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Quantification of munition compounds in the marine environment by solid phase extraction – ultra high performance liquid chromatography with detection by electrospray ionisation – mass spectrometry

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ABSTRACT

As a consequence of World War II, large amounts of munition have been deposited in coastal waters. Deterioration of the mines and bombs is resulting in a release of munition compounds (MCs) like trinitrotoluene to the surrounding marine environment, with potential implications to ecosystems. Analytical methods have thus far been unable to detect these compounds reliably in seawater. We present a highly sensitive method for the analysis of MCs in the marine environment. We combine preconcentration and sample clean up by solid phase extraction with separation and detection by ultra-high performance liquid chromatography – electrospray ionisation – mass spectrometry (UHPLC-ESI-MS) for the detection of MCs dissolved in filtered (< 0.2 µm) seawater. For biota, dried and ground samples were extracted in acetonitrile and analysed after simple dilution. Eleven MCs were detected by UHPLC-ESI-MS with limits of detection between 0.01 and 25 pg. For the first time, we used heavy isotopes of trinitroluene and dinitrobenzene to improve quantification in environmental samples. We detected 7 MCs in waters sampled at a known munition disposal site in the Baltic Sea after a 1000-fold preconcentration and using an injection volume of 25 µL. Trinitrotoluene and dinitrobenzene were the most abundant MCs, occurring at concentrations between 0.1 and 11.8 ng L^{-1}. We observed 10 MCs at concentrations up to 24 µg g^{-1} dry weight in benthic organisms sampled from the site. The enhanced sensitivity of our method allowed us to detect MCs at concentrations relevant for assessment and management of munitions disposal sites in the marine environment.

1. Introduction

Historical use and disposal of munitions in marine environments has led to direct contamination of seawater, sediments, and organisms by munition compounds (MCs) [1]. The North and Baltic Seas alone contain more than 1.6 million metric tons of dumped conventional explosives and the risk of contamination of marine waters with MCs is increasing with time, as the metal housings of dumped munitions and unexploded ordnance progressively corrode [1,2]. The issue of submerged munitions is also made more urgent by increasing offshore human activities such as oil and gas pipelines and wind farms.

A vast array of explosive chemical compositions has been used in various conventional munitions by different nations over time [3], but the predominant compounds of importance in the marine environment include nitroaromatic compounds (e.g., 2,4,6-trinitrotoluene, TNT; 1,3-dinitrobenzene, DNB; tetryl) and nitramines (e.g., hexahydro-1,3,5-trinitro-1,3,5-triazine, RDX). MCs accumulate in tissues of a range of organisms [4,5] and have been reported to be toxic to macro algae, benthic invertebrates, and fish [6]. As a consequence of their toxicity to marine organisms and potential for exposure to humans through seafood consumption, there is a need to understand the release, mobility, and fate of MCs from submerged munitions.

Contamination of marine environments with MCs is determined by the dissolution rate from solid explosive materials and the sum of removal processes including particle-water partitioning and microbial degradation. The solubility of MCs is relatively low, typically on the order of 10–100 mg L^{-1} [7], and is approximately 20% lower in seawater than freshwater [8]. Dissolution rates of munition material range typically between 0.5 and 50 mg cm^{-2} d^{-1} [1]. Perhaps as a result of slow release and rapid removal of MCs in

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seawaters, these compounds have only rarely been detected at marine sites that are impacted by unexploded or discarded munitions. For example, no MCs were detected in water or sediment samples at munitions disposal sites in Canada [9], USA [10,11], Scotland [12], or Sweden [13]. At a site in Puerto Rico, Porter and co-workers [14] showed that dissolved MCs were near saturated levels at the surface of breached munitions, but concentrations were undetectable only tens of centimetres from the source. Low concentrations of MCs were detected only occasionally in sediments in Canada [15] and Puerto Rico [14]. These studies make it clear that in order to thoroughly asses the scale and extent of contamination by MCs in the marine environment a method that combines excellent sensitivity with unambiguous compound identification in a complex matrix is required.

A variety of analytical methods has been reported for detection of MCs in environmental samples [16–18], which vary in their specificity, ease of use, and detection limits. Both gas chromatography and liquid chromatography have been applied to separate MCs prior to detection by mass spectrometry (reviewed in [18]). A widely used method for dissolved MC analysis uses solvent extraction (e.g. Refs. [9,11,14]), separation by HPLC, and spectrophotometric detection to achieve detection limits in the $\mu g\,L^{-1}$ range (US EPA Method 8330) [19]. However, this method does not allow definitive compound identification or analysis of poor light-absorbing MCs such as nitroglycerine (NGL) or [3-Nitrooxy-2,2-bis(nitroxymethyl)propyl] nitrate (PETN). Furthermore, mobile phase conditions can result in poor peak separation and shifts in retention time, making peak identification problematic in complex matrices such as seawater. More recently, mass spectrometry (MS) [18], coupled with gas chromatography [13] or with liquid chromatography and ionisation by atmospheric pressure chemical ionisation (APCI) [16,17,20], electrospray ionisation (ESI) [21] and electron impact (EI) [22,23] has been successfully applied for the analysis of MCs and shown enhanced sensitivities and specificity, either via detection of masses with high resolution ($>$ 100,000) [17,20] or via the detection of characteristic product ions [16]. A highly sensitive method for MC analysis employing laser desorption with high resolution mass spectrometry with detection limits of $9\,ng\,L^{-1}$ for TNT and that requires no chromatographic separation has also been developed for analysis of water samples [16]. However, to our knowledge the method has not been applied to seawater and did not include analysis of nitrobenzenes and the microbial metabolites of TNT (amino-dinitrotoluenes), which could be important in marine environments.

Analysis of compounds in seawater is typically challenging because of the high salt matrix, the presence of thousands of potentially interfering organic compounds [24] and the very low concentration of analytes of interest [1]. Solid phase extraction (SPE) has been shown to be effective for removal of the salt matrix and analyte preconcentration [25] and is routinely used for the analysis of organic compounds in seawater by mass spectrometry (e.g. [26]). However, recoveries can be variable and quantification thus problematic. The purpose of the current work was to develop a highly sensitive analytical method for the determination of MCs in the marine environment that can also provide unequivocal compound identification. For detection of MCs in seawater, we combined SPE for preconcentration and salt matrix removal, chromatographic separation in order to increase confidence in compound identification and reduce matrix effects, and high resolution mass spectrometry (within 5 ppm) for reduced signal to noise ratio and unequivocal compound identification [20]. For the first time, we employed the heavy isotopes of two analytes of interest (TNT and 1,3-dinitrobenzene, 1,3-DNB) as isotopic spikes to account for preconcentration factors and improve the accuracy and precision of quantification. The developed method was applied to environmental samples from a contaminated site in the Baltic Sea and to the direct analysis of MCs extracted from biota.

2. Methods

2.1. Materials

High purity water (MQ, $18.2\,M\Omega\,cm^{-1}$, Milli Q, Millipore) was used throughout. Methanol (MeOH) and acetonitrile (ACN) were of LCMS grade (Optima, Fisher Scientific). Stock standards ($1\,mg\,L^{-1}$), made from commercially available mixed standard solutions in ACN (EPA 8330B, $1000\,mg\,L^{-1}$, Restek, Germany), were diluted in ACN and kept at −20 °C. Working standards were prepared in 50:50 MQ: MeOH (v:v) and used within 24 h. Isotopically labelled trinitrotoluene (^{13}C, ^{15}N) and dinitrobenzene (^{13}C) standards in ACN ($1000\,mg\,L^{-1}$) were obtained from Cambridge Isotopes (LGC Standards, Germany). The complete list of compounds used in this study is given in Table 1. Brown borosilicate laboratory grade glass bottles were used for sample handling, storage and manipulation, in order to limit potential photolysis reactions.

Polystyrene divinylbenzene (6 mL, 200 mg) cartridges with a pore size of 50 Å and a particle diameter of 80 µm (Chromabond Easy, Machary-Nagel, Germany) were used for SPE. Preconcentration was undertaken with an automated SPE instrument (Autotrace, Telemark). The eluate was evaporated under vacuum with a centrifugal evaporator (Speedvac, Thermo). Samples were analysed with a biocompatible ultra-high performance liquid chromatographic system (UHPLC, Ultimate 3000, Thermo) consisting of a binary high pressure pump, a temperature controlled auto sampler, a column oven and an ultraviolet (UV) -visible diode array detector. Analytes were separated using a 150 × 2.1 mm Acclaim Explosives E2 column (Thermo, 3 µm pore size). The eluate from the UV detector was injected, via a divert valve, into a heated electrospray ionisation source and then into a high resolution quadrapole/orbitrap mass analyser (HESI-MS, Q Exactive, Thermo). The UHPLC-HESI-MS was controlled with Xcalibur and Chromeleon software.

2.2. Recovery experiments and incorporation of internal standards

Seawater sampled from surface waters near station W2 (Fig. S1) in Dec 2016, March 2017 and Jun 2017 was used to examine recoveries and matrix effects. For determination of process efficiencies (PE), matrix effects (ME) and recoveries (RE), seawater samples collected in June 2017 were pooled and then split into 7 × 1 L aliquots. Four aliquots were spiked with 100, 200, 300 and $400\,ng\,L^{-1}$ MCs and the remaining 3 aliquots preconcentrated without spiking. Aliquots were preconcentrated onto SPE columns after a column preconditioning step with 4 mL ACN followed by 4 mL MQ (gravity flow). Following results obtained from preliminary experiments on SPE optimisation (Figs. S2 and S3) samples were loaded at a flow rate of $8\,mL\,min^{-1}$ with the automated preconcentration system. After loading, SPE columns were rinsed with 10 mL MQ under gravity flow, and analytes eluted with 3.5 mL ACN. For sample elution, ACN was first loaded onto the column and left for ca. 5 min, prior to collection in a vial via gravity flow. Fifty µL of MQ was added to a 1.75 mL aliquot of the eluate as a keeper solvent and the solution subsequently evaporated to near dryness (ca. 50 µL) under vacuum with a centrifugal evaporator. The remainder was diluted to 0.5 mL with 50:50 MQ: MeOH (vol:vol) and mixed for approximately 20 s with a vortex mixer. Samples were transferred to amber HPLC vials and kept at 4 °C prior to analysis. The preconcentration factor was thus ca. 1000-fold. Process efficiency was determined by comparison of the samples spiked before preconcentration with standards in MQ: MeOH. Matrix effects were assessed by spiking MQ: MeOH fractions with 0, 100, 200, 300 and $400\,\mu g\,L^{-1}$ mixed MC standard. Recoveries were calculated according Ref. [27]. To examine the potential variability in ME, archived samples (n = 6–8, stored in the dark at 4 °C) from Dec 2016 and March 2017 were pooled and preconcentrated to make five equivalent MQ: MeOH fractions, which were spiked as for the June 2017 sample.

Table 1
Full list of compounds, monoisotopic masses, major ions and retention times observed for munition compounds (MCs) examined in this study.

Abbreviation	Name	Empirical formula	Monoisotopic mass	Major ions (*m/z*)	Detected ions[a]	Retention time (min)
HMX	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine	$C_4H_8N_8O_8$	296.0456	358.035	$(M+NO_3)^-$	4.1
				341.045	$(M+FA-H)^-$	
				331.016	$(M+Cl)^-$	
RDX	1,3,5-trinitroperhydro-1,3,5-trazine	$C_3H_6N_6O_6$	222.0348	284.0233	$(M+NO_3)^-$	6.6
				257.0043	$(M+Cl)^-$	
				267.033	$(M+FA-H)^-$	
				485.011	$(M+NFPA)^-$	
PETN	[3-Nitrooxy-2,2-bis(nitroxymethyl) propyl] nitrate	$C_5H_8N_4O_{12}$	316.014	378.0022	$(M+NO_3)^-$	18.2
NGL	Nitroglycerin	$C_4H_7N_3O_9$	227.0026	227.003	M^-	9.4
Tetryl	N-methyl-N-2,4,6-tetranitrolaniline	$C_7H_5N_5O_8$	287.0138	288.0224	$(M+H)^-$	12.0
				225.0263	$(M-NO_3)^-$	
				318.0327	$(M+MeOH-H)^-$	
				349.0021	$(M+NO_3)$-	
1,3,5-TNB	1,3,5-trinitrobenzene	$C_6H_3N_3O_6$	213.0022	183.0041	$(M-NO)^-$	6.9
				214.0101	$(M+H)^-$	
				213.0024		
				211.9949	M^-	
				198.0156	$(M-H)^-$	
					$(M-O+H)^-$	
1,3-DNB	1,3-dintrobenzene	$C_6H_4N_2O_4$	168.0171	138.0197[b]	$(M-NO)^-$	8.7
				153.031*		
				168.0168	$(M-O+H)^-$	
					M^-	
h1,3-DNB	^{13}C 1,3-dintrobenzene	$^{13}C_6H_4N_2O_4$	174.038	144.0397*	$(M-NO)^-$	8.7
				174.0377	M^-	
				159.050	$(M-O+H)^-$	
NB	Nitrobenzene	$C_6H_5NO_2$	123.032	UV		9.6
3,5-DNA	3.5-dintroanaline	$C_6H_5N_3O_4$	183.0280	182.020	$(M-H)^-$	14.1
TNT	2,4,6-Trinitrotoluene	$C_7H_5N_3O_6$	227.0184	226.0103	$(M-H)^-$	11.4
hTNT	$^{13}C^{15}N$ 2,4,6-Trinitrotoluene	$^{13}C_7H_5{}^{15}N_3O_6$	237.0324	236.0251	$(M-H)^-$	11.4
2,4-DNT	2,4-dinitrotoluene	$C_6H_6N_2O_4$	182.0333	181.025	$(M-H)^-$	11.9
2,6-DNT	2,6-dinitrotoluene		182.0333	181.025	$(M-H)^-$	13.5
2-NT	2-nitrotoluene	$C_7H_7NO_2$	137.047	UV		14.6
4-NT	4-nitrotoluene		137.047	UV		15.4
3-NT	3-nitrotoluene		137.047	UV		13.2
2-A-4,6-DNT	2-Amino-4,6-dinitrolouene	$C_7H_7N_3O_4$	197.0442	196.0359	$(M-H)^-$	16
4-A-2,6-DNT	4- Amino-2,6-initrolouene		197.0442	196.0359	$(M-H)^-$	16.4

[a] FA: formic acid; MeOH: methanol; NFPA: nonafluoropentanoic acid.
[b] isobaric interference with a background ion.

Heavy isotopes of TNT and DNB were tested as internal standards. The impact of sample matrix on the response of TNT, DNB, and their heavy isotopes was examined by preparing samples with nominal analysed concentrations in the range 10–40 $\mu g\,L^{-1}$ with spiked (0.08 $\mu g\,L^{-1}$ to 0.24 $\mu g\,L^{-1}$) and then preconcentrated artificial seawater. Non-linear mass bias effects were examined by varying the ratio of light to heavy isotopes by varying the concentrations of both isotope and standard [28].

2.3. Sample preparation

2.3.1. Study site

Kolberger Heide is located in the Southwest Baltic Sea near Kiel, Germany. Samples were collected from within an area restricted to marine traffic, centred around 54.4°N, and 10.3°E (Fig. S1). The study area is situated in an area that is ca. 10 m deep and is located three to five nautical miles off the coast. The area is a region of historical munitions disposal and is known to contain both German and British ordnance from World War II [5]. Approximately 30,000 t of munition including mines and depth charges were originally dumped in the area. The munitions at the site are thought to contain mainly RDX, TNT, and DNB but are also likely to contain impurities from the manufacturing process. Sub-sites within the Kolberger Heide represent intact and corroded munitions as well as completely exposed munition solids resulting from low-order detonation during blow-in-place (BIP) methods of in situ munition disposal [29]. Here we report data from seawater and biota samples collected from an area centred on a large pile of sea mines [30] on March 13, 2017. Sampling was conducted as part of a project (UDEMM) developing environmental impact assessment strategies related to future robotic munition removal approaches.

2.3.2. Dissolved MCs

Seawater samples were collected by pumping water from different depths using a Teflon bellows pump (Almatec A15) connected to a weighted polyvinyl chloride (PVC) hose. On collection, samples were directly filtered (0.2 µm, Acropak, Pall, Germany) into amber glass 1 L sample bottles and transferred to the laboratory. Samples were stored at 4 °C, spiked with 30 $\mu g\,L^{-1}$ hTNT and hDNB and preconcentrated as soon as possible (within 4 days) after collection following the procedure described for the recovery experiments.

2.3.3. Munition compounds in biota

Marine organisms (whole or partial) were collected manually by divers within 1 m of submerged munitions, and processed using a slight adaptation of published methods [4,19]. Organisms were sorted and identified to at least class level. Biota samples were frozen at −20 °C and then lyophilized. Biota was ground to a coarse powder using a stainless steel grinder for large samples or glass rod for small samples. Ca. 100–500 mg of

Table 2
Limits of detection, limits of quantification and sensitivities obtained for analysis of munition compounds.

Munition compound	Detection limit (pg)	Limit of quantitation (pg)	Sensitivity (ion counts or AU pg^{-1})
HMX	0.005	0.015	$4.0 \pm 0.3 \times 10^5$
RDX	0.28	0.67	$3.2 \pm 0.2 \times 10^4$
NGL	14	30	$1.8 \pm 0.5 \times 10^4$
PETN	0.18	0.54	$8.1 \pm 1.1 \times 10^3$
Tetryl	25.2	67	$1.1 \pm 0.1 \times 10^5$
1,3,5-TNB	0.54	1.19	$2.2 \pm 0.1 \times 10^5$
1,3-DNB	0.15	0.5	$7.7 \pm 0.9 \times 10^3$
NB[a]	10.2	509	13.3 ± 0.5
3,5-DNA	0.014	0.03	$1.4 \pm 0.5 \times 10^6$
TNT	0.036	0.10	$2.2 \pm 0.1 \times 10^5$
2,6-DNT	14.9	38	$4.6 \pm 0.4 \times 10^3$
2,4-DNT	1.05	2.7	$5.1 \pm 0.4 \times 10^4$
4-NT[b]			
2-NT[a]	26	1279	8.4 ± 0.2
3-NT[a]	27	1362	8.2 ± 0.8
4-A-2,6-DNT	0.05	0.15	$6.9 \pm 0.6 \times 10^5$
2-A-4,6-DNT	0.03	0.09	$1.8 \pm 0.2 \times 10^6$

[a] Detection by UV spectrophotometry.
[b] Co-elution with 2,4-DNT and lack of suitable MS signal excluded detection of 4-NT.

tissue was extracted in one or two mL of ACN. The lower volume of ACN was used when available sample mass was < 150 mg. Samples were sonicated at 40 kHz, 120 W in an ultrasonic bath (Pallsonic, Allpax, Germany) for 15 min at room temperature, and extracts filtered using 0.2 µm polytetrafluorethylene syringe filters (Whatman GD/X). Extracts were diluted with MQ to 30% ACN for analysis.

2.3.4. Sample analysis

Analytes were separated using high performance liquid chromatography. Mobile phases were (A) 95:5 MQ: MeOH (vol: vol) and (B) 100% MeOH. The flow rate was set to 0.3 mL min^{-1}. The gradient program consisted of a linear gradient from 33% to 50% B over 15 min, followed by a steep increase to 100% B over 5 min, a column wash (100% B, 2 min) prior to a return to the starting conditions (2 min) and re-equilibration of the column (3 min). The injection volume was 25 µL, and samples and standards were kept at 5 °C prior to injection. The column oven temperature was 31 °C.

The UV–visible detector channel A was set to 254 nm. Optimisation of HESI-MS conditions was undertaken by flow injection (injection volume 25 µL) into the HPLC eluent (50:50 A: B) at a flow rate of 0.3 mL min^{-1} with the source operated in the negative ion mode. Optimised conditions utilised an auxiliary gas temperature of 300 °C and an ionisation potential of −4 kV. The capillary temperature was set to 350 °C. The sheath gas flow rate was 40 arbitrary units and the auxiliary gas flow rate 10 arbitrary units. The mass detector was operated at a nominal resolution of 70,000 at *m/z* 200 with a scan range from *m/z* 100–750. Iodide (*m/z* 126.9050) and nonafluoropentanoic acid (*m/z* 162.9824, 262.9760) were used as lock masses. The instrument was mass calibrated every 3 days according to the manufacturer's instructions. The divert valve function was used to prevent the injection of high salt concentrations and potential contaminants during the elution of the solvent front. Thus the HPLC eluate was directed to waste until 2 min after injection, at which point the divert valve switched the eluate into the mass analyser.

3. Results and discussion

3.1. HESI-MS detection of MCs

Preliminary HPLC-HESI-MS analysis of the EPA 8330B standard mixture showed that HESI-MS detection was possible for 11 of the 13 different compounds, consistent with previous reports [17]. Nitrobenzene and NT could only be observed in the UV signal. Example extracted mass chromatograms for the compounds detectable by HESI-MS are provided in the Supplementary information (Fig. S4). Our priority compounds TNT, RDX, and the amino-DNTs produced strong ionisation signals. MCs with higher substitutions produced higher responses than those with fewer substitutions, so that ESI-MS of DNB and DNT resulted in lower ion counts than TNB and TNT. Quantification of 4-NT was problematic due to co-elution with 2,4-DNT and the lack of suitable MS signals.

Ionisation of toluenes resulted in production of deprotonated $(M\text{-}H)^-$ product ions (Table 1). However, ionisation of the non-toluene MCs was more complex, as previously reported [16,18,31,32]. The non-aromatic MCs HMX, RDX, Tetryl and PETN all showed a strong tendency to form multiple adducts with anions (e.g. nitrate, chloride and formate, Table 1, Fig. S5) [17,21,32]. The most abundant ion observed for RDX, HMX and PETN resulted from addition of NO_3^- [32,33]. For Tetryl, a product ion with *m/z* = 225.026 (Table 1, Fig. S5) resulted from the loss of NO_3^- and suggests Tetryl was subject to thermal decomposition within the electrospray source. Formation of M^- ions was observed for NGL, TNB and DNB. TNB and DNB self-decomposed in the source resulting in loss of a NO group (Table 1), presumably coupled to a rearrangement to form nitrophenols (e.g. 3,5-dinitrophenol: $(M\text{-}H)^-$ = 183.0047 was formed from TNB, Fig. S5).

We did not find any evidence for homolytic cleavage of RDX and HMX, despite the rather high source and capillary temperatures applied in our study, which were selected following preliminary experiments that indicated our source temperature conditions had only a minimal impact on ion intensities and ion formation (results not shown). The source temperatures applied here are routinely used in our laboratory to reduce the build-up of poorly volatile marine dissolved organic matter during sample analysis. The lack of homolytic cleavage may be linked to increased stability as a result of the formation of anionic adducts [32,33]. Thus, whilst our source conditions may not be ideal for certain analytes (e.g. Tetryl, RDX), our chosen conditions represented a compromise between sensitivity and a robust analysis pipeline suitable for routine analysis of marine samples.

3.2. Analytical detection limits, linear range and sensitivity

Limits of detection (LOD) and quantitation (LOQ) for each compound were calculated following injection of a 50:50 MeOH: H_2O mixture (n = 6) of the standard mixture [34]. Detection limits for compounds detected with MS (Table 2) ranged from 0.005 to 27 pg (0.21–1000 ng L^{-1} with a 25 µL injection volume). Detection limits were thus similar to those obtained previously for methods for analysis of explosive compounds in aqueous environmental samples (Table S1, [16,17]). The detection limits calculated for most of our important target compounds RDX, TNT and the A-DNT metabolites were all below 0.3 pg (12 ng L^{-1} with a 25 µL injection volume). The detection limits for Tetryl and 2,6-DNT were influenced by background contaminants with similar masses (isobaric interferences with < 5 ppm difference in monoisotopic mass). All compounds analysed produced linear responses up to our highest tested concentration of 10,000 pg (400 µg L^{-1} with a 25 µL injection volume). Sensitivities were relatively stable over time, with calibrations undertaken on six separate days over the course of two weeks resulting in relative standard deviation in sensitivities of < 10% for all compounds except Tetryl and TNB (rsd = 12%), and NGL (rsd = 27%).

3.3. Solid phase extraction

The low concentrations of MCs expected at munition disposal sites necessitate preconcentration of MCs. We used a polymeric styrene divinylbenzene stationary phase, as these effectively preconcentrate MCs [17]. Recovery efficiencies, ME and PE for MCs spiked into seawater

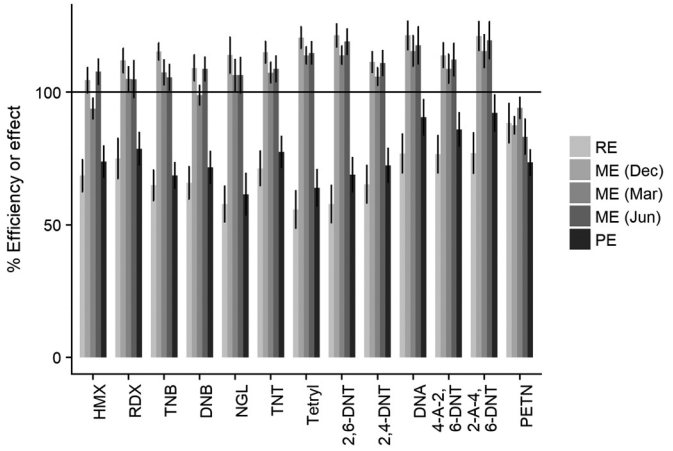

Fig. 1. Recovery efficiency (RE, n = 4), matrix effects (ME, n = 4) observed for samples collected in December, March and June, and process efficiency (PE, n = 4) obtained for munition compounds extracted from seawater and analysed by UHPLC-HESI-MS.

(salinity = 18) collected from the Baltic Sea were assessed (Fig. 1) [27]. Process efficiencies were lower for NGL and Tetryl (61–64%), and higher for DNA and the A-DNTs (86–92%). Unusually, matrix effects resulted in a maximum 20% increase in signal responses compared to those observed for MQ: MeOH. No consistent seasonal ME was observed across the MCs. The calculated recoveries were thus lower for Tetryl, NGL, and 2,6-DNT (55–58%), and higher for PETN (88 ± 7%).

3.4. Addition of internal standards

Natural seawater contains compounds that impact the preconcentration and detection of MCs. Inclusion of internal standards can be expected to improve the confidence of quantification by accounting for variability in PE. Nevertheless previous studies have shown that mass bias effects can be non-linear for ESI-MS [28]. We examined the mass bias for hTNT and hDNB over a range of concentrations relative to TNT and DNB, respectively (Fig. S6). Expected versus observed isotopic ratios ($R_{expected}/R_{observed}$) showed a linear response for TNT (slope = 1.18, r^2 = 0.999). Dinitrobenzene has one of the lowest ESI-MS sensitivities of the standard mixture and has a low PE (Fig. 1). The low sensitivity of DNB meant that a non-linear response was observed when $R_{expected} \geq 1.5$ and hDNB was < 20 μg L^{-1} (where R is the ratio of light to heavy isotope, Fig. S6).

As there are no certified reference materials available for the analysis of MCs in environmental samples, we tested the accuracy and precision of the internal standard method on spiked samples. Artificial seawater (1100 mL) was spiked with 50 ng L^{-1} TNT and DNB, and 30 ng L^{-1} hTNT and hDNB. The TNT concentration determine via isotope dilution was 50.7 ± 1.9 ng L^{-1}, which was both more accurate and more precise than the concentration of 40.8 ± 4.3 ng L^{-1} (n = 6) obtained using an external calibration curve (without accounting for the 77 ± 6% PE observed for TNT). For DNB, quantification via the isotope spike resulted in a concentration of 53 ± 2 ng L^{-1}against a value of 44 ± 3 ng L^{-1} obtained using the external calibration (not accounting for 72 ± 6%PE). In addition, we observed notable day to day variability in the calibration factor for DNB (e.g. k = 1.18 on 14.3.2017 vs k = 0.81 on the 8.6.2017), so that a single calibration factor could not be applied. The variability in the calibration factor was related to variability in the relative abundance of the DNB ions used in quantification (Fig. S7). DNB formed three ions via self-decomposition, however we excluded 138.0197 $(M\text{-}NO)^-$ from our routine detection method because of isobaric interference with a background ion. The relative abundance of the two remaining ions varied from day to day. As heavy isotopes are extremely useful for accurate determination of MCs the availability of further heavy isotope standards would be highly beneficial for the determination of MC concentrations in the environment.

3.5. Quantification of MCs in seawater and biological material

For seawater samples our developed protocol incorporated an isotopic spike to the sample of TNT and DNB of 30 ng L^{-1} and preconcentration of 1 L of seawater. TNT and DNB concentrations were calculated using the added isotopes (Fig. S8). Since external calibration was used for the quantification of the remaining MCs, results reported here are not fully quantitative, with PE (Fig. 1) suggesting a ca. 10–20% underestimation for ADNTs and RDX respectively. Application of standard addition or inclusion of further isotopes is to be recommended for future studies.

We demonstrated our analytical method in a preliminary survey of the distribution of MCs at a munition disposal site in the Baltic Sea. Recent work in the area has shown accumulation of TNT and ADNTs in naive mussels in a biomonitoring study [5]. Here we present results for MCs determined in filtered seawater samples collected from water several depths at two stations (W2 and W7) within 25 m of discarded mines and a BIP crater site respectively, and from two depths at one site 1 km to the north (W8). Furthermore, we present results from a screening study of benthic biota collected close to the disposal site and the crater.

We detected low concentrations of TNT and DNB (ca. 1–15 ng L^{-1}) and trace (< ng L^{-1}) levels of RDX, TNB, DNA, 2A-4,6-DNT, and 4A-2,6-DNT in the waters at Kolberger Heide (Table 2.). The levels of MCs even in contaminated waters were below those required for direct analysis of MCs [16]. We detected RDX, DNB, TNT, 2A-4,6-DNT, and 4A-2,6-DNT in every sample analysed, whilst TNB, and DNA were detected intermittently (Table 2). Some variability was observed for samples collected at the same stations and depths (e.g. site W7, 8 m), which likely related to collection of consecutive samples into 1 L bottles via pumping, as this meant that samples were subject to small scale temporal and spatial variability. Dinitrotoluene, NGL, HMX, and PETN were all below the LOD in these samples. The lack of HMX likely relates to its limited use during WWII [35]. All detected concentrations were several orders of magnitude below EC_{50} levels for marine organisms [6] (e.g., EC_{50} TNT for mysids = 0.26 mg L^{-1}) and concentrations of TNT detected at the site were lower than previously reported concentrations (see ref [1] for review) (Table 3).

The presence of low levels of ADNTs throughout our study area was likely a consequence of microbial degradation of TNT [4]. DNB was found in seawater samples at levels similar to TNT. DNB was also used extensively in ammunition, with some forms of ordnance containing up to 50% DNB by weight [3].

We also present results from a preliminary survey of benthic organisms found at the study site (Fig. 2). Organisms were collected close to the mine mound and from within the crater site. In addition to the seven compounds identified in the water column, TNB, DNA, and PETN were observed in at least one organism. NGL appeared to be the most abundant MC in *Alga* and *Tunicata* at the crater site, whilst RMX was the most abundant at the mine mound. Of the three types of organisms examined, *Asteroidea* sp. (starfish) contained the highest levels of MCs, with 24 μg g^{-1} TNT in the starfish collected at the crater site. These high concentrations of TNT could have arisen from direct contact with exposed munitions, since *Asteroidea* are benthic feeders. TNT toxicity to *Asteroidea* has not been assessed to our knowledge [6], but the high levels of TNT measured in this specimen require further investigation. *Alga* and *Tunicata* also contained higher concentrations of MCs (1–100 ng g^{-1} RDX, DNB, NGL, TNT, DNT and ADNT) at site W7, consistent with higher levels of TNT and ADNT observed in *Asteroidea*. The higher concentrations of MCs observed in these organisms at this site could be linked to the employment of BIP munitions disposal, which can result in unexploded residuals [36] and increased environmental exposure to MCs.

Table 3
Concentrations of MCs obtained for seawater samples collected in the vicinity of Kolberger Heide, a known munition dump site. Italicised numbers denote the range observed for samples preconcentrated in duplicate.

	Depth m	RDX[a] ng L^{-1}	TNB[a] ng L^{-1}	DNB[b] ng L^{-1}	TNT[b] ng L^{-1}	DNA[a] ng L^{-1}	4A-2,6-DNT[a] ng L^{-1}	2A-4,6-DNT[a] ng L^{-1}
W2	2	< d.l	< d.l	1.22	0.10	0.0022	0.192	0.066
(Mine site)	5	< d.l	< d.l	9.46	0.63	0.0033	0.285	0.095
	8	0.23	< d.l	6.34	1.11	0.0012	0.107	0.048
		0.03		*0.04*	*0.07*	*0.0006*	*0.010*	*0.003*
W7	2	0.36	0.27	5.73	0.90	0.0045	0.152	0.067
(Crater site)				*0.38*	*0.01*	*0.0001*	*0.007*	*0.001*
	5	0.28	0.23	6.03	0.95	0.0049	0.173	0.080
		0.03	*0.01*	*0.42*	*0.14*	*0.0003*	*0.019*	*0.008*
	8	1.9	< d.l	5.41	10.6	0.0031	0.081	0.0051
		1.5		*1.36*	*4.4*	*0.0014*	*0.006*	*0.014*
W8	2	0.80	0.24	9.15	0.59	0.0045	0.146	0.011
		0.28	*0.03*	*0.6*	*0.02*	*0.0034*	*0.006*	*0.002*
	8	0.02	< d.l	0.47	0.13	0.0033	0.459	0.114
				0.18	*0.01*	*0.0006*	*0.001*	*0.001*

[a] Process efficiencies not accounted for.
[b] Quantified by isotope dilution.

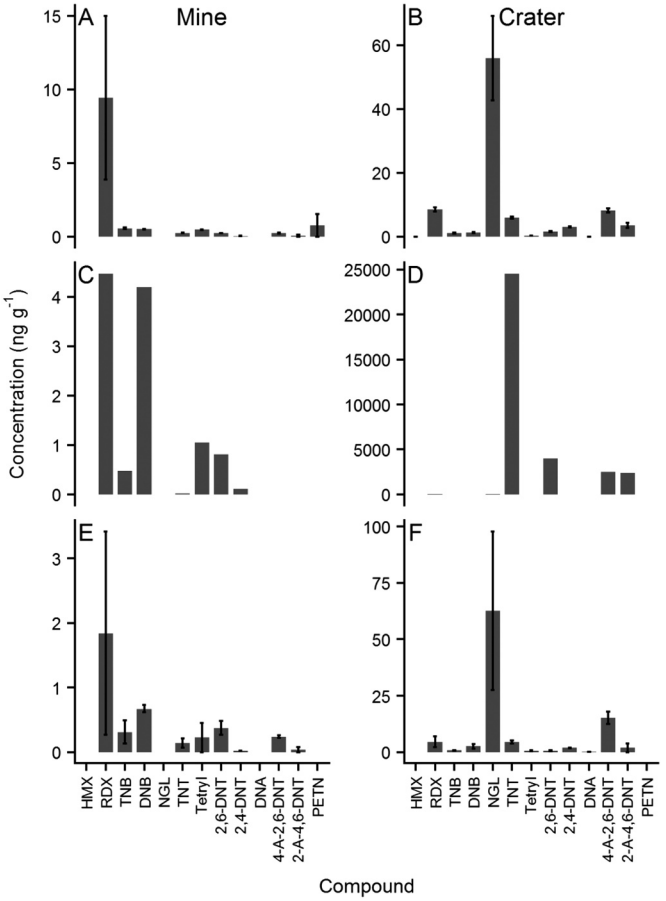

Fig. 2. Concentrations of munition compounds detected in *Alga* (A, B, n = 2), *Asteroidea* (C, D, n = 1), and *Tunicata* (E, F, n = 2) collected from the mine site (A, C, E) and from the crater site (B, D, F). Error bars represent the sample range.

4. Conclusion

We have shown that the application of HPLC with detection by high resolution ESI/MS combined with SPE results in a sensitive and selective procedure for the analysis of MCs in seawater. Isotope dilution is shown to further improve quantification of MCs when heavy isotopes are available. Whilst other techniques may offer greater sensitivity for particular analytes, they have yet to be applied to the analysis of seawater or to metabolites of MCs such as the amino dinitrotoluenes. Determination of MCs at environmental concentrations will allow for screening for MCs in possibly contaminated sites. As munitions manufactured in different countries and at different times in history contain varying compositions of primary explosives, the ability to reliably identify MCs in the environment also opens up the potential for MC fingerprinting. Furthermore, detection of MCs in water samples allows for sampling strategies that do not directly disturb sediments and potentially unexploded munitions. A reliable method for MC determination also allows for a more accurate assessment of MC fluxes and fate at contaminated sites. Further studies of MCs in the marine environment will help constrain risks associated with disposal sites, assess potential contamination resulting from remediation operations, and contribute to improved management of historical munitions disposal sites.

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Declaration of Interest

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2019.03.050.

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