DAIMON Work Package 4: Management strategies for marine munitions

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Activity 4.4: Safety risk for humans

Lead: Thünen Institute of Fisheries Ecology (TI-FI)

Description of finalised (main) output

1 Toxicity thresholds for seafood

In the framework of the DAIMON project, comprehensive chemical method development and subsequent analyses were carried out on hazardous substances originating from dumped munitions in wild marine organisms, mainly fish. These largely focused on key compounds, i.e. chemical warfare agent (CWA) related phenylarsenic chemicals and on trinitrotoluene (TNT) as well as their main degradation products.

For analyses on CWAs, the key target species was Atlantic cod (*Gadus morhua*) from dumpsites and reference areas in the Baltic Sea (Niemikoski et al. in prep.). Additional species analysed in a Skagerrak CWA dumpsite located in the Måseskär deep were saithe (*Pollachius virens*), witch flounder (*Glyptocephalus cynoglossus*), Norway pout (*Trisopterus esmarkii*) and a crustacean, Norway lobster (*Nephrops norvegicus*) (Niemikoski et al. 2017). Chemical analyses by means of a liquid chromatography-heated electrospray ionization/tandem mass spectrometry (LC-HESI/MS/MS) focused on phenylarsenic compounds from marine biota samples. The target chemicals were oxidation products of Adamsite (DM[ox]), Clark I (DPA[ox]), and triphenylarsine (TPA[ox]). From the Måseskär area, a total of ten fish samples and one lobster sample were analyzed.

For the first time, the DAIMON project provided evidence of the presence of CWA-related chemicals in marine biota samples. Trace concentrations of arsenic-containing CWAs were detected in three of ten fish and one lobster samples during the DAIMON project and the elemental composition of oxidized form of Clark I and/or II was confirmed by LC-HESI/HRMS. However, the concentrations detected were low (below the limit of quantification, LOQ). Similar results were obtained in a study on phenylarsenic compounds in the bottom-dwelling species Atlantic hagfish (*Myxine glutinosa*) from the Arendal dumpsite in the Skagerrak. In this case, the majority of samples were positive (19 out of 20), indicating a significant uptake of CWA-related compounds in this species (Niemikoski et al. in prep.). The same phenylarsenic substances found in the Skagerrak samples were also detected in 13 % of cod collected in the Bornholm CWA dumpsite (fillets were analysed), while only 3 % of cod from a reference area was positive (Niemikoski et al. in prep.).

The DAIMON project also focused on chemical analysis of explosives (i.e. TNT) and their degradation products in fish. Studies concentrated on the flatfish species dab (*Limanda limanda*) collected in a dumpsite for conventional munitions located in the western Baltic Sea at Kolberger Heide, Kiel Bight. A method was developed for analyzing bile samples which is the key matrix for substances with a high metabolic rate (Goldenstein et al. in prep.).

The results provided clear evidence that fish from the dumpsite are exposed to explosives and that traces of TNT and TNT-related compounds, such as 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), trinitrobenzoic acid (TNBA), dinitrobenzoic acid (DNBA), 2,4-dinitrotoluene (2,4-DNT), 2,5-dinitrotoluene (2,5-DNT) and 2,2,6,6-tetranitro-4,4-azoxytoluene (TNAzoxyT) can be found in bile samples from dab. Additionally, the explosives 1,3,5-trinitro-1,3,5-triazinane (RDX) and 1,3,5,7-tetranitro-1,3,5,7-tetrazoctane (HMX) were found in few bile samples from the Kolberger Heide dumpsite. A total of 90 out of 115 bile samples from dab caught in the Kolberger Heide contained explosives (TNT, RDX, HMX) or TNT-related compounds. In a neighboring reference site free of munitions (approx. 10 nm away from the dumpsite), only 3 out of 114 bile samples analysed contained explosive compounds. Interestingly, only 4 out of 229 bile samples contained TNT itself, and the degradation products accounted for the vast majority of the explosive compounds detected. This implies either that TNT taken up from the environment is metabolized by fish or that the fish directly take up the metabolites and not the TNT itself (Goldenstein et al. in prep.). Recent analytical measurements of seawater samples from Kolberger Heide prove that TNT and 1,3-dinitrobenzene (DNB) are the most abundant explosive compounds, but also known TNT degradation products like 2-ADNT and 4-ADNT were found in the water (Gledhill et al. 2019).

Interestingly, in dab from the dumpsite (the same fish that were used for chemical analysis), an elevated prevalence of liver tumours was detected in DAIMON (Straumer et al., in prep.) and it, thus, cannot be excluded that a link between the high tumour rate and the uptake of TNT and its metabolites exist.

In order to investigate the uptake of explosive compounds by fish directly exposed to the dumped munition, caging experiments within the Kolberger Heide area were carried out as a case study. For this purpose, dab from the same munitions-free reference site used to study the uptake in wild fish were exposed for three weeks in cages in close vicinity of the dumped munition. At the end of the experiment, all of the 11 cage fish contained explosive compounds in much higher concentrations than the investigated wild fish from the Kolberger Heide. 2-ADNT and 4-ADNT were found in the ng/l range, whereas these compounds were one to two orders of magnitude lower in wild fish from the dumpsite. The highest TNT concentration in all measured bile samples (0.017ng/l) was also found in a fish from the caging experiments. These results indicate that direct exposure of fish to the munition compounds is measurable and that varying levels of exposure are also detectable in the concentration of explosive compounds in bile. Another case study in which blue mussels (*M. edulis*) in the Kolberger Heide were exposed to the dumped munition proved that other marine biota also accumulate explosive compound in their tissue (UDEMM project, Strehse et al. 2017). After 3 month of exposure the mussels contained high concentrations of ADNTs (> 100 ng/g wet weight) and TNT itself was found in six mussels with a mean concentration of 31 ng/g.

Within DAIMON, a range of toxicity experiments was carried out, addressing both the toxicity of CWA and TNT and its metabolites. Toxicity of CWA was analysed by using an established *Daphnia* sp.

toxicity test (OECD Test No. 202: *Daphnia* sp. acute immobilisation test). Effects of six phenylarsenic CWAs parent compounds and four degradation products were tested. Despite their low solubility, all CWA parent compounds induced negative effects on *Daphnia magna*, while highly soluble CWA degradation products showed no effects at all. Three compounds turned out to be very toxic, including phenyldichloroarsine and Lewisite (Czub et al. in prep). For TNT and its main metabolites (2-ADNT, 4-ADNT) toxicity test with zebrafish embryos (*Danio rerio*) were carried out (Koske et al. in prep). After 120 hours exposure time, TNT revealed a higher toxicity (LC50: 4.5 mg/l) than its degradation products 2-ADNT (LC50: 13.4 mg/l) and 4-ADNT (LC50: 14.4 mg/l). A striking sublethal endpoint during the experiment was the relatively high proportion of chorda deformations during embryogenesis. In order to investigate the genotoxicity of nitroaromatic compounds, primary cells of zebrafish embryos exposed to 2-ADNT, 4-ADNT and TNT for 48 h were studied in the alkaline comet assay. The lowest concentrations tested for ADNTs (1 mg/l) and TNT (0.1 mg/l) revealed significantly higher genotoxicity compared to the corresponding controls, suggesting that exposure of fish to explosives is associated with a genotoxic threat and possible ultimate implications such as carcinogenic effects. Exposure experiments with blue mussels and dissolved TNT showed clear behavioral changes of mussels. Animals exposed to higher concentrations (>2.5 mg/L) close shells and isolate themselves from toxic environments. Mussels take up the dissolved TNT in relation to the exposure concentration (>2 mg/g 4-ADNT). Tissue analysis revealed that also mussels quickly metabolise the TNT, since in most individuals only 2-ADNT and 4-ADNT could be detected. First negative health effects (accumulation of lipofuscin and neutral lipids) were detected at concentrations between 0.65 and 1.25 mg/L (Schuster et al. in prep.).

Exposure of laboratory raised marine flatworms (*Macrostomum lignano*) revealed that feeding was reduced in concentrations of 3,3 mg/L 2ADNT and 4ADNT, and the ABC transporter activity (a cellular first line defense) was increased in concentrations higher then 330µg/L (Bickmeyer et al in prep).

Summary: Based on the DAIMON results of the comprehensive and methodologically challenging chemical analyses of compounds originating from dumped chemical and conventional munitions in biota and from the toxicity experiments carried out on effects of these substances, it can be stated that there is now clear evidence that

a) wild organisms, e.g. a number of commercially relevant fish species in munitions dumpsites are exposed,

b) CWA and explosives and their degradation products, resp., are taken up and can be detected and partly quantified, e.g. in the edible part,

c) these substances are partly toxic as shown in various toxicity experiments and as suggested by health studies of wild fish.

However, it has to be emphasized that concentrations found in wild fish were low; concentrations of CWA related phenylarsenic compounds in marine biota were below the limit of quantification (1.3 ng/g – 2.1 ng/g in fish and lobster tissue) (Niemikoski et al. 2017); concentrations of detected explosive compounds in bile from dab were within the ng/l range and partly below the limit of quantification (Goldenstein et al. in prep.).

During the DAIMON project only for the arsenic containing CWA analytic methods were developed and therefore only these compounds could be detected/analysed in biota. However, at all dumping sides also other CWAs were dumped which were not considered for method development during DAIMON. Further, analysis focused on oxidation products of arsenic CWA compounds, others degradation products and/or metabolites were not known at the stage of investigation. Thus, given concentration in measured biota are conservative measures rather underestimating the total concentrations of CWA related compounds in biota.

Toxic effects in the experiments were recorded at much higher concentrations, but cover only relatively short exposure scenarios and do not clarify the possible effects of chronic exposure to CWA-related compounds and explosive compounds.

With regard to seafood safety for consumers, there is currently no reason to assume that the concentrations found during DAIMON in marine biota is a reason of concern. However, it is clear that more studies are needed to provide more conclusive data that may lead to the establishment of threshold values for human consumption. These studies can now be done in a systematic manner because DAIMON succeeded to develop appropriate analytical methods and tool that can be applied by veterinary institutes. However, investments into infrastructure and education will be required to enable such institutions to carry out the sophisticated analytical work.

2 List of symptoms in fish, which should be checked for CWA-related contaminants

In the framework of the DAIMON project (and its predecessor projects CHEMSEA, MODUM and DAIMON), the health status of wild marine fish species collected at dumpsites of chemical munitions/warfare agents (Atlantic cod, *Gadus morhua*) and at a dumpsite of conventional munitions (common dab, *Limanda limanda*) in the Baltic Sea was studied and compared to fish from reference sites considered to be free of dumped munitions. The results revealed the presence of a range of well-known externally and internally visible infectious and non-infectious diseases/ patho-morphological changes as well as parasites in fish from the dumpsites and the reference areas.

Besides providing information on diseases/pathomorphological changes recorded, the questions were addressed (a) if the diseases and their symptoms identified can be used during routine veterinarian fish quality inspection as indicators of exposure to toxic compounds leaking from dumped munitions and (b) if such findings would warrant a subsequent targeted chemical analysis of toxic munitions compounds in fish in order to minimize potential consumer risks for food safety.

The following **conclusions** were drawn:

- Diseases/pathomorphological changes and parasites recorded in cod and dab from the munitions dumpsites and reference areas of the in Baltic Sea are well-known and are not regarded as specific indicators of exposure to or effects of munitions compounds.
- None of the externally visible diseases/pathomorphological or parasites changes occurred at a statistically significant elevated prevalence in the dumpsites compared to the reference sites. However, high levels of liver tumors were observed in dab from the dumpsite for conventional munitions in Kiel Bight.

- The approach to screen fish during official controls of competent authorities and own checks of food business operators at landing sites for certain disease conditions and to initiate subsequent chemical analysis of munitions compounds in fish with symptoms is not considered as feasible because of the non-specificity of the diseases/pathomorphological changes identified and practical constraints associated with standard official controls of competent authorities and own checks of food business operators.

A full report is available: DAIMON Output 4.4.3: List of disease symptoms in fish, which should be checked for contaminants related to dumped chemical or conventional munitions (authors Lang T., Straumer K., Neuhaus H., Heemken O.)

3 Cost-effective and fast analytical methods for veterinarian purposes

Analytical methods that can be used for veterinarian purposes have been developed in DAIMON and are detailed in the DAIMON Ecotox Toolbox and associated Fact Sheets (see output of DAIMON Activity 2.5 Assessment of munitions' impact on biota). These include methods for chemical analysis of CWA and explosives as well as methods to analyse biological effects of chemical munitions compounds.

4 Review of state-of-art literature about carcinogenic and other long-term effects of warfare agents on humans as seafood consumers

Summary: The results of the review (a full report is available, authors Bickmeyer et al.) reveal that published literature confirms that TNT is toxic to humans and ecosystems. Since the leakage rate in water and solubility are relatively low and since TNT is quickly metabolized by marine organisms, the exposure toxicity in comparison to industrial exposure in former times and nowadays (e.g. in munitions-producing factories) is low.

Information on the toxicity of TNT metabolites and their stability in marine organisms is clearly limited. However, some information on the main metabolites, the derivatives 2-ADNT and 4-ADNT has been published. New studies with blue mussels (*Mytilus* sp.) has demonstrated that TNT derivatives (mainly 4-ADNT) accumulate in tissues in areas with dumped munitions. These data give some insight into bioaccumulation in exposed organisms.

There is indication from DAIMON toxicity studies and from the literature that TNT and its derivatives are genotoxic to exposed organisms, implying a potential risk of exposure in human consumers, since for genotoxicity, no safety levels (thresholds) can be given so far. Since genotoxicity and toxicity of derivates of warfare agents have not been exhaustively investigated so far, no risk assessment can be done. This is true for both compound groups CWA and explosives.

Heavy metals originating from sea-dumped munitions and, amongst these, especially the methylated forms of mercury (MeHg) are very dangerous compounds as they bioaccumulate in the foodweb with humans as end consumers in concentrations up to hundreds of pg/g d.w.

In **conclusion**, there is currently no sufficient body of literature concerning long-term effects of warfare agents on humans as seafood consumers, except for MeHg, which is found together with explosives. Since the review focused on sea-dumped munitions in the Baltic, new forms of warfare agents such as biological, chemical and other types of explosives than TNT have not been considered.

Durability of the main output/investment

The output of DAIMON A4.4 is of interest to food safety authorities on the regional/national and the international level (e.g. EU). Although DAIMON provided groundbreaking new findings on the contamination of marine organisms including human food resources with hazardous substances originating form dumped chemical and conventional munitions, the data generated are not sufficient to propose or even establish threshold values for human consumption. More studies are therefore required, for which the basis was laid in the DAIMON project through providing first data and methodological guidelines.

References

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