):163–180, 2008. ISSN 1532-0383.
- J. S. Petrick, F. Ayala-Fierro, W. R. Cullen, D. E. Carter, and H. Vasken Aposhian. Monomethylarsonous Acid (MMA<sup>III</sup>) Is More Toxic Than Arsenite in Chang Human Hepatocytes. *Toxicology and Applied Pharmacology*, 163(2):203–207, 2000. ISSN 0041008X. doi: 10.1006/taap.1999.8872.
- T. Pohlert. The pairwise multiple comparison of mean ranks package (PMCMR). *R package*, 27, 2014.
- J. W. Porter, J. V. Barton, and C. Torres. Ecological, Radiological, and Toxicological Effects of Naval Bombardment on the Coral Reefs of Isla de Vieques, Puerto Rico. In *Warfare Ecology: A New Synthesis for Peace and Security*, pages 65–122. Springer, 2011.
- A. Preuss, J. Fimpel, and G. Diekert. Anaerobic transformation of 2,4,6-trinitrotoluene (TNT). *Archives of Microbiology*, 159(4):345–353, 1993. ISSN 0302-8933.
- R Core Team. R: A Language and Environment for Statistical Computing, 2019.
- H. Rapp-Wright, G. McEneff, B. Murphy, S. Gamble, R. Morgan, M. Beardah, and L. Barron. Suspect screening and quantification of trace organic explosives in wastewater using solid phase extraction and liquid chromatography-high resolution accurate mass spectrometry. *Journal of Hazardous Materials*, 329:11–21, 2017. ISSN 0304-3894.

- A. A. Ratushnyak, M. G. Andreeva, and M. V. Trushin. Effects of type II pyrethroids on *Daphnia magna*: Dose and temperature dependences. In *Biology Forum/Rivista di Biologia*, volume 98, 2005. ISBN 1825-6538.
- G. Reddy, A. E. G. Hampton, J. Amos, and M. Major. Metabolism of 1,3,5-Trinitrobenzene (TNB) *in vitro*. In *35th Annual Meeting of the Society for Toxicology*, pages 10–14, 1996.
- G. Reddy, T. V. Reddy, H. Choudhury, F. Bernard Daniel, and G. J. Leach. Assessment of Environmental Hazards of 1,3,5-Trinitrobenzene. *Journal of Toxicology and Environmental Health*, 52(5):447–460, 1997. ISSN 0098-4108.
- D. E. Rickert. Metabolism of Nitroaromatic Compounds. *Drug Metabolism Reviews*, 18(1):23–53, 1987. doi: 10.3109/03602538708998299.
- D. E. Rickert and R. M. Long. Metabolism and excretion of 2,4-dinitrotoluene in male and female Fischer 344 rats after different doses. *Drug Metabolism and Disposition*, 9(3):226 LP – 232, may 1981.
- P. Ricketts and P. Harrison. Coastal and Ocean Management in Canada: Moving into the 21st Century. *Coastal Management*, 35(1):5–22, 2007. ISSN 0892-0753.
- A. D. Rijnsdorp, A. D. Vethaak, and P. I. Van Leeuwen. Population biology of dab *Limanda limanda* in the southeastern North Sea. *Marine Ecology Progress Series*, pages 19–35, 1992. ISSN 0171-8630.
- C. Ritz and J. C. Streibig. Bioassay Analysis using R. *Journal of Statistical Software*, 12(5), 2005.
- K. S. Ro, A. Venugopal, D. D. Adrian, D. Constant, K. Qaisi, K. T. Valsaraj, L. J. Thibodeaux, and D. Roy. Solubility of 2,4,6-Trinitrotoluene (TNT) in Water. *Journal of Chemical & Engineering Data*, 41(4):758–761, 1996. ISSN 0021-9568.
- C. D. Robinson, L. Webster, C. Martínez-Gómez, T. Burgeot, M. J. Gubbins, J. E. Thain, A. D. Vethaak, A. D. McIntosh, and K. Hylland. Assessment of contaminant concentrations in sediments, fish and mussels sampled from the North Atlantic and European regional seas within the ICON project. *Marine Environmental Research*, 124:21–31, 2017. ISSN 0141-1136.
- P. J. Rodacy, S. D. Reber, P. K. Walker, and J. V. Andre. *Chemical Sensing of Explosive Targets in the Bedford Basin, Halifax, Nova Scotia*. Sandia National Laboratories, 2001.

- D. Ronisz and L. Förlin. Interaction of isosafrole,  $\beta$ -naphthoflavone and other CYP1A inducers in liver of rainbow trout (*Oncorhynchus mykiss*) and eelpout (*Zoarces viviparus*). *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 121:289 – 296, 1998.
- G. Rosen and G. R. Lotufo. Bioaccumulation of explosive compounds in the marine mussel, *Mytilus galloprovincialis*. *Ecotoxicology and Environmental Safety*, 68(2): 237–245, 2007. ISSN 0147-6513.
- G. Rosen and G. R. Lotufo. Fate and Effects of Composition B in Multispecies Marine Exposures. *Environmental Toxicology and Chemistry*, 29(6):1330–1337, 2010. ISSN 07307268. doi: 10.1002/etc.153.
- G. Rosen, G. R. Lotufo, R. D. George, B. Wild, L. K. Rabalais, S. Morrison, and J. B. Belden. Field Validation of POCIS for Monitoring at Underwater Munitions Sites. *Environmental Toxicology and Chemistry*, 37(8):2257–2267, 2018. ISSN 0730-7268.
- H. K. Rossland, A. Johansen, T. E. Karsrud, M. P. Parmer, A. Larsen, A. Myran, and S. V. Nordås. Contamination From Dumped Conventional Munitions in the Aquatic Environment - Preliminary Investigation. *FFI-Report-2010/00239, Norwegian Defence Research Establishment, Kjeller, Norway*, 2010.
- W. M. S. Russell, R. L. Burch, and C. W. Hume. *The Principles of Humane Experimental Technique*. 1959.
- G. Sabbioni and R. Rumler. Biomonitoring of workers cleaning up ammunition waste sites. *Biomarkers*, 12(6):559–573, 2007. ISSN 13665804. doi: 10.1080/13547500701456206.
- G. Sabbioni, O. Sepai, H. Norppa, H. Yan, A. Hirvonen, Y. Zheng, H. Järventaus, B. Bäck, L. R. Brooks, and S. H. Warren. Comparison of biomarkers in workers exposed to 2,4,6-trinitrotoluene. *Biomarkers*, 12(1):21–37, 2007. ISSN 1354-750X.
- R. Saborowski and F. Buchholz. Annual changes in the nutritive state of North Sea dab. *Journal of Fish Biology*, 49(2):173–194, 1996. ISSN 0022-1112.
- H. Sanderson, P. Fauser, M. Thomsen, and P. B. Sørensen. Screening level fish community risk assessment of chemical warfare agents in the Baltic Sea. *Journal of Hazardous Materials*, 154(1-3):846–857, 2008. ISSN 0304-3894.

- H. Sanderson, P. Fauser, M. Rahbek, and J. B. Larsen. Review of environmental exposure concentrations of chemical warfare agent residues and associated the fish community risk following the construction and completion of the Nord Stream gas pipeline between Russia and Germany. *Journal of Hazardous Materials*, 279:518–526, 2014. ISSN 0304-3894.
- D. Schiedek, B. Sundelin, J. W. Readman, and R. W. Macdonald. Interactions between climate change and contaminants. *Marine Pollution Bulletin*, 54(12):1845–1856, 2007. ISSN 0025-326X.
- D. Schlenk, M. Celander, E. P. Gallagher, S. George, M. James, S. W. Kullman, P. van den Hurk, and K. Willett. Biotransformation in Fishes. In *The Toxicology of Fishes*, pages 153–234. CRC Press Boca Raton, 2008.
- A.-C. Schmidt, R. Herzsuh, F.-M. Matysik, and W. Engewald. Investigation of the ionisation and fragmentation behaviour of different nitroaromatic compounds occurring as polar metabolites of explosives using electrospray ionisation tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, 20(15):2293–2302, 2006. ISSN 0951-4198.
- S. Schmidt, W. Busch, R. Altenburger, and E. Küster. Mixture toxicity of water contaminants-effect analysis using the zebrafish embryo assay (*Danio rerio*). *Chemosphere*, 152:503–512, 2016. ISSN 0045-6535.
- Y. Shinkai, Y. Nishihara, M. Amamiya, T. Wakayama, S. Li, T. Kikuchi, Y. Nakai, N. Shimojo, and Y. Kumagai. NADPH-cytochrome P450 reductase-mediated denitration reaction of 2,4,6-trinitrotoluene to yield nitrite in mammals. *Free Radical Biology and Medicine*, 91:178–187, 2016. ISSN 18734596. doi: 10.1016/j.freeradbiomed.2015.09.011.
- U. Siegenthaler and J. L. Sarmiento. Atmospheric carbon dioxide and the ocean. *Nature*, 365(6442):119–125, 1993. ISSN 1476-4687.
- J. G. Sims and J. A. Steevens. The role of metabolism in the toxicity of 2,4,6-trinitrotoluene and its degradation products to the aquatic amphipod *Hyalella azteca*. *Ecotoxicology and Environmental Safety*, 70(1):38–46, 2008. ISSN 0147-6513.
- N. Singh, M. McCoy, R. Tice, and E. Schneider. A Simple Technique for Quantitation of Low Levels of DNA Damage in Individual Cells. *Experimental Cell Research*, 175(1):184–191, 1988. ISSN 00144827. doi: 10.1016/0014-4827(88)90265-0.

- R. W. Smith, P. Vlahos, J. K. Böhlke, T. Ariyaratna, M. Ballentine, C. Cooper, S. Fallis, T. J. Groshens, and C. Tobias. Tracing the Cycling and Fate of the Explosive 2,4,6-Trinitrotoluene in Coastal Marine Systems with a Stable Isotopic Tracer,  $^{15}\text{N}$ -[TNT]. *Environmental Science & Technology*, 49(20):12223–12231, 2015. ISSN 15205851. doi: 10.1021/acs.est.5b02907.
- R. J. Spanggord, K. E. Mortelmans, A. F. Griffin, and V. F. Simmon. Mutagenicity in *Salmonella typhimurium* and Structure-Activity Relationships of Wastewater Components Emanating from the Manufacture of Trinitrotoluene. *Environmental Mutagenesis*, 4(2):163–179, 1982. ISSN 0192-2521.
- J. J. Stegeman and M. E. Hahn. Biochemistry and Molecular Biology of Monooxygenases: Current Perspectives on Forms, Functions, and Regulation of Cytochrome P450 in Aquatic Species. In *Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives*, volume 87, page 206. CRC Press, Boca Raton, Fla, USA, 1994.
- U. Strähle, S. Scholz, R. Geisler, P. Greiner, H. Hollert, S. Rastegar, A. Schumacher, I. Selderslaghs, C. Weiss, and H. Witters. Zebrafish embryos as an alternative to animal experiments - A commentary on the definition of the onset of protected life stages in animal welfare regulations. *Reproductive Toxicology*, 33(2):128–132, 2012.
- K. Straumer and T. Lang. Studies on the health status of dab (*Limanda limanda*) from a dumpsite of conventional munitions in Kiel Bight, Baltic Sea. 2019. URL [https://www.researchgate.net/publication/335230806\\_Studies\\_on\\_the\\_health\\_status\\_of\\_dab\\_Limanda\\_limanda\\_from\\_a\\_dumpsite\\_of\\_conventional\\_munitions\\_in\\_Kiel\\_Bight\\_Baltic\\_Sea](https://www.researchgate.net/publication/335230806_Studies_on_the_health_status_of_dab_Limanda_limanda_from_a_dumpsite_of_conventional_munitions_in_Kiel_Bight_Baltic_Sea).
- J. S. Strehse, D. Appel, C. Geist, H.-J. Martin, and E. Maser. Biomonitoring of 2,4,6-trinitrotoluene and degradation products in the marine environment with transplanted blue mussels (*M. edulis*). *Toxicology*, 390(September):117–123, 2017. ISSN 0300483X. doi: 10.1016/j.tox.2017.09.004.
- W. Strober. Trypan Blue Exclusion Test of Cell Viability. *Current Protocols in Immunology*, 21(1):A.3B.1–A.3B.2, mar 1997. ISSN 1934-3671. doi: 10.1002/0471142735.ima03bs21.
- K. T. Suzuki, T. Tomita, Y. Ogra, and M. Ohmichi. Glutathione-conjugated Arsenics in the Potential Hepato-enteric Circulation in Rats. *Chemical Research in Toxicology*, 14(12):1604–1611, 2001. ISSN 0893-228X.



- A. Szarejko and J. Namieśnik. The Baltic Sea as a dumping site of chemical munitions and chemical warfare agents. *Chemistry and Ecology*, 25(1):13–26, feb 2009. ISSN 0275-7540. doi: 10.1080/02757540802657177.
- L. Szinicz. History of chemical and biological warfare agents. *Toxicology*, 214(3):167–181, 2005. ISSN 0300-483X.
- S. S. Talmage, D. M. Opresko, C. J. Maxwell, C. J. E. Welsh, F. M. Cretella, P. H. Reno, and F. B. Daniel. Nitroaromatic Munition Compounds: Environmental Effects and Screening Values. In *Reviews of Environmental Contamination and Toxicology*, pages 1–156. Springer, 1999.
- S. Taylor, A. Hewitt, J. Lever, C. Hayes, L. Perovich, P. Thorne, and C. Daghlian. TNT particle size distributions from detonated 155-mm howitzer rounds. *Chemosphere*, 55(3):357–367, 2004. ISSN 0045-6535.
- A. Temming. Migration and mixing of dab (*Limanda limanda*) in the Baltic. *Rapports et Procès-Verbaux des Réunions du Conseil International pour l’Exploration de la Mer*, 190:25–38, 1989.
- D. J. Thomas, M. Styblo, and S. Lin. The Cellular Metabolism and Systemic Toxicity of Arsenic. *Toxicology and Applied Pharmacology*, 176(2):127–144, 2001. ISSN 0041-008X.
- D. J. Thomas, S. B. Waters, and M. Styblo. Elucidating the pathway for arsenic methylation. *Toxicology and Applied Pharmacology*, 198(3):319–326, 2004. ISSN 0041-008X. doi: 10.1016/j.taap.2003.10.020.
- V. Tornero and G. Hanke. Chemical contaminants entering the marine environment from sea-based sources: A review with a focus on European seas. *Marine Pollution Bulletin*, 112(1-2):17–38, 2016. ISSN 0025-326X. doi: 10.1016/j.marpolbul.2016.06.091.
- J. A. Tørnes, A. M. Opstad, and B. A. Johnsen. Determination of organoarsenic warfare agents in sediment samples from Skagerrak by gas chromatography-mass spectrometry. *Science of the Total Environment*, 356(1-3):235–246, 2006. ISSN 00489697. doi: 10.1016/j.scitotenv.2005.03.031.
- U. University of Hawaii. Final Follow-Up Investigation Assessment Report, Ordnance Reef (HI-06), Wai’anae, O’ahu, Hawaii. Technical report, Johnstown, PA, 2014.
- A. f. T. S. U.S. Department of Health and Human Services and D. Registry. Toxicological Profile for 2,4,6-Trinitrotoluene, 1995.

- US Environmental Protection Agency. Method 8330A: Nitroaromatics and nitramines by high performance liquid chromatography (HPLC), 2007.
- R. Valskienė, J. Baršienė, L. Butrimavičienė, W. Grygiel, V. Stunžėnas, K. Jokšas, and M. Stankevičiūtė. Environmental genotoxicity and cytotoxicity levels in herring (*Clupea harengus*), flounder (*Platichthys flesus*) and cod (*Gadus morhua*) inhabiting the Gdansk Basin of the Baltic Sea. *Marine Pollution Bulletin*, 133:65–76, 2018. ISSN 0025-326X.
- P. J. van Bladeren. Formation of toxic metabolites from drugs and other xenobiotics by glutathione conjugation. *Trends in Pharmacological Sciences*, 9(8):295–299, 1988. ISSN 0165-6147.
- A. D. Vethaak and T. Ap Rheinallt. Fish disease as a monitor for marine pollution: the case of the North Sea. *Reviews in Fish Biology and Fisheries*, 2(1):1–32, 1992. ISSN 0960-3166.
- Ø. A. Voie and E. Mariussen. Risk Assessment of Sea Dumped Conventional Munitions. *Propellants, Explosives, Pyrotechnics*, 42(1):98–105, 2017. ISSN 15214087. doi: 10.1002/prop.201600163.
- P. J. Vuorinen, M. Keinänen, H. Vuontisjärvi, J. Baršienė, K. Broeg, L. Förlin, J. Gercken, J. Kopecka, A. Köhler, and J. Parkkonen. Use of biliary PAH metabolites as a biomarker of pollution in fish from the Baltic Sea. *Marine Pollution Bulletin*, 53(8-9):479–487, 2006. ISSN 0025-326X.
- W. J. Waddell. Comparisons of thresholds for carcinogenicity on linear and logarithmic dosage scales. *Human & Experimental Toxicology*, 24(6):325–332, 2005. ISSN 0960-3271.
- C. H. Walker and D. R. Livingstone. *Persistent Pollutants in Marine Ecosystems*. Elsevier, 2013. ISBN 148328767X.
- T. Watanabe and S. Hirano. Metabolism of arsenic and its toxicological relevance. *Archives of Toxicology*, 87(6):969–979, 2013. ISSN 03405761. doi: 10.1007/s00204-012-0904-5.
- L. Webster, M. Russell, P. Walsham, L. A. Phillips, I. Hussy, G. Packer, E. J. Dalgarno, and C. F. Moffat. An assessment of persistent organic pollutants in Scottish coastal and offshore marine environments. *Journal of Environmental Monitoring*, 13(5):1288–1307, 2011.

- M. Wei, T. Yamada, S. Yamano, M. Kato, A. Kakehashi, M. Fujioka, Y. Tago, M. Kitano, and H. Wanibuchi. Diphenylarsinic acid, a chemical warfare-related neurotoxicant, promotes liver carcinogenesis via activation of aryl hydrocarbon receptor signaling and consequent induction of oxidative DNA damage in rats. *Toxicology and Applied Pharmacology*, 273(1):1–9, 2013. ISSN 0041008X. doi: 10.1016/j.taap.2013.08.022.
- H. v. Westernhagen, G. Krüner, and K. Broeg. Ethoxyresorufin O-deethylase (EROD) activity in the liver of dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.) from the German Bight. EROD expression and tissue contamination. *Helgoland Marine Research*, 53(3):244–249, 1999. ISSN 1438-387X. doi: 10.1007/s101520050027.
- J. R. Wheeler, K. M. Y. Leung, D. Morritt, N. Sorokin, H. Rogers, R. Toy, M. Holt, P. Whitehouse, and M. Crane. Freshwater to saltwater toxicity extrapolation using species sensitivity distributions. *Environmental Toxicology and Chemistry*, 21(11): 2459–2467, 2002. ISSN 0730-7268.
- C. Woodley and C. Downs. Ecological Risk Assessment of Munitions Compounds on Coral and Coral Reef Health. Technical report, 2014.
- X. Xu, M. Koeberg, C.-J. Kuijpers, and E. Kok. Development and validation of highly selective screening and confirmatory methods for the qualitative forensic analysis of organic explosive compounds with high performance liquid chromatography coupled with (photodiode array and) LTQ ion trap/Orbitrap mass spectrometric detections (HPLC-(PDA)-LTQOrbitrap). *Science & Justice*, 54(1):3–21, 2014. ISSN 1355-0306.
- C. Yan, Y. Wang, B. Xia, L. Li, Y. Zhang, and Y. Liu. The retrospective survey of malignant tumor in weapon workers exposed to 2,4,6-trinitrotoluene. *Chinese Journal of Industrial Hygiene and Occupational Diseases*, 20(3):184–188, 2002. ISSN 1001-9391.
- L. J. Yoo, G. R. Lotufo, and A. B. Gibson. Toxicity and bioaccumulation of 2,4,6 trinitrotoluene in fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry*, 25(12):3253–3260, 2006.
- R. A. Zakharyan and H. V. Aposhian. Enzymatic Reduction of Arsenic Compounds in Mammalian Systems: The Rate-Limiting Enzyme of Rabbit Liver Arsenic Biotransformation is MMA<sup>V</sup> Reductase. *Chemical Research in Toxicology*, 12(12):1278–1283, 1999. ISSN 0893-228X.
- S. Zbaida. Nitroreductases and Azoreductases. In *Enzyme Systems that Metabolise Drugs and Other Xenobiotics*, volume 4, pages 555–566. 2002. ISBN 0471894664.



## A. Appendix A - Genotoxicity of nitroaromatic compounds

**Table A.1.:** Nominal and mean measured concentrations of nitroaromatic compounds for testing acute toxicity to zebrafish embryos. Mean concentrations were measured in freshly prepared test solutions of three individual tests by HPLC-MS; standard deviation is given in parentheses.

Nominal concentration (mg/L)	Mean measured concentration (mg/L)
<b>TNT exposure</b>	
0.5	0.49 (0.09)
0.82	0.76 (0.17)
1.36	1.39 (0.23)
2.24	2.14 (0.44)
3.68	3.47 (0.49)
6.07	5.58 (0.4)
10	9.43 (0.96)
<b>2-ADNT exposure</b>	
3.5	3.49 (0.42)
4.86	4.59 (0.59)
6.74	6.28 (0.27)
9.35	8.65 (1.1)
12.98	9.79 (0.96)
18.02	13.49 (2.58)
25	20.46 (2.47)
<b>4-ADNT exposure</b>	
3.5	4.1 (0.21)
4.86	5.41 (0.39)
6.74	6.93 (0.39)
9.35	10.36 (0.58)
12.98	12.82 (0.1)
18.02	18.27 (0.89)
25	23.87 (4.03)

**Table A.2.:** Nominal and mean measured concentrations of nitroaromatic compounds used in the comet assay with zebrafish embryos. Mean concentrations were measured in freshly prepared test solutions of three individual tests by HPLC-MS; standard deviation is given in parentheses.

Nominal concentration (mg/L)	Mean measured concentration (mg/L)
<b>TNT exposure, comet assay</b>	
0.1	0.12 (0.03)
0.27	0.24 (0.04)
0.73	0.68 (0.15)
2	1.56 (0.47)
<b>2-ADNT exposure, comet assay</b>	
1	1.34 (0.12)
2.29	2.8 (0.09)
5.24	6.08 (0.33)
12	12.39 (0.37)
<b>4-ADNT exposure, comet assay</b>	
1	1.41 (0.08)
2.29	3.05 (0.13)
5.24	6.98 (0.34)
12	13.05 (0.33)

## B. Appendix B - *In vitro* metabolism of TNT

**Table B.1.:** EROD and MROD activity (pmol/min/mg) measured in dab liver samples after exposure to different concentrations of TNT (0-15 mg/L).

Conc. TNT (mg/L)	Liver 2		Liver 4		Liver 6		Liver 7		Liver 8		Liver 10		Liver 11		Liver 12	
	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD
0	73.6	20.3	42.8	17.5	29.1	7.4	35.5	14.7	66.6	18.4	14.7	6.3	17.8	8.2	53.6	17.8
0.15	70.8	19.6	41.1	16.0	29.5	6.8	29.9	13.0	67.1	17.9	12.2	6.6	16.9	7.4	49.9	15.7
0.3	68.9	19.4	39.3	14.8	28.7	8.2	30.1	11.3	63.7	17.4	12.2	7.0	16.7	7.1	45.5	14.8
0.6	72.3	22.2	34.4	13.7	29.5	7.2	28.9	10.9	64.8	18.7	11.8	5.9	16.4	7.7	41.5	15.2
2	59.3	17.3	33.6	12.0	26.6	6.6	27.2	9.9	58.8	17.4	11.8	6.6	14.2	6.3	41.9	13.8
7	47.6	12.7	29.7	9.8	18.4	4.5	21.9	6.6	39.6	10.8	10.0	4.4	12.3	4.4	34.0	11.0
15	33.1	7.6	26.5	7.3	12.7	2.5	15.9	5.2	28.9	7.6	7.4	2.6	7.9	1.9	23.4	5.8

**Table B.2.:** EROD and MROD activity (pmol/min/mg) measured in flounder liver samples after exposure to different concentrations of TNT (0-15 mg/L).

Conc. TNT (mg/L)	Liver 21		Liver 22		Liver 23		Liver 24		Liver 25		Liver 29		Liver 31		Liver 32		Liver 32	
	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD
0	35.3	7.3	8.0	1.1	19.5	3.0	15.4	2.4	14.6	2.5	7.6	1.2	10.4	1.8	38.8	7.5		
0.15	34.4	7.4	7.1	1.1	15.7	2.6	14.6	2.6	12.9	2.2	7.3	1.2	9.7	1.3	33.2	6.8		
0.3	34.0	8.0	6.9	1.1	16.9	2.6	13.4	1.9	11.8	2.0	6.9	0.8	8.5	1.0	32.7	7.3		
0.6	34.1	6.8	6.6	1.1	17.1	2.6	13.4	2.1	10.7	1.9	6.9	0.8	7.7	0.8	26.2	5.1		
2	27.0	5.6	5.7	0.7	14.6	2.8	12.2	2.1	8.9	1.5	6.2	0.5	7.1	0.6	25.1	4.8		
7	21.4	4.9	4.1	0.4	10.6	1.6	7.9	1.2	7.3	1.2	4.0	0.3	4.6	0.4	21.0	4.5		
15	13.3	2.8	2.0	0.2	7.2	0.6	3.9	0.3	5.4	0.4	1.9	0.2	2.8	0	13.3	2.1		

**Table B.3.:** EROD and MROD activity (pmol/min/mg) measured in plaice liver samples after exposure to different concentrations of TNT (0-15 mg/L).

Conc. TNT (mg/L)	Liver 41		Liver 43		Liver 45		Liver 46		Liver 49		Liver 50		Liver 52		Liver 54		Liver 54	
	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD	EROD	MROD
0	25.6	4.6	50.6	10.3	21.5	4.0	32.8	6.4	11.5	22.3	29.5	6.3	17.7	2.9	40.1	8.8		
0.15	24.8	4.0	43.6	10.2	20.5	4.0	31.5	6.0	11.3	18.4	30.9	6.6	15.8	2.9	33.2	8.2		
0.3	23.1	4.2	45.1	10.3	22.7	3.7	30.6	6.2	10.9	17.6	26.7	5.7	14.3	2.4	29.5	7.9		
0.6	21.2	4.2	48.5	10.5	23.6	3.9	28.2	5.6	9.8	15.7	24.1	5.4	13.3	2.4	29.6	7.9		
2	18.1	4.6	44.1	8.6	21.1	3.4	4.4	88.6	14.1	22.9	6.2	14.0	2.5	24.9	6.9			
7	15.0	2.5	33.6	6.6	17.4	2.5	4.1	67.9	12.0	15.8	3.7	11.0	1.2	17.4	4.6			
15	10.4	1.5	22.1	4.0	9.9	1.7	12.8	3.1	47.3	8.3	11.6	2.9	6.9	0.7	11.8	3.7		



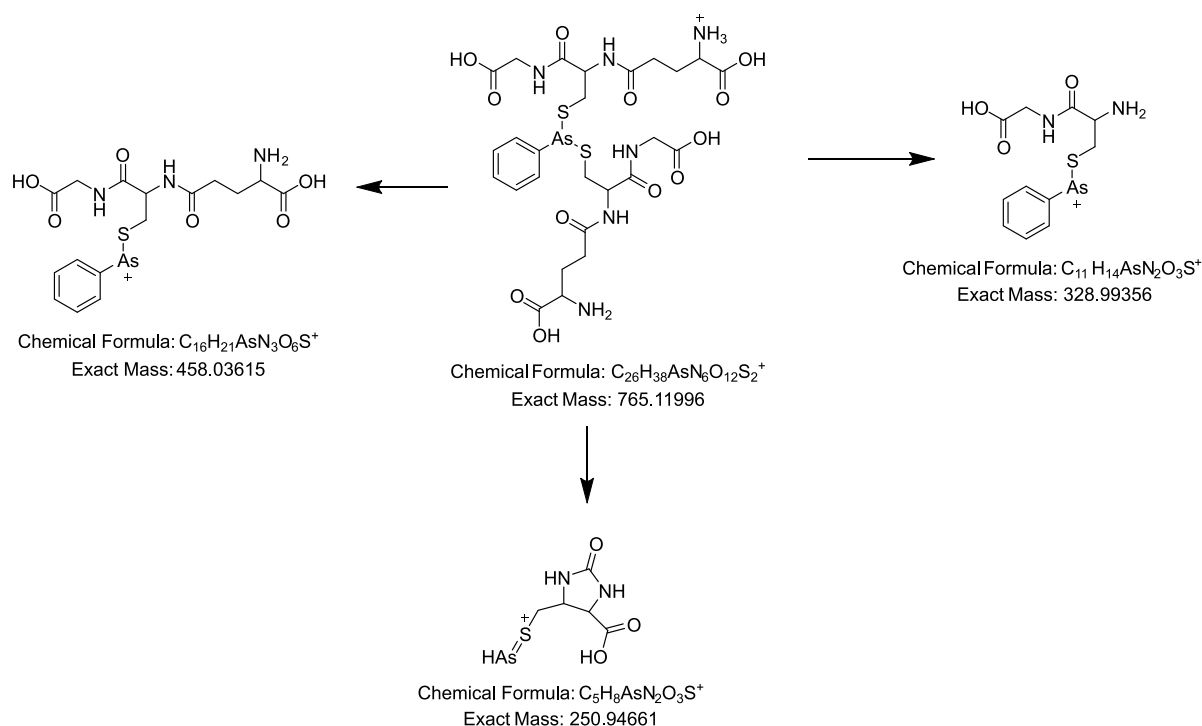
## C. Appendix C - *In vitro* metabolism of phenylarsenic chemicals

**Table C.1.:** HCD energies [%] for different metabolites detected in *in vitro* samples.

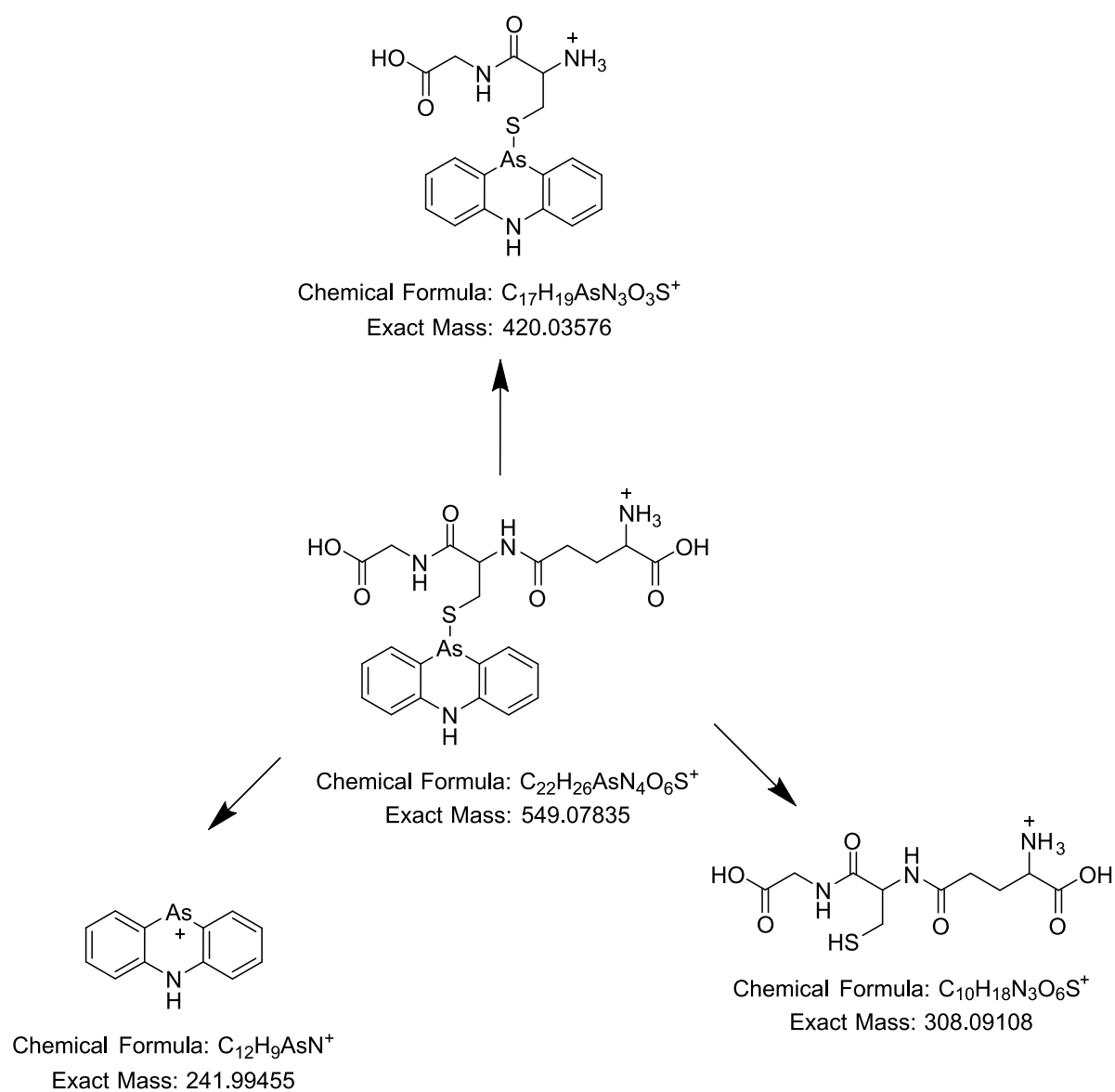
Metabolites	HCD [%]
PA-GSH <sub>2</sub>	10
DM-GSH	3
DPA-GSH	18
MPAA	50
10-M-5H-PA-10-O	30
MDPAO	50
PDCA[ox]-OH	40
DM[ox]-OH	35
DPA[ox]-OH	40
TPA[ox]-OH	60

**Table C.2.:**  $^1\text{H}$  NMR shifts and exact masses for synthesized reference chemicals.

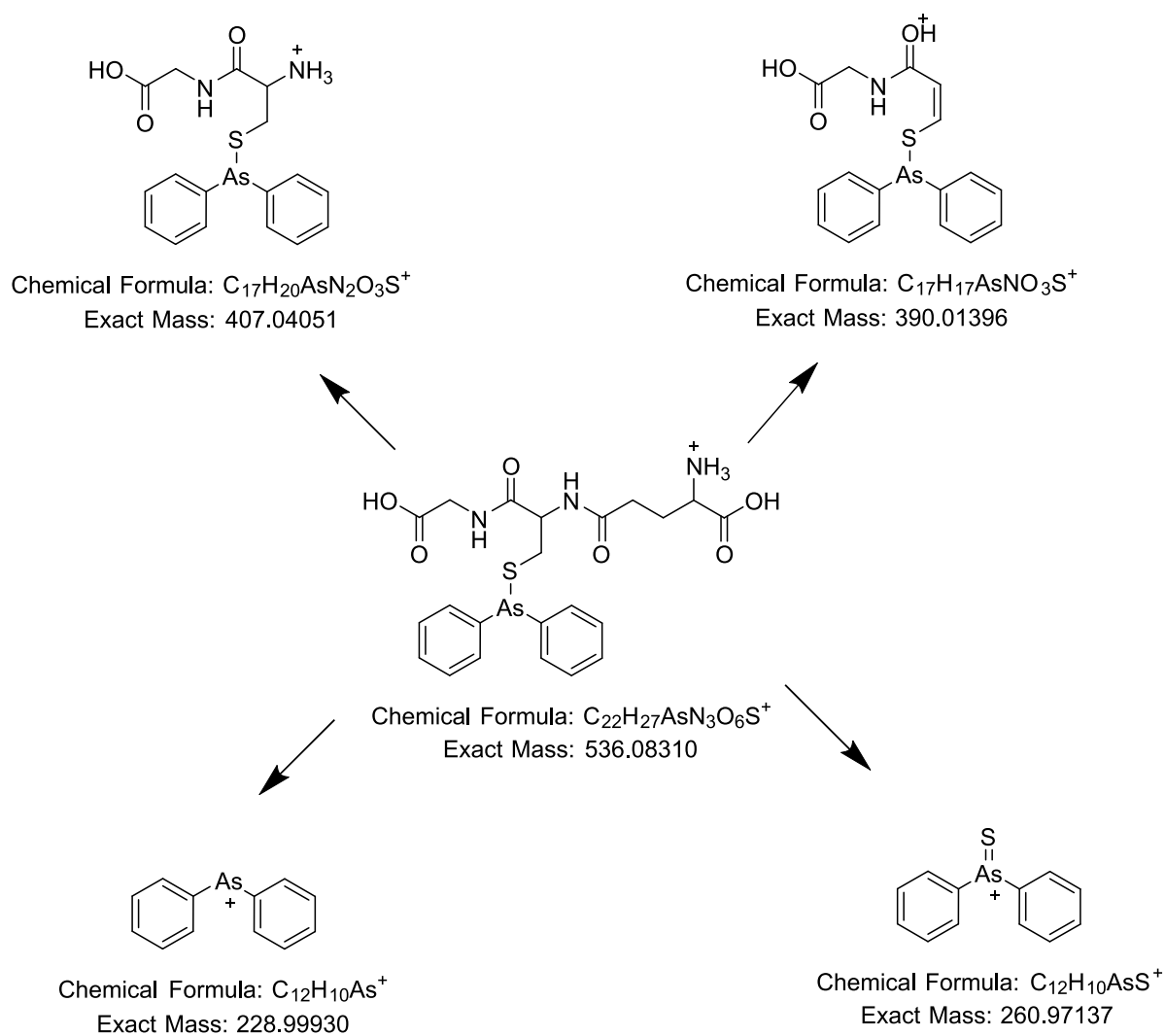
Compound	(Cys $\alpha$ )	(Cys $\beta'$ )	(Cys $\beta$ )	$[\text{M}+\text{H}]^+ m/z$
$\delta$ [ppm]				
PA-SG <sub>2</sub>	4.67, m, $^2\text{H}$	3.25, m, $^2\text{H}$	3.11, m, $^2\text{H}$	765.119 79
DM-SG	4.35, m, $^1\text{H}$	3.29, dd, (Cys $\beta'$ , $J_{\text{vic}}=5$ Hz, $J_{\text{gem}}=14$ Hz, $^1\text{H}$ )	3.11, dd, (Cys $\beta$ , $J_{\text{vic}}=9$ Hz, $J_{\text{gem}}=14$ Hz, $^1\text{H}$ )	549.07861
DPA-SG	4.62, m, $^1\text{H}$	3.29, dd, (Cys $\beta'$ , $J_{\text{vic}}=5$ Hz, $^1\text{H}$ )	3.11, dd, (Cys $\beta$ , $J_{\text{vic}}=9$ Hz, $J_{\text{gem}}=14$ Hz, $^1\text{H}$ )	536.00833



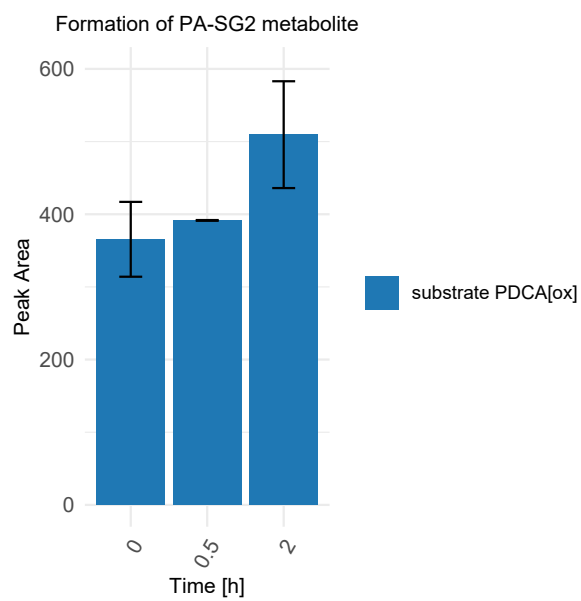
**Figure C.1.:** Proposed fragmentation pathway for PA-SG<sub>2</sub> metabolite.



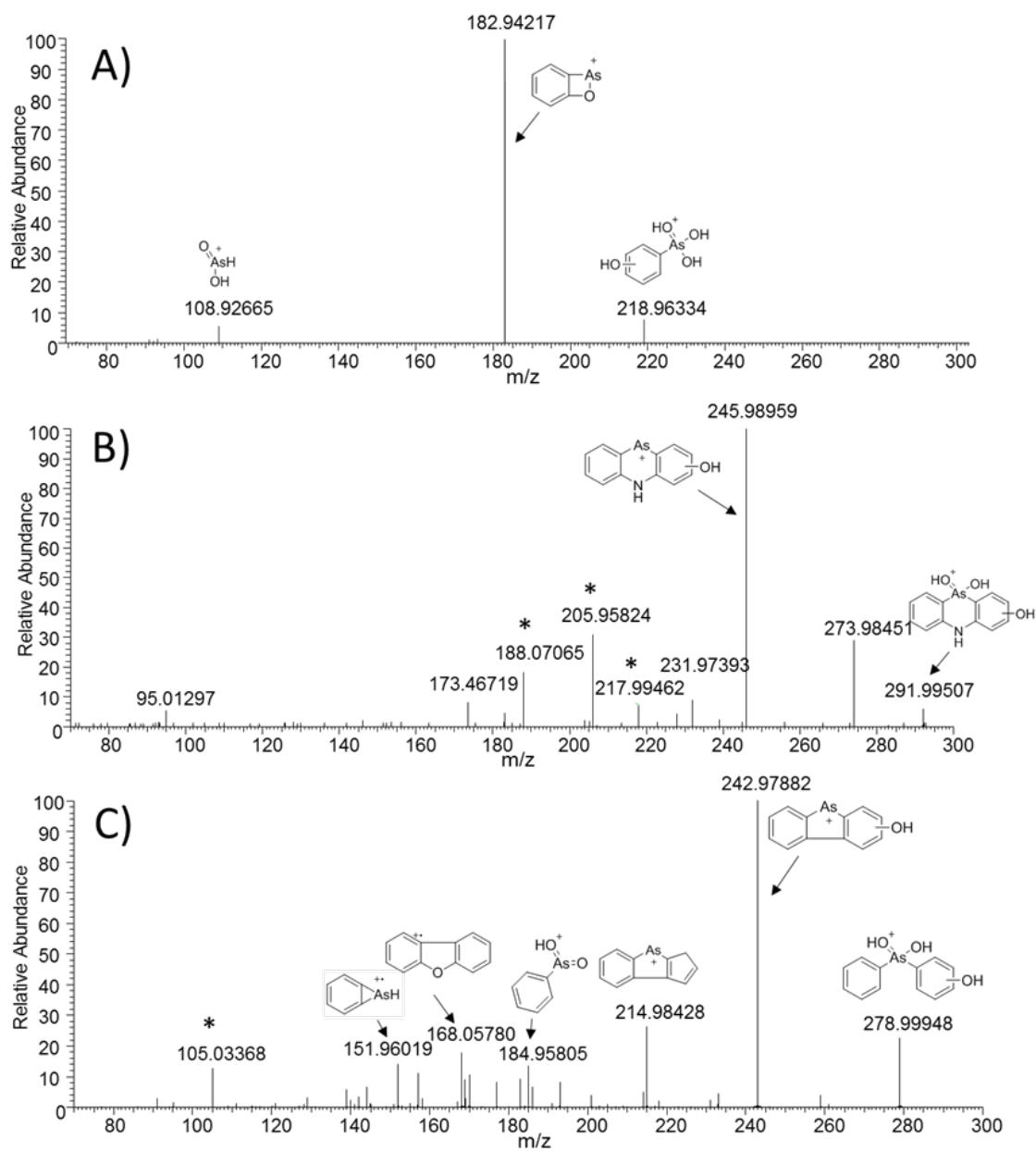
**Figure C.2.:** Proposed fragmentation pathway for DM-SG metabolite.



**Figure C.3.:** Proposed fragmentation pathway for DPA-SG metabolite.



**Figure C.4.:** Formation of PA-SG<sub>2</sub> in *in vitro* samples. Bars represent the mean value of the peak areas from two individual measurements. The error bars represent the lower and upper limits.



**Figure C.5.:** MS/HRMS spectra for hydroxylated metabolites PDCA[ox]-OH (A), DM[ox]-OH (B), and DPA[ox]-OH (C) formed in *in vitro* samples. \* Peaks are not identified (might be generated from the background).

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## **Eidesstattliche Erklärung**

Hiermit erkläre ich, dass die vorliegende Dissertation, abgesehen von der Beratung durch meinen Betreuer Prof. Dr. Reinhold Hanel, nach Inhalt und Form eine eigenständige Arbeit ist und nur mit den angegebenen Hilfsmitteln verfasst wurde. Die Dissertation ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft (DFG) entstanden. Diese Arbeit wurde weder ganz noch zum Teil an anderer Stelle im Rahmen eines Prüfungsverfahrens vorgelegt, veröffentlicht oder zur Veröffentlichung eingereicht. Mir wurde nie ein akademischer Grad entzogen.

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Kiel, 27. Juli 2020