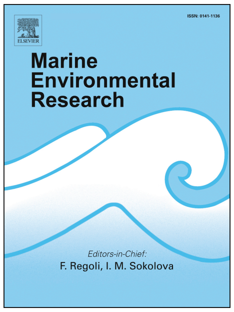

Identification and toxicological evaluation of cyclic sulfonium ion degradation products of sulphur mustard

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PII: S0141-1136(20)30199-9

DOI: https://doi.org/10.1016/j.marenvres.2020.105047

Reference: MERE 105047

To appear in: *Marine Environmental Research*

Received Date: 28 February 2020

Revised Date: 30 May 2020

Accepted Date: 9 June 2020

Please cite this article as: Hemström, P., Holmgren, K.H., Hammarström, B.E., Larsson, A., Östin, A., Identification and toxicological evaluation of cyclic sulfonium ion degradation products of sulphur mustard, *Marine Environmental Research* (2020), doi: https://doi.org/10.1016/j.marenvres.2020.105047.

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CRediT author statement

Hemström, P: Investigation-Analytical Chemistry, Writing - Original Draft-Chemistry

Holmgren, K. H: Investigation-Analytical Chemistry, Visualization

Hammarström, B. E: Investigation-toxicology, Writing - Original Draft-Toxicology

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Identification and toxicological evaluation of cyclic sulfonium ion degradation products of sulphur mustard

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Abstract

In the aftermath of WWII large amount seized German chemical munitions were dumped in the Baltic Sea by Allied forces. In this work, we have compared the chemical content of the solidified blocks of dumped WWII mustard gas collected from the Baltic Sea with solid precipitate from stored mustard gas, known as heel. We have identified the same cyclic sulfonium ions in both samples. In assessing the environmental and toxicological impact of dumped sulphur mustard munitions on the world's oceans the potential risk posed by cyclic sulphur mustard salts have so far not been incorporated.

The toxicity of 1-(2-chloroethyl)-1,4-dithiane and its hydrolysis product 1-(2-hydroxyethyl)- 1,4-dithiane was evaluated using three different cell lines. Their effect on released pro-inflammatory cytokines was also measured. The toxicity tests showed low toxicity and low pro-inflammatory response and we therefore conclude that the environmental threat posed by these compounds is low.

1. Introduction

In the aftermath of WWII, large amounts of seized German chemical weapons, mainly mustard gas, were disposed by dumping at sea (Beldowski et al., 2016; Duursma, 1999; Glasby, 1997; Greenberg, Sexton, & Vearrier, 2016) . Today, the dumped mustard gas is in the form of blocks, thought to have resulted from polymerisation processes that occur when slowly corroding containers rupture and the hydrophobic mustard gas is exposed to seawater. These blocks preserve some mustard gas internally for long times, the corrosion/degradation process could take several hundred years (Jurczak & Fabisiak, 2017). Thus, the presence of large amounts of mustard gas munitions in the sea pose a clear occupational hazard for workers at sea, *e.g.* fishermen who may accidentally catch remains of dumped munitions during trawling. When analysing Baltic Sea sediments, neither mustard gas nor its expected primary hydrolysis product thiodiglycol are generally found, if this reflects an absence or the difficulty of analysing sediments is unclear (Söderström, 2014). If mustard gas is detected, it will be at ppt levels close to leaking objects (Söderström, 2014). Instead, the cyclic products dithiane [28] [substance numbers according, table 1 and 2] and oxathiane [7] are commonly found and used as markers for potential leakage of mustard gas degradation products to the surrounding sediments (Black, Clarke, Cooper, Read, & Utley, 1993; Magnusson, Nordlander, & Ostin, 2016; Roen, Unneberg, Tornes, & Lundanes, 2010). The formation of dithiane [28] and oxathiane [7] is thought to proceed through a charged cyclic intermediate: 1-(2-chloroethyl)-1,4-dithiane [3] and 4-(2-chloroethyl)-1,4-oxathiane [D], respectively (Figures 1 and 2). In the USA, tests connected to the destruction of old one-ton containers of mustard gas, untouched for 50 years, showed that up to 50% of the content consisted of a solid precipitate, called 'mustard heel' (Rohrbaugh & Yang, 1997). We hypothesise that formation of polymerized blocks on the seafloor and mustard heel involve the same chemistry, in which the charged cyclic species 1-(2-chloroethyl)-1,4-dithiane [3] and 4-(2-chloroethyl)-1,4-oxathiane [D] are key intermediates. In the work reported here, we analysed the polar fraction of a polymerised mustard block dredged up by a trawler and relate the results to information on mustard heel.' The toxicity of the intermediates in the formation of dithiane [28], 1-(2-chloroethyl)-1,4-dithiane [3] and 1-(2-hydroxyetyl)-1,4-dithiane [2], were also evaluated.

2. Material & Methods

2.1. Chemicals

Solidified samples of sulphur mustard from dumped chemical munitions were caught accidentally by the fishing vessel *Tanja av Grebbestad* while fishing near Bornholm in the Baltic Sea. The lump analysed in this study was solid with a porous structure, and had been stored in a closed vial at room temperature since 1991.

Mustard heel was obtained from sulphur mustard stored in steel barrels since the 1950s by the Swedish Armed Forces. The barrels were emptied and the green tar-like residue was sampled with a hollow glass rod, the barrels were then rinsed with deionized water (10 mL), and a sample of the resulting solution was collected. This water extract formed a three phase system with a sulphur mustard phase, a water phase (fraction 2) and an emulsion phase. The mustard heel had similar consistency to moist sugar, it was soluble in water, and accounted for less than one percent of the initial mustard gas.

Mustard gas salts are difficult to find commercially. Therefore the reference substances for their chlorinated [D,3] [substance letter according figure 1] and hydrolysed forms [1,2] were synthesised following previously reported procedures with slight modifications (Davies & Oxford, 1931;

Stahmann, Fruton, & Bergmann, 1946), Figures 1 and 2. Final products were white solids [D,3] or colourless crystals [1,2].

All other chemicals were purchased from Sigma-Aldrich (www.sigmaaldrich.com) with at least *pro-analysi* purity.

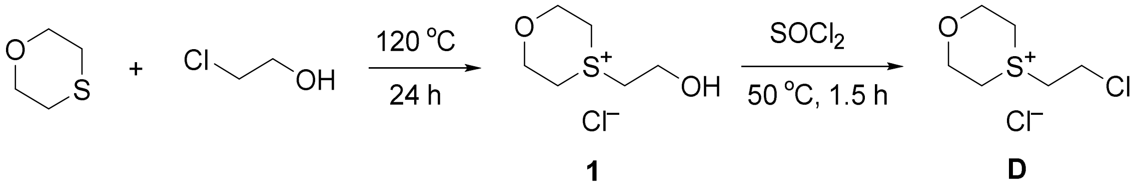


Figure 1. Synthesis of oxathian sulphur mustard salt, 4-(2-chloroethyl)-1,4-oxathian [D], and its hydrolysis product 4-(2-hydroxyethyl)-1,4-oxathian [1].

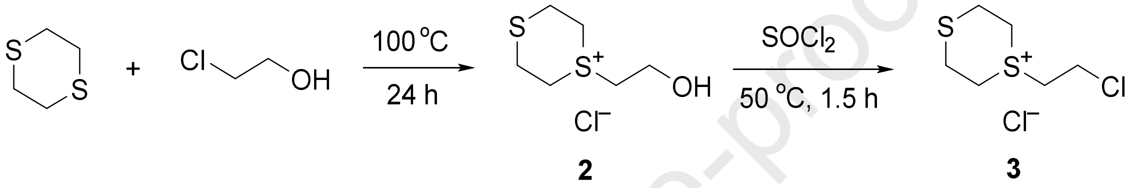


Figure 2. Synthesis of dithiane sulphur mustard salt, 1-(2-chloroethyl)-1,4-dithiane [3], and its hydrolysis product 1-(2-hydroxyetyl)-1,4-dithiane [2].

2.2. Toxicological analysis

Toxicological analysis were performed on 1-(2-chloroethyl)-1,4-dithiane [3], and its hydrolysis product 1-(2-hydroxyetyl)-1,4-dithiane [2].

In the statistical evaluation in the toxicological experiments below are the results expressed as mean values ± standard deviation (S.D). The data was analysed by one-way-ANOVA followed by Dunnett's post-hoc test. Data was considered significant at $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$). GraphPad Prism v.5.02 (GraphPad Software, San Diego, CA, USA) for Microsoft®, Windows, was used for the statistical analyses.

2.2.1.Analysis of cell toxicity

Cells of the human type II alveolar epithelial cell line A549 (ATCC CCL-185; American Type Culture Collection) and the mouse fibroblast cell line L929 (ATCC CCL-1) were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS; Hyclone, Perbio Science, Aalst, Belgium) and 50 µg/mL gentamicin. The human bronchial epithelial cell line BEAS-2B transformed by an adenovirus 12-SV40 hybrid (ATCC CRL-9609) was grown in serum-free bronchial epithelial cell basal medium with supplements (BEGM; Cambrex, Verviers, Belgium). BEAS-2B cells were cultivated in tissue culture flasks or on plates pre-coated with fibronectin, vitrogen and bovine serum albumin. All cells were maintained at 37° C in a humidified atmosphere containing 5% CO_2. For experiments, the cells were seeded in 24- or 96-well culture plates and allowed to attach overnight before exposure to sulphur mustard salt or sulphur mustard. The cells were exposed for 1 h, washed and incubated in fresh medium for another 18 h. Their viability (percentage of apparently living cells) was assessed fluorescently, using an AlamarBlue assay kit (Thermo Fisher), 24 h after exposure. The significance of differences between treated cells and controls (exposed to medium only) was also assessed.

2.2.2. Analysis of released pro-inflammatory cytokines

The release of the pro-inflammatory cytokines interleukin (IL)-8 and IL-6 into the culture media was measured using an immunoassay (ELISA). Lung epithelial cells were seeded at 5×10^4 in 24-well plates. After sulphur mustard salt or sulphur mustard exposure for 1 h the medium was removed. Fresh media was added in a total volume of 0.5 mL for an additional 23 h. The supernatants were then separated from the cells by centrifugation. IL-8 and IL-6 were measured in the cell-free fluid using the DuoSet ELISA Development kit (R&D Systems, Abingdon, UK) according to manufacturer's protocol. Stimulation with TNF-α was used as a positive control and exposure to complete medium served as negative control. Each experiment was performed with 4 replicates.

2.3. Sample preparation

2.3.1. Extraction of mustard gas lumps

A sample of 1.26 mg of a solid sulphur mustard lump was extracted with 2 mL dichloromethane (DCM) for 3 hours at room temperature. The extract was concentrated 10-fold then analysed by gas chromatography/ mass spectrometry with electron impact ionization (GC/MS EI: see section 2.4 for instrumental details). Trimethyl silanol (TMS) derivatives of the analytes were also prepared, prior to GC/MS analysis, by adding 10 µL of N,O-bis(trimethylsily)trifluoroacetamide (BSTFA) and 1% trimethylchlorosilane (TMCS) to 90 µL DCM extract and heating for 60 minutes at 60 °C.

The DCM-extracted sample was then subjected to a second extraction by incubation in 2 mL 50/50 ACN-water for 3 hours at room temperature. The extract was subsequently divided and half was evaporated to 500 µL and analysed by liquid chromatography-high resolution mass spectrometry (LC-HRMS), as described below.

A second lump (4.66 mg) was extracted first with 2 mL acetonitrile and then with 2 mL water for two hours each in an attempt to extract more compounds for LC-MS analysis. 1 mL of each extract was then combined and analysed by LC-HRMS (data not shown). The remaining part of the acetonitrile fraction was analysed by GC/MS after derivatization as described above.

2.3.2. Mustard heel

Hundred microliter of the aqueous phase of the mixture obtained by rinsing mustard heel, as described above (fraction 2), was diluted with 100 µL ACN then 500 µL DCM was added. DCM and ACN are miscible while the water forms a separate phase, which was removed, then the organic phase was analysed (in both native form and after silylation) by GC/MS, as described below.

The aqueous phase (fraction 2) was also diluted 1000-fold with 50/50 acetonitrile/water and subjected to LC-HRMS analysis, as described below.

2.4. Chemical analysis

Mustard heel from sealed long-term stored mustard gas containers and mustard lumps from dumped mustard gas were analysed for mustard gas-related products by GC/MS and LC/MS, as described below.

2.4.1. GC/MS

An Agilent Technologies 7890A gas chromatograph equipped with a 30 m, 0.25 mm id DB-5MS column (0.25 µm film) coupled to an Agilent 5975C mass selective detector was used for all GC/MS EI analyses. The carrier gas was helium at a constant 1.0 mL/min, and the samples were introduced via splitless injection of 1 µL samples at 250 ºC. The GC temperature program for analysis of non-polar fractions consisted of 40 ºC for 1 minute, followed by a linear increase of 10 ºC/min to 280 ºC, which was held for 5 minutes. The program for analysing polar silylated fractions was identical except that the initial temperature (held for the first minute) was 60 ºC. In both cases, the temperatures of the transfer line, ion source and quadrupole were 280, 230 and 150 ºC, respectively. The mass spectrometer was used in scan mode (29-500 m/z).

The analytes were identified using the deconvolution software AMDIS software (Automated Mass Spectral Deconvolution and Identification System, NIST, version 2.73, 2017) and the OPCW OCAD MS-library v 21 (OPCW central analytical database, 2019). An AMDIS net match factor of ≥ 80 and RI match of ± 30 units from a reference value were the identification criteria. An in-house AMDIS library including compounds related to sulphur mustard was also used. Peaks with fragmentation patterns similar to sulphur mustard-related compounds were sought in NIST databases.

2.4.2. LC-MS

The water-soluble fraction extracted from sulphur mustard containers was analysed by LC-HRMS. The extracts had a strong green colour, probably due to metal ions from the stainless steel containers, and they were too paramagnetic for NMR analysis.

Polar fractions were analysed using a Dionex Ultima 3000 chromatographic system fitted with a Waters AcQuity UPLC HSS C18 (1.8 µm) 2.1x100 mm column coupled to a Bruker Impact QTOF mass spectrometer operated in positive electrospray ionization (ESI) mode. The mobile phase consisted of 10 mM ammonium acetate + 0.1% formic acid (A) and 99% methanol containing 10 mM ammonium acetate + 0.1 % formic acid (B). The A:B ratio was held at 95:5 from 0 to 1 min, then changed linearly to 85:15 at 1.5 min, and 5:95 at 4.5 min. After a hold to 5 min, the column was re-equilibrated with the initial 95:5 A:B mixture from 5.1 to 6 min. The flowrate was 0.4 mL/min throughout each run. The MS settings were: capillary voltage 4500 V, end plate offset -500 V, dry gas flow 9 L/min, heater temperature 200 °C, scanned mass range 100-800 m/z. Sodium formate clusters injected at the start of analysis were used for internal calibration.

Mass spectra of the two hydroxyl ethyl compounds [1 and 2] were also compared to those of synthesized standards. Identified compounds are listed in Table 1, in which compounds with multiple plausible structures for the same formula are noted as tentative.

3. Results

3.1. Toxicological analysis

The toxicity of the mustard salts [2 and 3] in Figure 1, was tested following Karacsonyi (Karacsonyi, Shanmugam, & Kagan, 2009).

3.1.1. Analysis of cell toxicity

In order to test cell toxicity, three cell systems were used: pulmonary epithelial cell line A549, pulmonary epithelial cell line BEAS-2B, and L929 mice fibroblast cells. Exposure to concentrations ≥ 250 µM for 24 hours significantly reduced the viability of A549 and BEAS-2B pulmonary epithelial cells, and the highest test concentration (1000 µM) reduced their viability by ca. 20%. L929 mouse fibroblast cells were slightly more susceptible to exposure to the mustard salts, which reduced their survival by ca. 30% at 1000 µM. No difference in toxicity towards the three cell systems between the mustard salt [2] and [3] samples was detected. Exposure to mustard gas [30] at concentrations ranging from 0.19 to 100 µM caused a significant dose-dependent reduction in viability of both A549 and BEAS-2B cells. At 100 µM (the highest test concentration) it reduced these cells' viability by 53 and 50%, respectively (Figure 3).

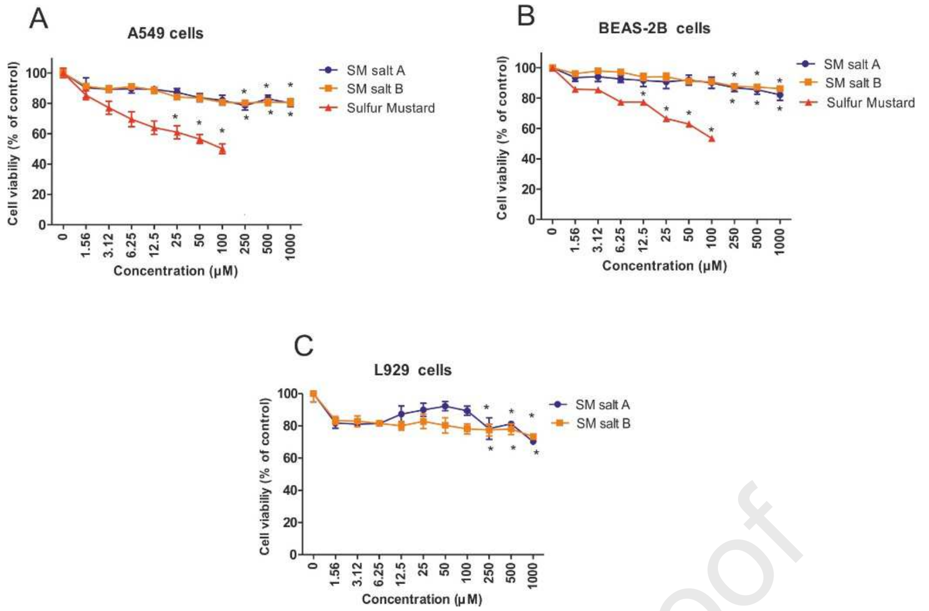


Figure 3. Effects of 1-(2-chloroethyl)-1,4-dithiane [3] and its hydrolysis product 1-(2-hydroxyethyl)- 1,4-dithiane [2] on viability of (A) an alveolar cell line (A549), (B) a bronchial epithelial cell line (BEAS-2B) and (C) a mouse fibroblast cell line (L929). Cell viability was assed using the AlamarBlue assay and the results are presented as percentages of the viability of unexposed control cells. The significance of differences between treated cells and controls (exposed to medium only) was assessed by one-way ANOVA and Dunnett's post-hoc-test. Differences that are significant at the p <0.05 level are marked in presented figures by one (*).

3.1.2.Analysis of released pro-inflammatory cytokines

The expression of pro-inflammatory cytokines in pulmonary epithelial cells A549 and BEAS-2B as a response to exposure to 1-(2-chloroethyl)-1,4-dithiane [3], and its hydrolysis product 1-(2-hydroxyetyl)-1,4-dithiane [2] was also measured and correlated to unexposed cells.

Both sulphur mustard salts induced a low upregulation of IL-8 secretion in the alveolar A549 cell line and in the bronchial BEAS-2B cell line compared to control. 1-(2-hydroxyetyl)-1,4-dithiane [2] had a slightly higher effect of IL-8 secretion in BEAS-2B cells compared 1-(2-chloroethyl)-1,4-dithiane [3], in samples at 500 µM (figure 4).

Exposure to sulphur mustard [30] however induced a significant dose-dependent production of IL-8 in both A549 and BEAS-2B cells. The highest concentration of IL-8 was achieved in supernatants at 25µM for A549 (4.3±0.07 fold increase compared to control) and for BEAS-2B (2.0±0.1 fold increase compared to control).

Neither of the sulphur mustard salt [2 and 3] samples tested nor sulphur mustard [30] samples induced IL-6 production above background levels, data not shown.

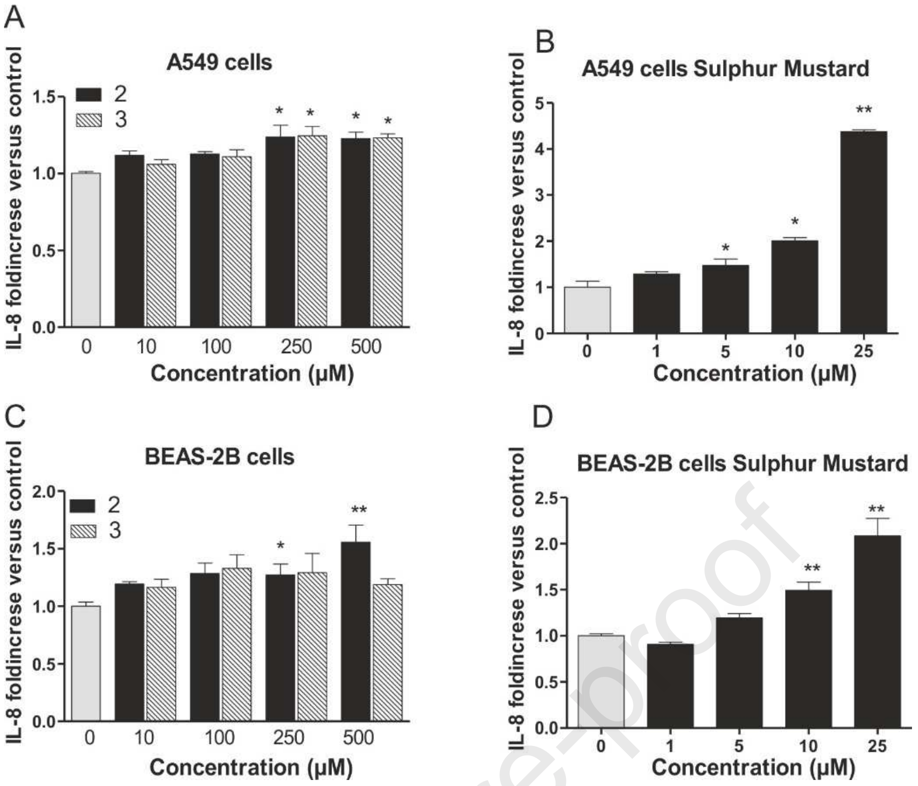


Figure 4. Release of pro-inflammatory cytokine IL-8 from (A- B) an alveolar cell line (A549) as a result of exposure to 1-(2-chloroethyl)-1,4-dithiane [3], and its hydrolysis product 1-(2-hydroxyetyl)-1,4-dithiane [2] (A) and mustard gas [30] (B), respectively. Release of pro-inflammatory cytokine IL-8 from (C-D) a bronchial epithelial cell line (BEAS-2B) as a result of exposure to 1-(2-chloroethyl)-1,4-dithiane [3], and its hydrolysis product 1-(2-hydroxyetyl)-1,4-dithiane [2] (C) and mustard gas [30] (B), respectively. Error bars indicate standard deviations. Statistical analysis was performed using one-way-ANOVA followed by Dunnett`s post- test testing significances vs. untreated cells (* $p < 0.05$, ** $p < 0.01$).

3.2. Identification of mustard gas degradation products with GC/MS

The GC/MS analysis of lump extracts detected sulphur mustard and a number of sulphur mustard-related compounds such as 1,2-bis(2-chloroethylthio)ethane [33], bis(2-chloroethyl) disulphide [32] and bis(2-chloroethylthioethyl)ether [15] (Figure 5). Three cyclic compounds were detected: 1,4-oxathiane [7], 1,4-dithiane [28] and 1,2,5-trithiepane [29]. The overall findings were similar to those previously reported (Mazurek, Witkiewicz, Popiel, & Sliwakowski, 2001). Following derivatization, thiodiglycol [34] and other hydrolysis products were detected in the acetonitrile extract of the mustard lump (Figure 6). Names, analytical data, formulae and structures of compounds identified in mustard lump and heel are presented in Table 2. Most of the compounds identified by GC/MS were found both in lump and heel.

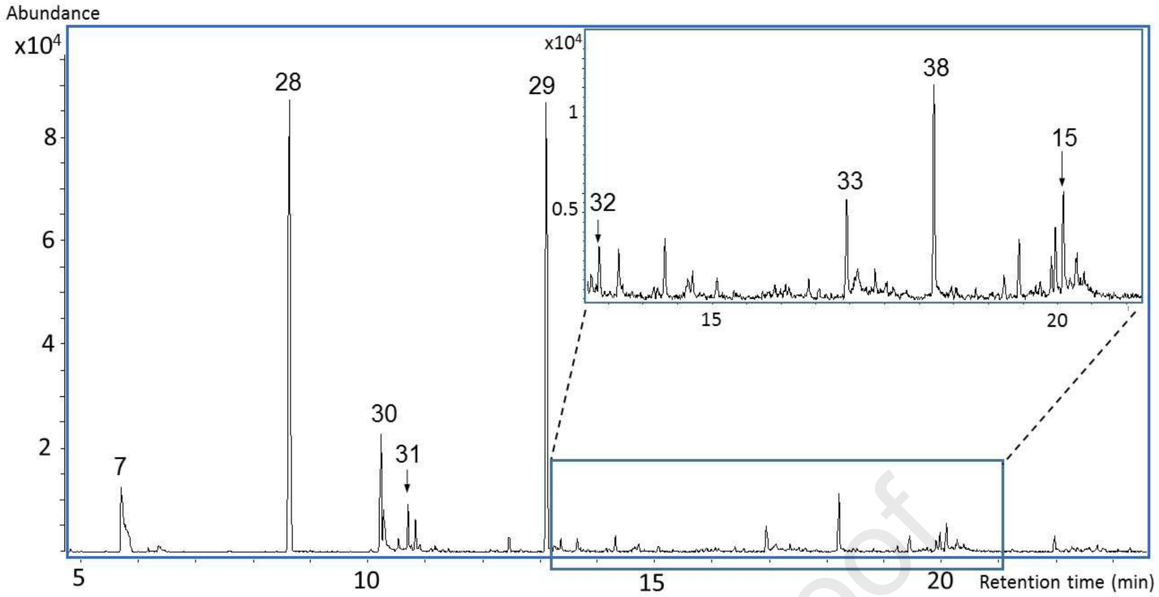


Figure 5. GC/MS Reconstructed ion chromatogram (m/z 104, 109, 210, 123, 152, 154, 182 and 190) of compounds related to sulphur mustard found in the dichloromethane extract of lump. (Identified compounds numbered according to Table 2).

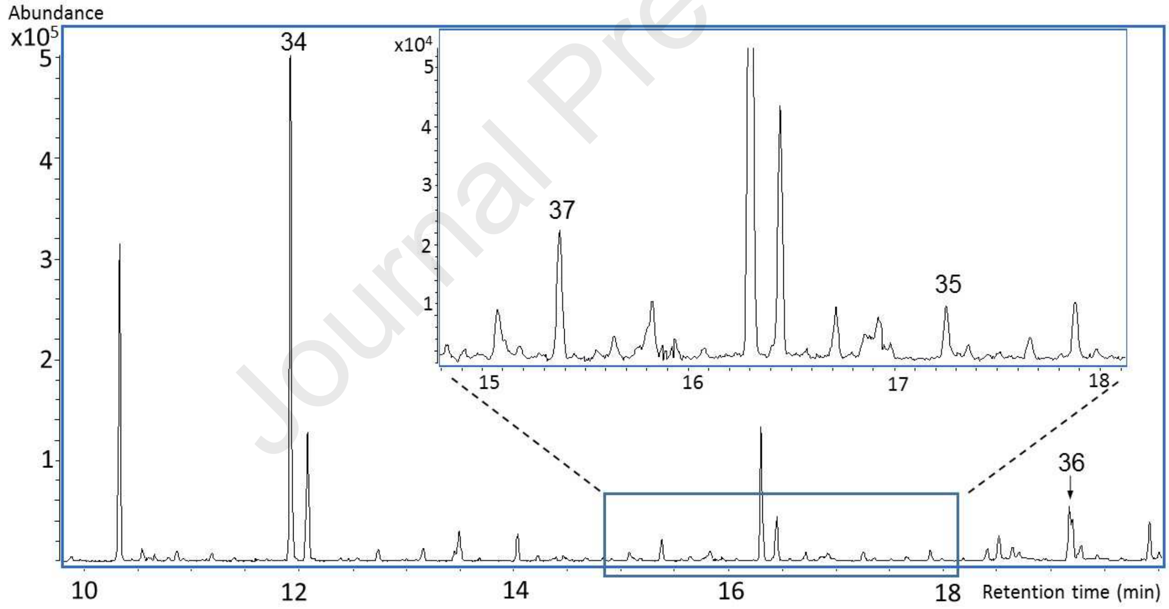


Figure 6. GC/MS extracted ion chromatogram (m/z 116, 283 and 298) of TMS-derivatized compounds related to sulphur mustard found in the acetonitrile extract of lump. (Identified compounds numbered according to Table 2)

3.3. Identification of mustard gas degradation products with LC-HRMS

Analysis of sulphur mustard degradation products by LC-HRMS detected numerous compounds. Most importantly, some of the most abundant constituents of both extracts of dumped munition blocks and stored sulphur mustard were hydrolysed cyclic sulphur mustard salts. The two hydrolysis products [1] and [2] in Figures 7 and 8 were identified by comparing their retention times and exact masses with those of synthesised standards as shown in Table 1. The other compounds were tentatively identified by exact masses, isotope patterns and manual spectra interpretation Table 1.

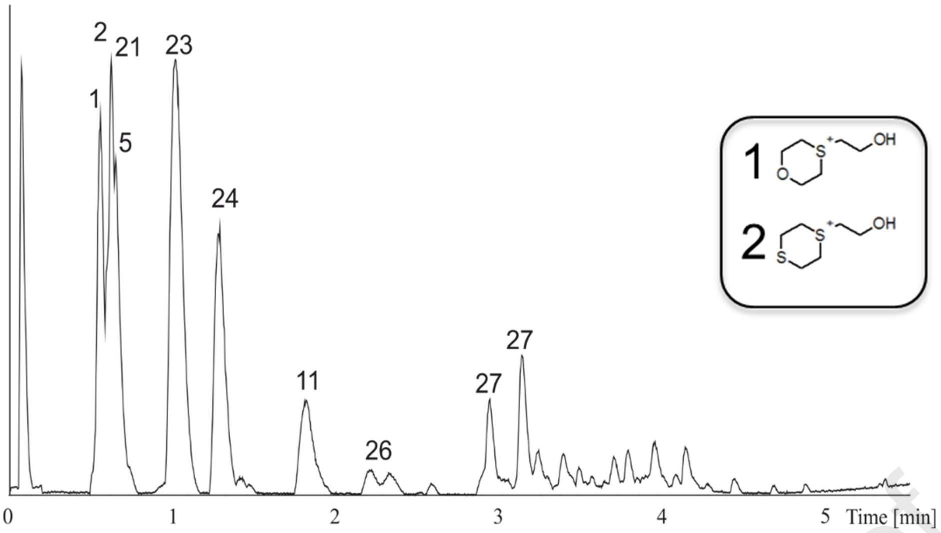


Figure 7. LC-HRMS chromatogram of water-extractable compounds (numbered according to Table 1) in a solid lump from dumped German WWII mustard gas munitions.

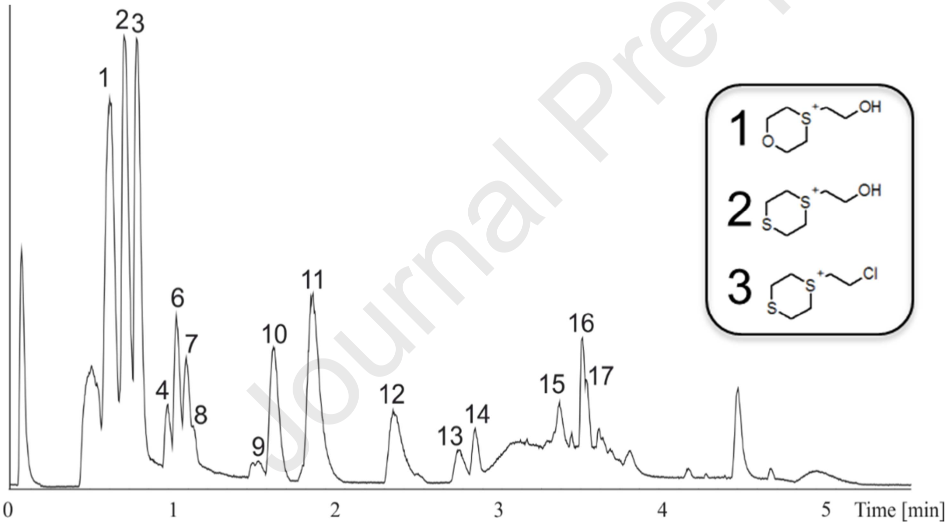
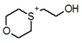
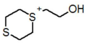
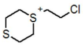
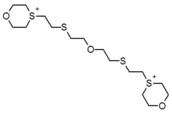
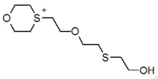
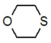
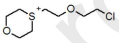
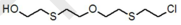
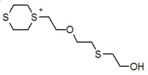
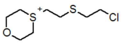
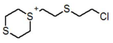
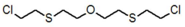
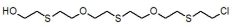
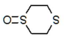
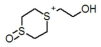
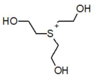
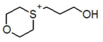
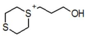
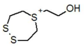
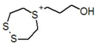
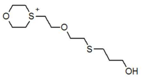
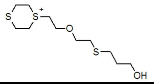


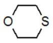
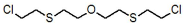
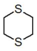
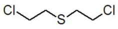
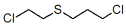
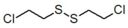
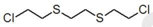
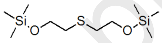
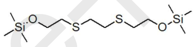
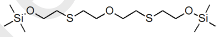
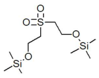
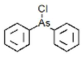
Figure 8. LC-HRMS chromatogram of compounds (numbered according to Table 1) extracted from sulphur mustard (heel) in storage containers.

Table 1. Sulphur mustard related compounds in lumps and heel detected by LC-HRMS analysis.

Comp.no.	Retention time	m/z	Formula[M+H]$^+$	CAS	Strucure	Found in sample
1	0.63	149.063	$C_6H_{13}O_2S$	255861-28-0	 (R)	Lumps and Heel
2	0.72	165.040	$C_6H_{13}OS_2$	255861-27-9	 (R)	Lumps and Heel
3	0.79	183.006	$C_6H_{12}S_2Cl$	199982-97-3		Heel
4	0.97	200.061	$C_{16}H_{32}O_3S_4$	N. A.		Heel
5	0.97	177.094	$C_8H_{17}O_2S$			Lumps and Heel
6	1.03	253.093	$C_{10}H_{21}O_3S_2$	N. A.		Heel
7	1.09	105.037	C_4H_9OS	15980-15-1		Heel
8	1.14	211.056	$C_8H_{16}O_2SCl$	N. A.		Heel
9	1.54	193.072	$C_8H_{17}OS_2$			Heel
10	1.63	245.043	$C_8H_{18}O_2S_2Cl$	150640-37-2		Heel
11	1.87	269.070	$C_{10}H_{21}O_2S_3$	N.A		Lumps and Heel
12	2.36	227.033	$C_8H_{16}OS_2Cl$	N. A.		Heel
13	2.76	243.010	$C_8H_{16}S_3Cl$	224949-02-4		Heel
14	2.86	331.107	$C_{12}H_{27}O_4S_3$			Heel
15	3.38	263.009	$C_8H_{17}OS_2Cl_2$	63918-89-8		Lumps and Heel
16	3.52	349.073	$C_{12}H_{26}O_3S_3Cl$	N. A.		Heel
17	3.54	373.100	$C_{14}H_{29}O_3S_4$			Heel
18	1.48	137.009	$C_4H_9OS_2$	19087-70-8		Heel
19	0.53	181.035	$C_6H_{13}O_2S_2$	N. A.		Lumps and Heel
20	0.54	167.074	$C_6H_{15}O_3S$	44910-50-1		Lumps
21	0.63	163.079	$C_7H_{15}O_2S$	N. A.		Lumps
22	0.66	179.056	$C_7H_{15}OS_2$	N. A.		Lumps
23	1.02	197.012	$C_6H_{13}OS_3$	N. A.		Lumps
24	1.29	211.028	$C_7H_{15}OS_3$	N. A.		Lumps
25	1.42	273.065	$C_9H_{21}O_3S_3$			Lumps
26	1.86: 2.22 and 2.33	267.109	$C_{11}H_{23}O_3S_2$	N. A.		Lumps
27	2.94 and 3.14	283.086	$C_{11}H_{23}O_2S_3$	N. A.		Lumps

R= structure confirmed by reference analysis. Remaining structures are tentative and based on elemental composition.

Table 2. Sulphur mustard related compounds in lumps and heel detected by GC/MS analysis.

Comp.no.	RI	Formula	CAS	Strucure	Found in sample
7	891	C_4H_8OS	15980-15-1		Lumps and Heel
15	1991	$C_8H_{16}OS_2Cl_2$	63918-89-8		Lumps and Heel
28	1077	$C_4H_8S_2$	505-29-3		Lumps and Heel
29	1382	$C_4H_8S_3$	6576-93-8		Lumps and Heel
30	1180	$C_4H_8SCl_2$	505-60-2		Lumps and Heel
31	1219	$C_5H_{10}SCl_2$	71784-01-5		Lumps and Heel
32	1402	$C_4H_8S_2Cl_2$	1002-41-1		Lumps and Heel
33	1701	$C_6H_{12}S_2Cl_2$	3563-36-8		Lumps and Heel
34	1413	$C_{10}H_{26}SO_2Si_2$	20486-03-7		Lumps
35	1872	$C_{12}H_{30}S_2O_2Si_2$	936623-39-1		Lumps and Heel
36	2148	$C_{14}H_{34}S_2O_3Si_2$	959082-71-4		Lumps and Heel
37	1672	$C_{10}H_{26}SO_4Si_2$	97916-04-6		Lumps
38	1811	$C_{12}H_{10}AsCl$	712-48-1		Lumps

4. Discussion

In order to understand the fate of sulphur mustard [30] in WWII dumped munition in the Baltic Sea we analysed a sample of solidified sulphur mustard lumps and identified the major degradation products. In our GC/MS study, sulphur mustard [30], four sulphur mustard analogues [15, 31-33] and arsine oil [38] were identified. These are components of the original weapons grade sulphur mustard where addition of arsine oil [38] often was used to lower the freezing point (Söderström et al., 2018). The hydrolysis product of sulphur mustard [30], thiodiglycol [34], as well as its oxidized form is observed [37] after silylation of the hydroxyl-groups. Furthermore, three cyclic degradation products are identified where dithiane [28] and oxathiane [7] is thought to form through the cyclic intermediates 1-(2-chloroethyl)-1,4-dithiane [3] and 4-(2-chloroethyl)-1,4-oxathian [D], as presented in Figure 9 for dithiane [3]. However, these intermediates are not analysable by GC/MS but require LC-MS analysis. In the GC/MS analysis the use of deconvolution program with combined spectra/retention index libraries will provide a good basis for identification. The LC-MS analysis is dependent on reference chemicals and HRMS for elucidation of elemental composition. In this work we use authentic reference standards and mustard heel, a product in long-term stored sulphur mustard that are known to form cyclic sulphur degradation products, to support the identification of the key intermediates in polymerised lumps of sulphur mustard from the Baltic Sea. Based on this, the hydrolysis products 1-(2-hydroxyetyl)-1,4-dithian [2] and 4-(2-hydroxyethyl)-1,4-oxathian [1] were identified in the sulphur mustard lump (Figure 7) while in the mustard heel also 1-(2-

chloroethyl)-1,4-dithiane [3] was identified (Figure 8). Further identifications in the LC-HRMS analysis lacks comparison with authentic reference and are dependent on manual interpretation and should therefore be considered tentative. In these products the major difference between lumps and heels is that almost all chloro-groups are hydrolysed to the corresponding hydroxyl group in the lumps.

Our analysis of sulphur mustard [30] degradation products in both mustard heel and lumps of solidified dumped sulphur mustard yields comparable results to previous investigations (Mazurek et al., 2001; Rohrbaugh et al., 1997). That our findings are so similar to the more dedicated work done by Mazurek et.al. on GC/MS analysis of sulphur mustard lumps indicates that the degradation processes and end products are relatively similar at least for munitions dumped in the same geographical area and likely from the same source. Bizzigotti et al. (Bizzigotti, Castelly, Hafez, Smith, & Whitmire, 2009) stated that cyclic mustard salts are likely formed in dumped sulphur mustard munitions and that they are water soluble and could thus potentially be released into the environment but that their fate once released was unknown. Neither sulphur mustard [30], nor the end product of sulphur mustard polymerisation are water soluble. However, parts of the sulphur mustard lump/heel fraction are water-soluble and could be major sources of products extracted by seawater from dumped sulphur mustard. In contact with water, the 1-(2-chloroethyl)-1,4-dithiane [3] and 4-(2-chloroethyl)-1,4-oxathiane [D] products will eventually be hydrolysed, resulting in the dithiane [28] and oxathiane [7] observed in sediment surrounding dumping sites(Beldowski et al., 2016; Magnusson et al., 2016). These compound could also be produced in hydrophobic conditions in the mustard heel. These two processes are shown for dithiane in figure 9.

Since 1-(2-chloroethyl)-1,4-dithiane [3] and its hydrolysed product could be major products leaking into the environment, we investigated their toxicity. Epithelial cells from the human respiratory tract are routinely used in *in vitro* studies of the toxicity of sulphur mustard and related skin-damaging substances(Karacsonyi et al., 2009). Here, the toxicity of the cyclic mustard salts, 1-(2-chloroethyl)-1,4-dithiane [3] and 1-(2-hydroxyetyl)-1,4-dithiane [2] were tested and the results compared with those of sulphur mustard [30]. The *in vitro* experiments for cell viability showed that the mustard salts are less toxic than mustard gas, a 50% cell viability is observed at 100 μM for sulphur mustard [30] compared to 90% for the compounds [2] and [3] at the same concentration. The released pro-inflammatory cytokines for 1-(2-chloroethyl)-1,4-dithiane [3] and 1-(2-hydroxyetyl)-1,4-dithiane [2] are only 1/10 or less as compared to the corresponding concentration of sulphur mustard [30] . Additionally, the toxicity of sulphur mustard [30] follows a dose-dependent manner while the response for [2] and [3] is low and dose-independent, similar to the effect on cell viability expressed by most organic compounds.

Toxicity observed in living systems and published toxicity data are highly relevant for marine systems. The latter include results of tests of the toxicity of the mustard gas salts towards the bacterium *Aliivibrio fischeri* (EC50, 75-400 mg/L)(Storgaard, Christensen, & Sanderso, 2018). Although these are very different taxa, the results confirm the lower toxicity of the salts, which is supported by the change in structure (involving elimination of the ability to form the reactive three membered sulfonium ion rings). Our toxicology studies indicate that the cyclic sulphur mustard salts are not overly toxic and their leaching from rusted sulphur mustard munitions is not likely to pose great environmental threats.

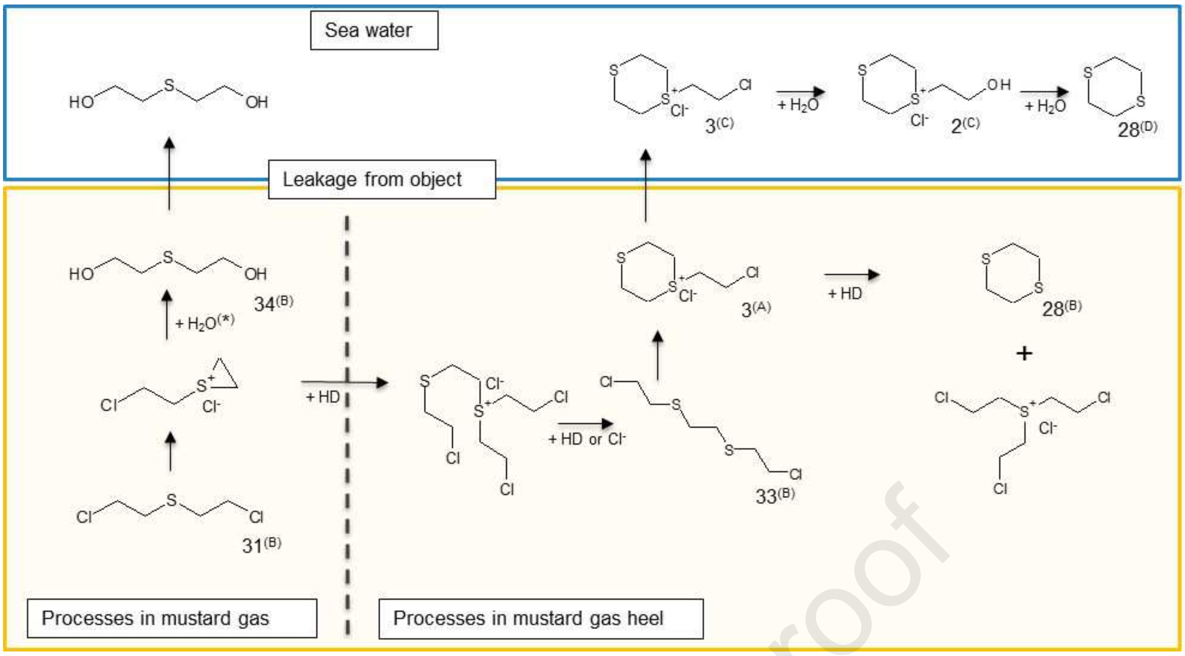


Figure 9. The formation of dithane [28] from sulphur mustard [30] may occur through two degradation pathways. In long-term storage of sulphur mustard the formation of 1-(2-chloroethyl)-1,4-dithiane [3] will result in a release of dithiane [28] as shown in the yellow box. The mustard gas salt may be extracted by sea water (blue box), and in contact with water 1-(2-chloroethyl)-1,4-dithiane [3] will be hydrolysed to the corresponding alcohol. These salts can be further hydrolysed to dithiane [28], which is frequently used as a marker for mustard gas leakage at dumping sites. (HD = sulphur mustard [30]). The numbers in the figure are according to table 1 and 2. A is detected by LC/MS analysis of heel, B is detection by GC/MS in lumps or heel, C is toxicity tested on alveaolar cells, bronchial cells and mouse fibroblast cell line and D is detected in analysis of sediment at duping sites (Magnusson et al., 2016).

5. Conclusion

Our GC/MS analysis results corroborate findings of an extensive analysis of the extractable hydrophobic volatile fraction of recovered polymerised mustard block by Mazurek et al. (Mazurek et al., 2001). This clearly showed that active mustard gas and related blister agents are still present in the polymerised block, and thus pose hazards for direct contact for marine organisms and occupational health problems for people encountering the object.

Our work supports the hypothesis that the degradation of sulphur mustard in both dumped and long-term stored mustard gas should be similar. To the best of our knowledge, no previous scientific investigations have been conducted to support this.

Our work support the hypothesis that cyclic sulphur mustard salts could constitute a large part of the release of sulphur mustard degradation products at the dumpsites to the environment where further degradation forms the dithiane and oxathiane that frequently are found in the surrounding sediment.

We have conducted a toxicological evaluation of these cyclic sulphur mustard salts and found them to possess some toxicity in cell viability assays and inflammatory response but nothing that is of special concern compared with the reactivity of mustard gas.

Our conclusion is that the release of these cyclic sulphur mustard salts are not of immediate environmental concern and that our finding do not warrant a re-evaluation of the environmental aspects of dumped sulphur mustard munitions.

Acknowledgement: the Swedish Ministry of Defence financed this work

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Highlights

- Analysis of dumped mustard gas revealed cyclic sulfonium ion degradation products
- The identified sulfonium ions may be the major source of leakage to the environment
- The identified cyclic sulfonium ion products possess low toxicity

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

No conflict of interest