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Tracking explosive contaminants from dumped munition in the western Baltic Sea via urine and bile analysis of three flatfish species

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Abstract

Background Dumped munitions in German coastal waters, particularly the explosive 2,4,6-trinitrotoluene (TNT), may pose significant environmental risks. TNT and its metabolites, such as 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT), contaminate marine organisms, including fish. These TNT metabolites bioaccumulate in fish tissues, serving as markers for environmental monitoring. Traditionally, fish bile has been a primary matrix to investigate TNT exposure; however, the present study is the first to explore the use of fish urine to detect TNT metabolites. Three flatfish species, common dab (*Limanda limanda*), European plaice (*Pleuronectes platessa*), and European flounder (*Platichthys flesus*) from three regions with munition dumping sites in the Western Baltic Sea were investigated.

Results Differences in the levels of contamination at the dumping sites are reflected in the concentrations of explosives found in the urine and bile. Fish from the Kolberger Heide dumping site in Kiel Bight exhibited the highest concentrations of explosive contaminants. In individual dab, contamination with 2-ADNT was recorded up to 26.356 ng/ml in bile and 36.120 ng/ml in urine. Concentrations of 4-ADNT ranged up to 95.908 ng/ml in bile and 26.877 ng/ml in urine. The patterns of TNT metabolites in urine and bile varied, and the concentrations of these metabolites in urine and bile did not always correspond in individual fish. However, the different mean contamination levels in the three regions were reflected in both: urine and bile. Contamination levels of explosives in the three regions decreased in the order Kiel > Schlei > Lübeck.

Conclusions TNT metabolites were detected in fish urine for the first time. Urine and bile can serve as useful matrices to assess environmental exposure of fish to TNT. Additionally, dab, plaice and flounder can be utilized in studies focusing on the analysis of explosives in bile or urine. The present study supports the development of fish urine usage for reliable and effective monitoring strategies for explosives.

Keywords Marine dumped munition, Explosives, TNT, Metabolites, Baltic Sea, Fish, Dab, Plaice, Flounder

Background

Marine dumped munition is an issue of global concern. Due to the ongoing corrosion of munition shells, munition compounds such as explosives, are leaking into the marine environments [12]. The western Baltic Sea is worldwide one of the hot spots of contamination with marine munition, due to military activities during the

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world wars and dumping of huge amounts of munition (approx. 300,000 t) after the wars [9]. The explosive 2,4,6-trinitrotoluene (TNT) is widely used in military and industrial applications and therefore present in many dumped conventional munition objects [4] and TNT is leaking from corroding munition objects dumped near the German coast [6]. Climate change is suspected to accelerate the deterioration of munition objects and consequently the leakage of munition compounds into the environment [42].

Organisms living in the vicinity of dumping sites can be contaminated with this explosive [3, 5, 31]. TNT is a nitroaromatic compound that, upon entering marine environments, undergoes biotic and abiotic degradation processes. These processes result in the formation of metabolites, including 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT). Marine fish are particularly vulnerable to the toxic effects of TNT and its metabolites due to their direct exposure through water, sediment and food: TNT and its metabolites can induce oxidative stress in marine fish by triggering the generation of reactive oxygen species [1, 16]. TNT leads to an increase in white blood cell counts and methaemoglobin levels in fish [15]. Sensini et al. [44] found that TNT exposure caused structural damage to the gills of European eel, including oedema, vascular congestion, and mucus hypersecretion. Behavioural changes in fathead minnows, such as lethargy were observed after exposure to 0.46 mg/l TNT [45]. The metabolites of TNT can interfere with cellular function and disrupt metabolic pathways in fish [11, 15]. Additionally, developmental abnormalities such as defects in heart formation, hatchability and malformations in fish larvae were observed in zebrafish exposed to TNT [17, 29]. TNT is a well-known genotoxic substance in humans [8]. TNT, along with its two main metabolites, 2-ADNT and 4-ADNT, has been shown to be genotoxic to zebra fish embryos [29] and cause liver cancer in humans [48].

TNT and its metabolites can bioaccumulate in marine fish, which leads to higher TNT concentrations in their body fluids and tissues than in the surrounding environment [4, 41]. This bioaccumulation may pose a risk not only to the fish themselves but also to their predators [7]. Consumption of seafood contaminated with explosives by humans, however, is yet regarded as not problematic due to the low concentration of explosives in the consumable parts [37].

It was shown before, that fish accumulate explosives in bile and tissues [16, 34]. In previous studies we revealed that common dab (*Limanda limanda*) caught in the dumping site Kolberger Heide [27], located in the western Baltic Sea and close to the German coastline, exhibited TNT metabolites in concentrations in bile up to 141 ng/ml 4-ADNT [31]. Also, dab from the North Sea caught near known dumping sites alongside the German coastline [24], as well as pouting (*Trisopterus luscus*) caught in the vicinity of contaminated ship wrecks, were observed to be contaminated with TNT metabolites [36].

For detoxification, TNT is transported to the liver of the fish, subjected to enzymatic metabolization and secreted into the bile fluid [30, 31]. Accordingly, before excretion of bile fluid via the gut, TNT metabolites accumulate in fish bile fluid, making it a valuable sample matrix for TNT exposure. In feeding experiments, bile fluid exhibited the highest concentrations of TNT metabolites compared to fish tissues [34]. Consequently, fish bile has been used to assess explosive contamination in coastal waters before [24, 31], thus providing insight into the bioavailability and biotransformation of TNT and aiding in ecological risk assessments and remediation efforts.

Also, muscle tissue of fish was used for explosive determination [4, 36]. The concentration of explosives in muscle is of special interest in fish used for human consumption. However, effective environmental monitoring requires high sample throughput, which is facilitated by a low matrix content (background, such as tissue protein) in the samples. Bile, a clear body fluid, offers this advantage along with relatively high contamination levels [34]. Similarly, fish urine—also a low-matrix body fluid—could potentially be as suitable for environmental monitoring as bile. However, studies on explosives in fish urine are currently lacking.

In human diagnostics, the detection of drug metabolites in urine is a critical aspect. Urine offers—at least for humans—a non-invasive medium for monitoring physiological and pathological states. Analysing urinary metabolites aids in disease diagnosis, in therapeutic drug monitoring, and in biomarker discovery [40]. Accordingly, fish urine could be a suitable matrix for the measurement of TNT metabolites too. However, to the authors' knowledge, fish urine has never been used for explosive analytics. Fish urine can be used as a non-invasive alternative to bile in environmental studies. Accordingly sampling of urine can help to reduce numbers of fish that need to be killed if ongoing and future leakage of explosive compounds from marine dumped munition is monitored. Furthermore, urine can add new information when the focus of the study lays on different tissue contamination combined with insight in potentially different metabolite patterns in the tissues. Urine analysis can be interesting for laboratory exposure experiments too, when metabolite formation kinetics are studied. The possibility of using urine as a non-invasive sampling method has the potential to facilitate the ethical acceptance of laboratory and

environmental studies on marine munition issues. Accordingly, in the present study explosive concentrations in fish urine were investigated for the first time.

The common dab (*L. limanda*), European plaice (*Pleuronectes platessa*), and European flounder (*Platichthys flesus*) are key flatfish species in German coastal waters, serving crucial roles in pollutant monitoring. These benthic fish accumulate contaminants from sediments and water, making them effective bioindicators of environmental health [32]. Dab, widely distributed in the North Sea and in the western Baltic Sea, is sensitive to various pollutants, including trace metals [22, 23, 32] and organic compounds [13, 21, 33, 38]. Plaice, prevalent in the North and the Baltic Sea, is used to assess hydrocarbon and pesticide contamination [28]. Flounder, found in estuaries and coastal areas, is a sentinel species for monitoring trace metals [39] and organic pollutants [21]. These species' presence in their ecological niches and corresponding pollutant accumulation make them indispensable for marine environmental assessments. Dab predominantly inhabit sandy and muddy substrates in deeper waters and feed on benthic invertebrates [20, 43]. Plaice favour sandy bottoms and exhibit strong site fidelity, consuming a diet mainly composed of polychaetes and molluscs [43]. Flounders are, more adaptable and occupy a range of substrates from estuaries to coastal zones, preying on small fish, crustaceans and molluscs [47]. These niche differentiations reduce interspecific competition, allowing coexistence and maintaining ecosystem balance in the diverse coastal habitats of Germany.

As the remediation of intentionally dumped munitions has begun in 2024 in the German waters of the western Baltic Sea [19], the question arises of how to establish an effective monitoring program to accompany this process. Remediation of marine munition is technically demanding, cost and labour intensive, and will take time. Effective monitoring of munition contamination is therefore also requested at dumping sites, which are not yet prioritized for remediation, to detect potential changes if their contaminants leak out, and to re-evaluate their prioritization. To achieve a reliable assessment of contamination with explosives, it is crucial to determine which fish species, and which of their tissues and body fluids are most appropriate for monitoring or which can be combined. The present study addresses the following questions:

1. Can TNT metabolites be detected in fish urine?
2. Is fish urine as effective as fish bile in indicating regional differences in explosive contamination in fish?
3. Do amounts of explosives in urine and bile from the same fish correlate?
4. Do TNT metabolites concentrations in urine and bile differ among the three flatfish species studied?
5. How can a future marine monitoring program for explosives benefit from these findings?

Methods

Study areas

Flatfish were collected with bottom gillnets during three separate expeditions of the German research vessel *Clupea* from 2021 to 2023 (Table S1). Since bottom gillnets require contact with the seafloor to function, fishing directly on munition dumping sites was avoided to mitigate any risks associated with munition contact. Nonetheless, sampling sites were chosen close to known munition disposal areas as identified by AmuCad.org [2]. High-resolution seafloor imaging carried out by GEOMAR Kiel assisted in determining the optimal locations for deploying bottom gillnets in the Kiel region (Kolberger Heide), Lübeck region, and Schlei region, maintaining a safety distance of at least one nautical mile from munition-contaminated areas. The sampling locations as well as the three sampling regions are depicted in Fig. 1 and detailed in Table S2.

Fish sampling, biological data acquisition

A total of 181 fish, including common dab (*L. limanda*) ($n=102$), European plaice (*P. platessa*) ($n=20$), and European flounder (*P. flesus*) ($n=59$), were caught using bottom gillnets in the western Baltic Sea (Fig. 1; Table 2; Table S2). The fish were sorted from the catch and transferred to tanks with a flow through of seawater prior to examination. Sampling of fish started immediately after sorting and all fish from one net were sampled consecutively, before the next net was taken up. The fish were anaesthetized using clove oil (400 µl/l sea water), weighed to the nearest gram, and their lengths were measured to the nearest centimetre. They were then euthanized by decapitation. The body cavity was opened, and urine and bile samples were collected using separate syringes (1 ml measuring syringe, Injekt®-F Luer Solo with needles Sterican® 0.60×25 mm 23 G×1″, BRAUN, Melsungen, Germany) for each type of fluid and each fish. The bile, respectively, the urinary bladder was pierced carefully with the needle, in order to prevent leakage of fluid. Fluid was aspirated slowly, avoiding collapse of the respective bladder, up to the maximum available volume or until the 1 ml filling of the syringe was reached. The latter was possible occasionally with urine, but collected volumes of bile were mostly <1 ml. Only fish providing sufficient body fluids were included in the present study. The minimum volume required was about 25 µl for either fluid. The samples were stored at −20 °C.

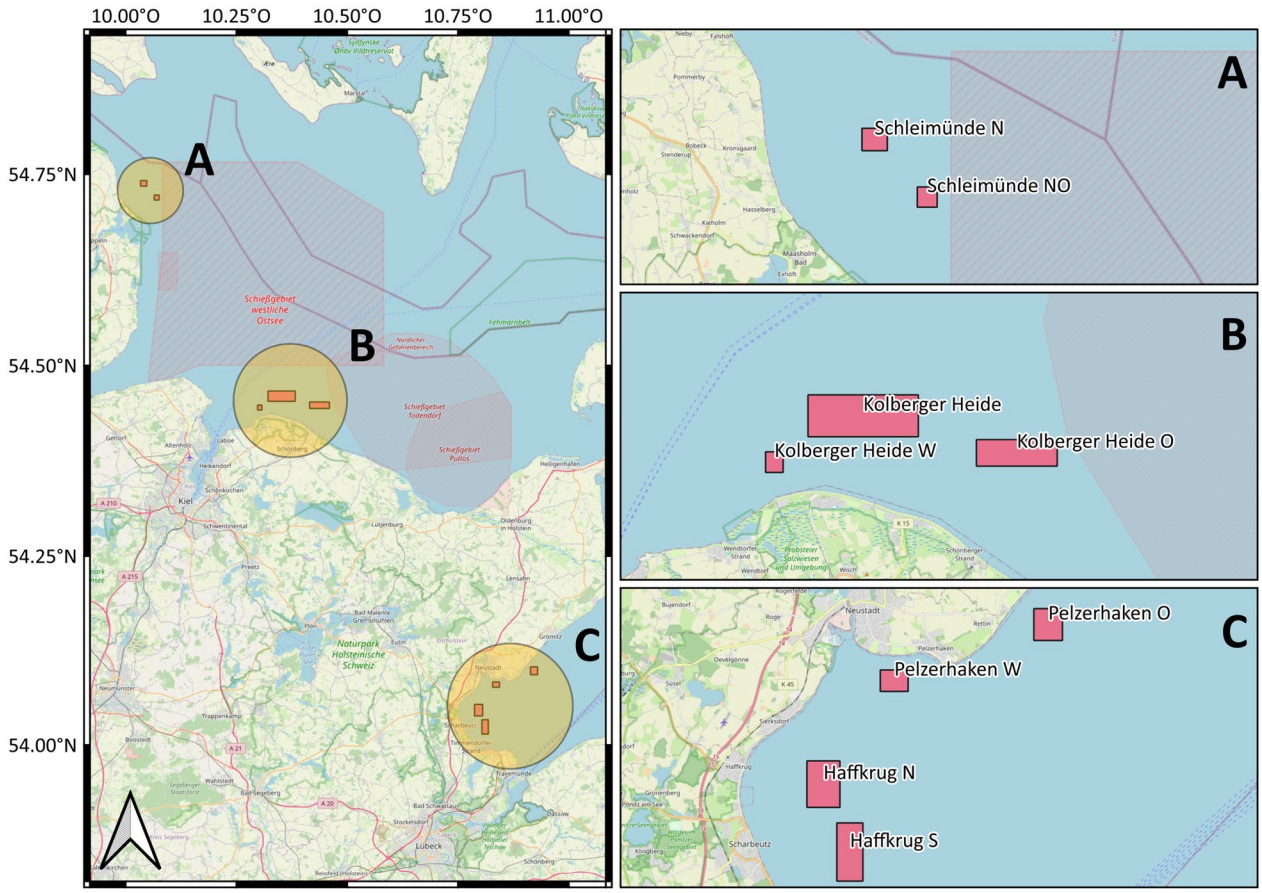

Fig. 1 Sampling areas of flat fish close to the German coastline in the Baltic Sea. The three sampling regions Schlei (**A**), Kiel (**B**) and Lübeck (**C**) are indicated by yellow circles and are shown in separate maps at the right. The individual sampling sites (red boxes) were named based on local place names and geographic direction (compare Table S2)

Chemicals and analytical materials

Acetonitrile (99.9%, HPLC grade), methanol (99.97%, UHPLC grade), and acetic acid (99%, analytical grade) were provided by Th. Geyer (Germany). Clove oil (natural origin, >80%) was sourced from Carl Roth (Germany). Ammonium acetate was supplied by Honeywell Fluka (USA). Certified reference standards for TNT, 2-ADNT, and 4-ADNT (Fig. S1), with purities greater than 99.0%, were obtained from AccuStandard (USA). β-Glucuronidase from Helix pomatia Type H-3 was acquired from Sigma-Aldrich (Germany). Chromabond Easy polystyrene-divinylbenzene-copolymer reversed-phase solid-phase extraction columns (1 ml/30 mg) were procured from Macherey and Nagel (Germany). Ultrapure water was prepared in the laboratory using a Purelab Flex 3 system (Elga Veolia, United Kingdom). The internal standard, isotopically labelled TNT (13C7 99%; 15N3, 98%), was purchased from Cambridge Isotope Laboratories, Inc. (USA).

Sample preparation and HPLC–MS/MS analysis

The processing of urine and bile samples was adapted from Gledhill et al., [18], Koske et al. [31], and Bünning et al. [10], with detailed methods described in Kammann et al. [24, 25]. Briefly, a sample volume of 25 µl of bile or urine was enzymatically treated with β-glucuronidase after the addition of 5 ng of the internal standard 13C15N-TNT. The final volume of 120 µl was loaded onto a Chromabond Easy solid-phase extraction column, rinsed with 500 µl of ultrapure water, and eluted four times with 100 µl of acetonitrile. Samples were then dried and reconstituted in 250 µl of acetonitrile/water (30:70, v/v). An injection volume of 20 µl was used for analysis on an Agilent 1290 Infinity High-Performance Liquid Chromatograph coupled with an AB Sciex QTrap 5500 Triple Quadrupole/Ion-Trap Mass Spectrometer (HPLC–MS/MS). The HPLC column used was an "Acclaim Explosives E2" (2.2 µm, 2.1 mm × 150 mm, Thermo Fisher Scientific) maintained

at 22 °C. The eluents consisted of (1) ultrapure water with 10 mM acetic acid and 10 mM ammonium acetate, and (2) methanol. The gradient started at 50% methanol and was increased in steps to 100% methanol over 25 min, with a constant flow rate of 0.22 ml/min. Ionization was performed in negative ion mode, and explosives and their metabolites were detected using the multiple reaction monitoring mode, based on characteristic MS/MS transitions optimized with commercially available standards. The following masses were used for quantification: TNT 226.0 *m*/*z*; 2-ADNT and 4-ADNT 196.0 *m*/*z*. Retention times were 10.7 min for TNT, 14.1 min for 2-ADNT and 13.6 min for 4-ADNT. Example chromatograms are presented in Fig. S2. Peak detection was carried out using MultiQuant™ Software Version 3.0.2 (Sciex).

Quality assurance and treatment of censored data

For quantification, the internal standard 13C15N-TNT was used alongside a 10-point external calibration curve for each analyte (Fig. S3), covering the expected concentration range. Limits of detection (LOD) and limits of quantification (LOQ) were calculated according to DIN [14] standards for each explosive compound. Matrix-specific LOD and LOQ were established using spiked bile samples. Uncontaminated bile samples from dab were prepared as described previously and spiked with the target explosive compounds and the internal standard at concentrations ranging from 0.05 to 50 ng/ml. No matrix spike was performed for urine due to logistical reasons. For urine the same LOD and LOQ were used as for bile. The detection and quantification of explosives were performed as outlined earlier [26]. Censored data, representing values below the LOD or LOQ (Table 1), were considered estimates. These values were used to calculate mean concentrations.

Statistics

Statistical analyses were conducted using Statistica Version 12.5 (Statsoft Europe, Hamburg, Germany). Two-factorial ANOVAs, without interaction between factors, were employed to test for potential differences in TNT metabolite contamination based on origin or species, followed by Fisher LSD post hoc tests, both with a 95% significance level. To compare single metabolite levels between individual fish, a paired-sample t-test was used. Linear regression was calculated with a level of confidence of 95%.

Table 1 Limits of detection (LOD) and quantification (LOQ) obtained for different munition compounds according to DIN [14] using matrix specific standards with bile for 2,4,6-trinitrotoluene (TNT), 2-amino-4,6-dinitrolouene (2-ADNT) and 4-amino-2,6-dinitrolouene (4-ADNT)

Substance	LOD (ng/ml)	LOQ (ng/ml)
TNT	3.255	9.765
2-ADNT	0.049	0.147
4-ADNT	0.071	0.213

Values are given in ng/ml urine or bile fluid. The same LOD and LOQ were used for bile and urine

Results

Biological data of fish and sample details

A total of 181 fish were studied, which included the flatfish species dab ($n=102$), flounder ($n=59$), and plaice ($n=20$). The length of the fish ranged from 20 to 36 cm, and their weight ranged from 86 to 478 g (Table 2). The largest fish in this study were dab from the Kiel region, while the smallest were dab from the Lübeck region. An ANOVA showed no significant differences in length among the three regions across all species. However, a post hoc LSD test revealed a significant difference in the length of dab between the Lübeck and Kiel regions. The same was true for the weight of these fish, as size and weight are closely correlated. There was no significant positive correlation between the weight of the fish and the concentration of any explosive metabolite in urine or bile (data not shown). Accordingly, explosive concentrations in urine and bile were not related to fish size or weight, and fish could be included in the study independent of their size/weight without biasing the results.

In the present study we collected urine and bile samples from the same individuals during two cruises (Table S1). We observed that less than 10% of fish produced bile but no urine and another 10% which produced urine but no bile. These amounts may vary due to several factors from study to study, but our results indicate that both matrices

Table 2 Length and weight of dab (*Limanda limanda*), flounder (*Platichtys flesus*) and plaice (*Pleuronectes platessa*)

Region	Species	*n*	Length (cm)	Weight (g)
Lübeck	Dab	23	24.4 (20–33)[a]	156.2 (86–261)[a]
	Flounder	52	29.6 (25–36)	277.9 (165–478)
	Plaice	9	27.7 (25–32)	234.7 (172–307)
Kiel	Dab	72	27.9 (21–35)[a]	214.6 (86–362)[a]
	Flounder	7	29.1 (26–32)	250.0 (175–316)
	Plaice	11	28.3 (25–33)	226.9 (148–277)
Schlei	Dab	7	26.0 (21–33)[a]	199.0 (93–340)[a]
All		181	27.9 (20–36)	227.5 (86–478)

Given are number (*n*), length and weight (mean and minimum–maximum including outliers) for investigated regions and species

[a] Data partly taken from Kammann et al. [25]. New dataset is available underKammann et al. [26]

were available from the majority of flatfish in the present study. Urine and bile samples together were taken from 104 fish, where bile fluid only was accessible from 69 fish. Urine only in sufficient amounts were obtained from a subset of 8 fish. In sum, 112 urine and 173 bile samples of 181 fish were included in the present study (Table 3). Lower numbers of urine samples are mainly due to the fact that the cruise "CLU355" covered bile samples only (Table S1).

TNT metabolites in urine and bile

The TNT metabolites 2- and 4-ADNT, but not TNT itself, were detected in urine and bile of dab, flounder and plaice as detailed in Table 3. This was presumably attributed to the fact that fish rapidly metabolize TNT to the less toxic and better excretable ADNTs [30]. The mean values of 2-ADNT in dab bile varied significantly across regions, ranging from below the limit of detection (<LOD) in the Lübeck region to 1.008 ng/ml in the Schlei region, and up to 3.694 ng/ml in the Kiel region (Table 3). In dab urine, the mean concentrations of 2-ADNT were similar to those found in bile, ranging from <LOD in the Lübeck region to 1.815 ng/ml in the Kiel region. For individual dab of the Kiel region, the contamination levels of 2-ADNT reached up to 26.356 ng/ml in bile and 36.120 ng/ml in urine, while 4-ADNT levels reached up to 95.908 ng/ml in bile and 21.186 ng/ml in urine. Several samples showed concentrations below LOD for at least one metabolite in either matrix, thus were considered negative (Table S3). In the Lübeck region, 60% of urine samples and 49% of bile samples over all tested flat fish species were below the LOD. In the Schlei region, no sample was below the LOD, whereas in Kiel Bay, 19% of urine samples and only 1% of bile samples were negative (<LOD). Detailed results are given in Table S3. The percentage of negative samples has previously been used to describe contamination levels of dab with TNT metabolites [24].

When examining TNT metabolite contamination in urine and bile on the level of individual fish, correlations were evident between the two matrices (2-ADNT: $p < 0.01$, $r^2 = 0.21$, Fig. 2); (4-ADNT: $p < 0.01$; $r^2 = 0.20$; data not shown). However, in some cases negative result in urine corresponded with explosive contamination in bile of the same fish. While the opposite, negative result in bile, but explosive contamination in urine was not observed. This might be attributed to faster turnover rates of urine compared to bile. Interestingly, the metabolite patterns in urine and bile differed: in bile, the concentration of 4-ADNT was often higher than that of 2-ADNT, while in urine, the concentrations of the two metabolites were similar. Significant differences in the TNT metabolite pattern in urine and bile were observed in dab from the Kiel region, but less pronounced in the lower contaminated Lübeck region (Figs. 3, 4). We observed an enrichment of 4-ADNT compared to 2-ADNT in bile and urine of flounder from the Kiel region. For dab and plaice higher 4-ADNT levels compared to 2-ADNT were observed in bile only. However, dab and plaice from the Kiel region did not show this difference in the metabolite pattern in urine (Fig. 4).

Data on TNT contamination above the limit of detection (>LOD) combined from all tested flatfish species (dab, flounder, plaice) illustrated significantly higher levels of TNT contamination in urine and bile in the Kiel region compared to the Lübeck and Schlei regions (ANOVA $p < 0.01$, Fig. 3). In urine samples from the Kiel region, 2-ADNT and 4-ADNT levels differed between

Table 3 TNT metabolites 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) in urine and bile of dab (*Limanda limanda*), flounder (*Platichtys flesus*) and plaice (*Pleuronectes platessa*)

Region	Species	*n*	Urine		*n*	Bile	
			2-ADNT (ng/ml)	4-ADNT (ng/ml)		2-ADNT (ng/ml)	4-ADNT (ng/ml)
Lübeck	Dab	18	<LOD (<LOD–0.189)	<LOD (<LOD–0.220)	19	<LOD (<LOD–<LOQ)[a]	<LOQ (<LOD–0.295)[a]
	Flounder	47	<LOD (<LOD–0.192)	0.058 (<LOD–0.239)	52	<LOQ (<LOD–0.212)	<LOQ (<LOD–0.655)
	Plaice	0	n.d	n.d	9	<LOD (<LOD–0.157)	<LOQ (<LOD–0.655)
Kiel	Dab	36	1.815 (<LOD–36.120)	1.544 (<LOD–21.186)	68	3.694 (<LOD–26.356)[a]	14.561 (<LOD–95.908)[a]
	Flounder	4	7.820 (0.519–11.042)	12.179 (0.856–26.877)	7	3.992 (1.322–7.717)	18.963 (8.934–39.103)
	Plaice	6	4.361 (0.939–8.195)	4.805 (1.120–8.211)	11	2.770 (0.166–10.433)	9.936 (0.708–44.995)
Schlei	Dab	1	<LOQ	<LOD	7	1.008 (0.251–1.742)[a]	4.786 (1.088–11.671)[a]
All		112	1.118 (<LOD–36.120)	1.221 (<LOD–26.877)	173	1.853 (<LOD–26.356)	7.387 (<LOD–95.908)

Given are number of samples (*n*), mean (minimum–maximum including outliers) concentrations for different regions and species

New dataset is available under Kammann et al. [26]

LOD limit of detection, *LOQ* limit of quantification, *n.d.* not determined

[a] Data partly taken from Kammann et al. [25]

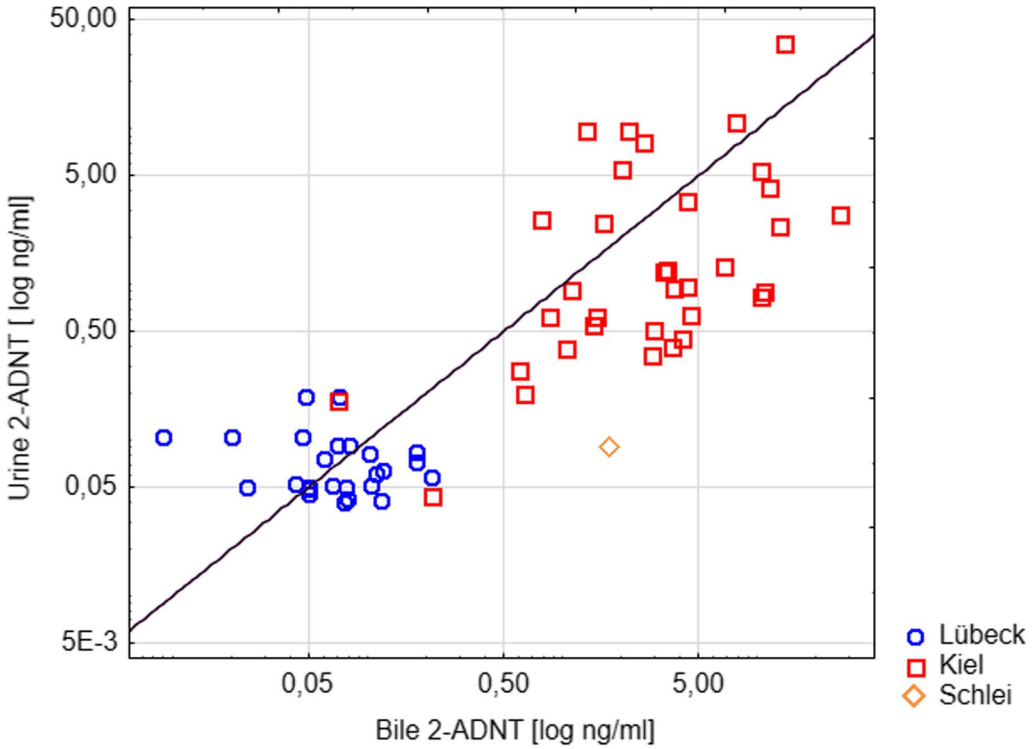

Fig. 2 Concentrations of 2-ADNT in urine and bile of individual fish grouped for the regions Lübeck, Kiel and Schlei. Black line: 1:1 equality relation

dab and flounder and 4-ADNT varied between plaice and flounder (ANOVA $p < 0.05$ Fig. 4). No significant differences in 2-ADNT and 4-ADNT concentrations were observed in bile samples from the Kiel region across species (ANOVA $p > 0.05$, Fig. 4).

Data on TNT contamination above the limit of detection (>LOD) combined from all tested flatfish species (dab, flounder, plaice) illustrated significantly higher levels of TNT contamination in urine and bile in the Kiel region compared to the Lübeck and Schlei regions (ANOVA $p < 0.01$, Fig. 3).

In the Kiel region, no significant differences in 2-ADNT and 4-ADNT concentrations were observed in bile across species ($p > 0.05$, Fig. 4). However, in urine, 2-ADNT and 4-ADNT levels differed significantly between dab and flounder and 4-ADNT varied between plaice and flounder when comparing each metabolite between the species by using ANOVA combined with Fisher LSD post hoc tests, both with a 95% significance level. In the Lübeck region, both 2-ADNT and 4-ADNT concentrations in bile differed significantly between dab and flounder, as well as between plaice and flounder using the same statistical test as described before. Dab and plaice, however, had comparable concentrations of these metabolites in bile as in the Kiel region. In terms of urine, there were no significant differences in 2-ADNT and 4-ADNT levels (ANOVA and Fisher LSD post hoc) between dab and flounder in the Lübeck region. Data are given in Table 3.

Discussion

In the present study, we observed that explosives leaking from dumped munitions in coastal waters of the Baltic Sea are detectable in fish urine and bile in comparable amounts across different flat fish species and differently contaminated regions. To the best of our knowledge, fish urine was used with the present study for the first time for environmental monitoring of TNT contamination of fish. Comparison of urine and bile samples from individual fish revealed that urine could be used to describe local differences in TNT contamination in a similar manner as bile. Our present findings in fish urine and bile are consistent with previous results in dab from the western Baltic Sea [31], near-shore North Sea areas [24, 35] and in fish caught near munition-loaded wrecks in the North Sea [36]. Measurements of the present study align with those of Beck et al. [4], Koske et al. [31], and Kammann et al. [24, 25], who reported that dab caught near the dumping area "Kolberger Heide" (Kiel region) in the Baltic Sea were contaminated with 1.60–7.80 ng/ml of 2-ADNT and 6.84–28.53 ng/ml of 4-ADNT in their bile.

The present study showed the suitability of fish urine to describe regional and species differences in explosive

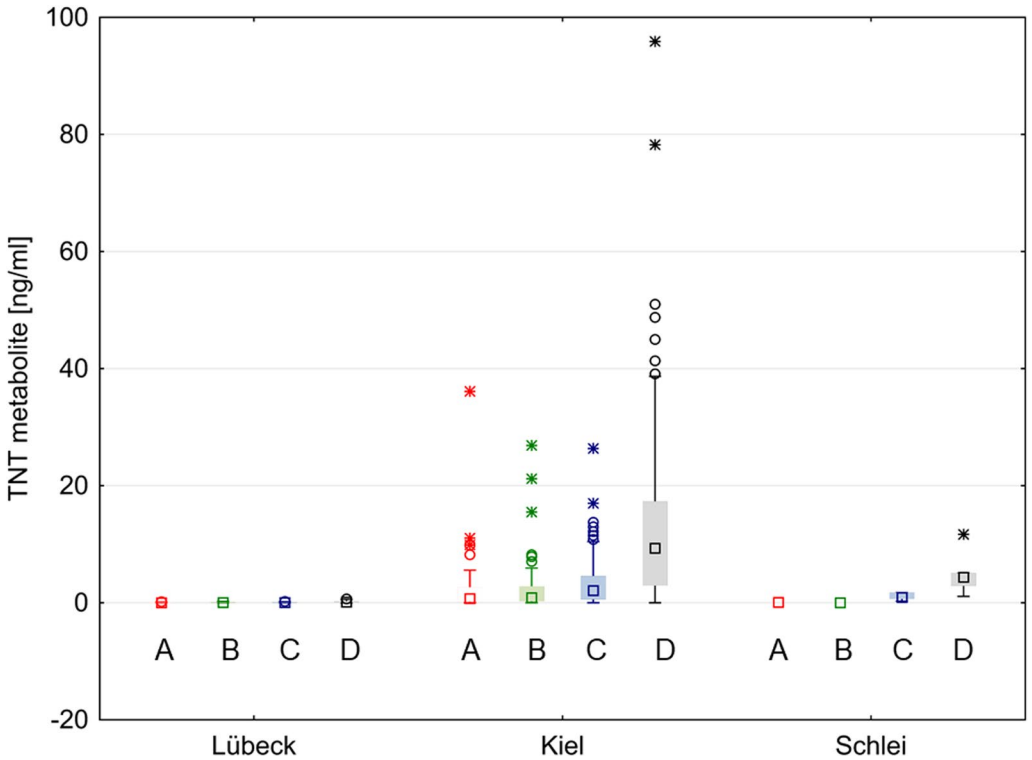

Fig. 3 TNT metabolite patterns in different regions combined for of dab ($n = 102$), plaice ($n = 20$) and flounder ($n = 59$). A: 2 ADNT ng/ml urine; B: 4-ADNT ng/ml urine; C: 2-ADNT ng/ml bile; D: 4-ADNT ng/ml bile. Shown are medians, 25–75% percentiles (box) and minima/maxima (whiskers), as well as outliers (circle) and extremes (asterisks)

contamination. Fish bile is a well-established indicator for contamination with TNT and its metabolites due to their accumulation in bile following detoxification activity of the livers. However, fish urine, analysed here, showed its potential as an easily obtainable sample matrix of fish. Our findings indicate that fish urine contains levels of TNT metabolites similar to those in bile.

Further, our comparative analysis across three differently munition-contaminated regions in the western Baltic Sea revealed that urine reflects contamination patterns in a similar manner as bile. This suggests that urine could be an effective alternative to bile for monitoring regional differences in contamination of marine fish with explosives. However, comparison of urine and bile samples from the same individual fish revealed that some fish contained TNT metabolites in their bile, while being negative (<LOD) for TNT metabolites in urine. So, TNT metabolites in urine do not exactly mirror the contamination in bile of the same fish. This might be attributed to higher turnover rates of urine versus bile, resulting in quicker expulsion of TNT metabolites via urine. We speculate that TNT metabolites might occur earlier in urine than in bile, which might be an advantage for some monitoring questions.

Nevertheless, the big picture of contamination differences across regions was well reflected by urine as well as bile (Fig. 3). The contamination status of 2-ADNT in urine of plaice, dab and flounder as shown in Fig. 5, was quite similar to results in dab bile published earlier [24]. Fish urine is—like bile—an easy to sample body fluid, which is completely homogenous and has little organic matrix that would interfere with chromatography, compared to fish tissue (e.g., muscle). Therefore, determination of explosives compounds in urine and bile is relatively quick and easy, compared to tissues. However, depending on the physiological and nutritional status of each fish, urine and bile are not necessarily available in sufficient quantities from every fish. It might therefore be advantageous to sample urine and bile from each fish, to increase the likelihood to obtain at least one of the body fluids in sufficient amounts. However, we do not recommend to sample both urine and bile in parallel for environmental monitoring because this would double the effort but not the results.

An alternative advantage of urine is the possibility to develop it into a non-invasive contamination indicator, since it would be possible to catheterize urine without killing and dissecting the fish. This approach, though

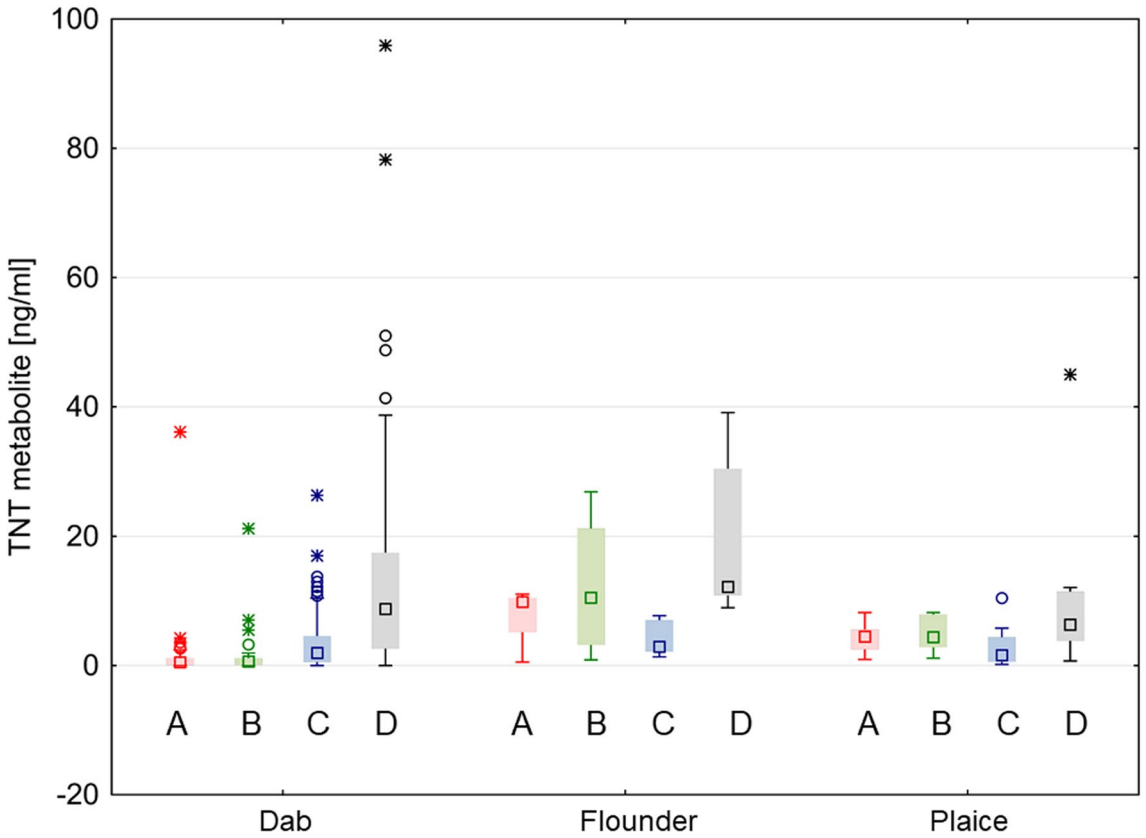

Fig. 4 TNT metabolite pattern in urine and bile of dab ($n = 36/68$), flounder ($n = 4/7$) and plaice ($n = 6/11$) from Kiel region. A: 2 ADNT ng/ml urine; B: 4-ADNT ml/urine; C: 2-ADNT ng/ml bile; D: 4-ADNT ng/ml bile. Shown are medians, 25–75% percentiles (box) and minima/maxima (whiskers), as well as outliers (circles) and extremes (asterisks)

not part of the present study, may gain attention if regular monitoring of marine munition contamination with minimal invasive impact is required. Mariussen et al. [34] found that TNT metabolites accumulated in bile of Atlantic salmon (*Salmo salar*) after 48 h of exposure with a bioconcentration factor (BCF) of nearly 2000, whereas gills, blood, liver, kidney, muscle, brain and intestine had BFCs below 100. Since the fish were not fed in the study by Mariussen et al. [34], the bile was not excreted into the gut and TNT concentrations remained high up to 50 h after the end of exposure. In mammals, comparative studies showed that the main excretion of TNT through urine takes place as glucuronide conjugates [46]. The same is true for TNT metabolites analysed in urine of TNT-exposed workers [40]. In fish it appears that a major portion of the TNT is metabolized followed by a phase II conjugation with glucuronides and biliary excretion [34]. However, our study shows that fish excrete TNT via urine too. Based on the results of the present study we hypothesize that BCF of 2-ADNT in urine and bile of fish reach comparable levels. The contamination level in both, urine and bile mirror the TNT exposure of the fish during the last hours or few days, depending on feeding events or loss of urine.

Accordingly, a fish could leave the region of contamination, while keeping its body fluids, and when caught at a less contaminated site this fish would increase variation of results. Therefore, analysing a suitable number of individual fish—we estimate 10—in a given region is recommended. The minimal number of individuals to be analysed depends on the fish-to-fish variation and on the level of difference to be detected. The combined group of dab and plaice investigated in the Kiel and Lübeck regions was well above 10 (Table 2). However, fewer samples were available from the Schlei region. We point out that one urine sample (Table 3; Fig. 5) was by far not enough to describe the contamination level in this region. It can be taken as an exemplary result, which needs substantiation by further analysis of more individuals.

We also addressed the question if explosive concentrations differ between three flat fish species, plaice, common dab and flounder. All three species have in common that they have a strictly benthic live style, potentially near munition on the sea floor, and that they do not exhibit

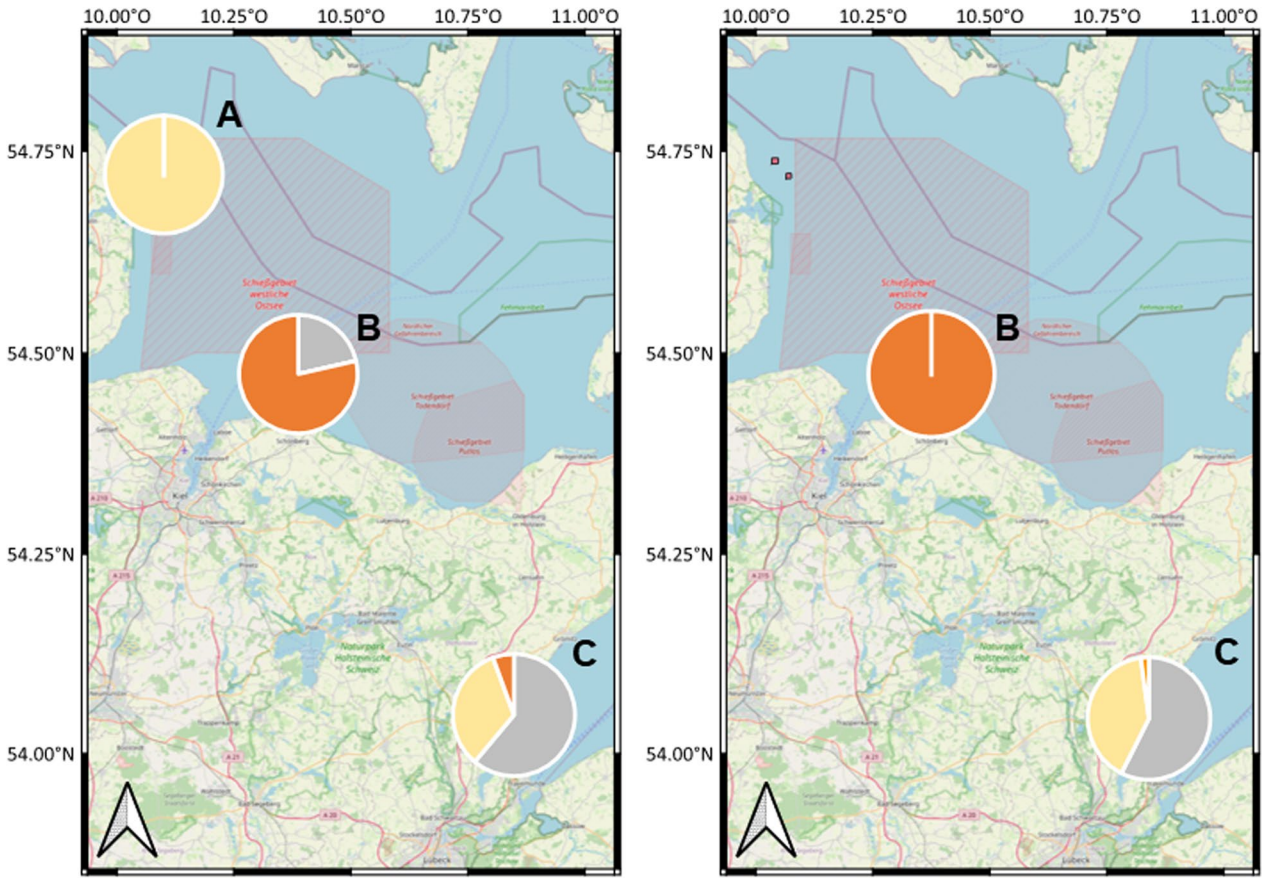

Fig. 5 Spatial distribution of TNT metabolite 2-amino-4,6-dinitrotoluene (2-ADNT) in fish urine. Left side: Common dab (*Limanda limanda*) and European plaice (*Pleuronectes platessa*); right side European flounder (*Platichthys flesus*). The three regions are Schlei (**A**), Kiel (**B**) and Lübeck (**C**). Given are percentages of individuals per sampling area below limit of detection (<LOD, grey), between LOD and LOQ (limit of quantification; yellow), and above LOQ (orange)

extensive migratory activity, thus would display in their body fluids what they had been exposed to in the last hours and days.

The comparison of TNT metabolite contamination in urine and bile of the three flatfish species in the Kiel region revealed that TNT contamination in dab and plaice could be combined in future studies, but that explosive contamination in flounder significantly differed from the other two species. This fact might be explained by different ecological niche occupation of flounder compared to plaice and dab. Beside the statistical differences between the species, regional contamination in flounder urine showed no big difference to dab and plaice, when results are grouped as in Fig. 5. However, numbers of fish are low in the present study. So, it cannot be excluded, that bigger data sets may lead to other results concerning species comparability. Additionally, we want to point out that the flounder is an excellent species for explosive monitoring because it is found in coastal areas where dab and plaice may be absent. Especially in the Baltic Sea, where dab are inhabiting only the western part with relatively high salinity, alternative fish species like flounder and plaice, more tolerant to decreasing salinity towards the eastern Baltic Sea are needed for spatial coverage of monitoring.

Future monitoring of explosives contamination could benefit from the usage of several fish species comparable in the TNT metabolite concentration. This would reduce the sampling effort, while maintaining the desired number of analysed fish, since these species can be collected with the same fishing gear (here bottom gillnets). With the present study and the first detection of explosives in fish urine we present a novel avenue for environmental monitoring of munition contamination. This approach offers insights into the presence and distribution of explosives in aquatic environments and complement traditional indicator matrices like fish bile and fish muscle tissue. The findings of the present study are crucial for advancing methods in environmental monitoring and assessing effectiveness of future

remediation efforts focused on mitigating the impacts of munition dumping in coastal waters.

Conclusions

Urine and bile can both be used as matrix to monitor environmental exposure of fish to TNT. Concentrations of TNT metabolites in both, urine and bile, revealed local differences. Accordingly, both matrices are suitable to detect local differences in the severity of environmental contamination with explosives. Such information is crucial for the prioritization of munition remediation measures. Further on, dab and plaice can be combined in monitoring studies of bile or urine. Also, flounder is a suitable species for environmental monitoring of explosives. We propose to conduct exposure studies with fish to calculate BCF of TNT metabolites in fish urine. We suggest to develop fish urine as matrix for environmental monitoring of munition contamination, since urine samples can be obtained with minimal invasive measures. For classical environmental studies with a spatial focus, however, it is sufficient to analyse explosives in one matrix only: bile or urine. Bile is the matrix that has been used successfully in a number of studies, which is clearly an advantage. Urine is a good alternative and provides the advantage of non-invasive sampling with less ethical concerns. Accordingly, urine might become the matrix of choice for future environmental monitoring studies.

Abbreviations

2-ADNT	2-Amino-4,6-dinitrolouene
4-ADNT	4-Amino-2,6-dinitrolouene
BCF	Bioconcentration factor
LOD	Limit of detection
LOQ	Limit of quantification
PCA	Principal component analysis
RV	Research vessels
TNT	2,4,6-Trinitrotoluene

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-025-01074-0.

Additional file 1. Table S1: Sampling cruises in German coastal waters of the south-western Baltic Sea of the fishery research vessel Clupea from 2021 to 2023. Table S2: Sampling area names and coordinates in German coastal waters of the Baltic Sea from 2021 to 2023, region, number of cruise, mean depth and area size. Table S3: Explosives 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) in urine and bile of dab (Limanda limanda), flounder (Platichtys flesus) and plaice (Pleuronectes platessa). Given are number of samples (n), number of samples < limit of detection (< LOD), numbers of samples >LOD and < LOQ (limit of quanification) and number of samples >LOQ. For details on LOD and LOQ see Tabel 1. Fig. S1: Chemical structures of trinitrotoluene (TNT), 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT). Fig. S2. Chromatograms of 2-amino-4,6-dinitrotoluene (2-ADNT, top left, blue peak) and 4-amino-2,6-dinitrotoluene (4-ADNT; top right, blue peak) analysed in a real fish bile sample compared to blanks (lower line). Fig. S3: Multi-point calibrations for trinitrotoluene (TNT, blue), 2-amino-4,6-dinitrotoluene (2-ADNT, pink) and 4-amino-2,6-dinitrotoluene (4-ADNT, orange).

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Author contributions

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

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Availability of data and materials

The dataset generated and analysed during the current study are available in the OpenAgrar repository, https://doi.org/https://doi.org/10.3220/DATA20241022124456-0.

Declarations

Ethics approval and consent to participate

All procedures were conducted in accordance with European directive 2010/63/EU on the protection of animals used for scientific purposes.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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