CHEMSEA
CHEMICAL MUNITIONS SEARCH & ASSESSMENT

ANALYSIS OF CHEMICAL WARFARE AGENT RELATED CHEMICALS IN *IN VIVO* EXPOSED MUSSELS

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Studied dumping areas

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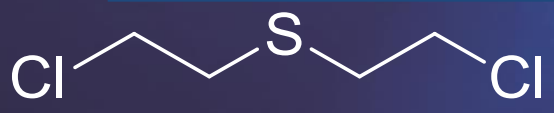
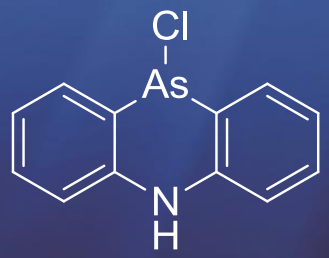
Major chemicals

and amounts dumped in Bornholm



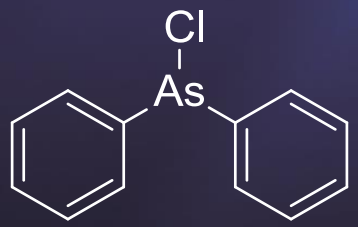
Sulphur mustard (H)

7 000 tons



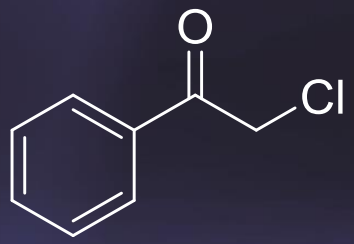
Adamsite (DM)

1 500 tons



Clark I (DA)

~1 000 tons



α-Chloroacetophenone (CN)

500 tons

Introduction

- Samples from mussel exposure experiment (FOI/SYKE/AWI)
  - The main aim was to evaluate biological responses in blue mussels (*Mytilus edulis* L.) induced by chemical warfare agents (CWA) mixtures at environmentally relevant concentrations
  - Mussels were exposed to 11 different mixtures containing the arsenic containing warfare agents Diphenylarsinechloride (Clark I, DA), 10-Chloro-5-hydrophenarsazine (Adamsite, DM) and the tear gas α-Chloroacetophenone
  - Different concentrations (low, medium, high) were selected for each compound ranging from 1.25 to 50 µg/L based on the assumed toxicity of each chemical
  - Each mixture group consisted of 56 mussels that were distributed among eight glass aquaria (12L; 7 mussels per aquarium)
  - A separate exposure was conducted with bis(2-chloroethyl)sulfide (mustard agent) only

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Schedule for *in vivo* exposure studies Experiment set-up based on multivariate design to enable modeling of results

	8 tanks - each containing 7 mussels
	Sampling day

Totally 56 mussels/mixture group
Whole experiment lasted for 6 weeks

Experimental design for the testing of:
one species (cultivated blue mussels, 2nd year, 2.5-3.5 cm)
Present examble is a setup for:
11 mixtures (+ 2 for the middle point) = 13 treatments
32 aquaria, 6 (+ 1 as back up) **individuals per aquaria**
Sampling of 0-Group before start of experiment
Daily water exchange, feeding once a week

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Adamsite, Clark, α-Chloroacetophenone and Mustard Gas added at environmentally relevant concentrations

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Background

- No published data of metabolism or detoxification rates of CWAs in mussel

- For the evaluation of the risks of dumped CWAs, chemicals which pose the highest realistic risk to marine biota are:
  - Sulfurd mustard (H)
  - Adamsite (DM)
  - Clark I (DA)
  - Triphenyl arsine (TPA)

- Probable metabolic reactions are hydrolysis and oxidation

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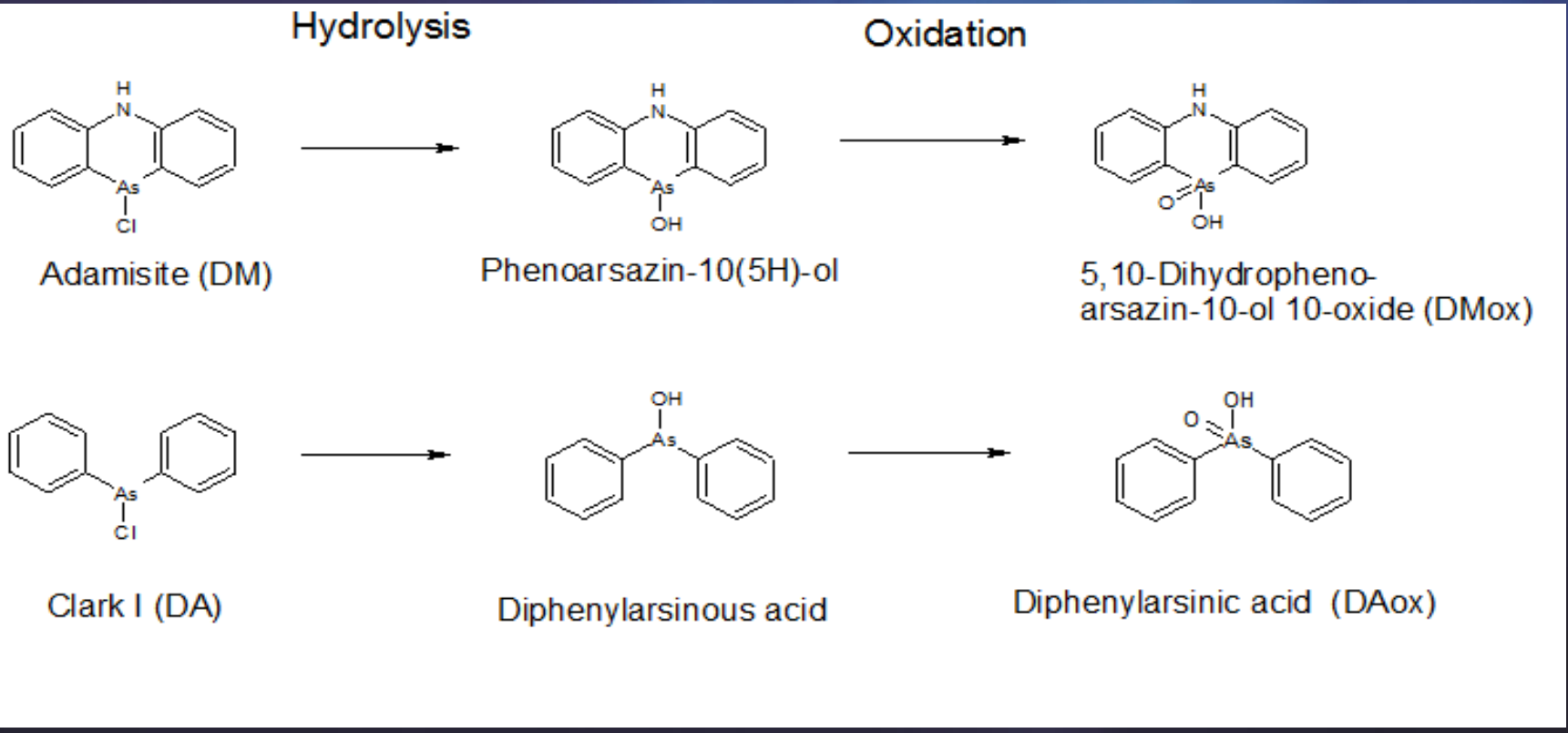

α-Chloroacetophenone has been reported to undergo hydrolysis slowly (Missianen et al 2010).

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Adamsite and Clark related products in water from mussel exposure experiments

- The measured concentrations of DMox and DAox (after oxidation) were generally lower than the nominal concentrations.
- There were no significant differences in concentration before and after the exposure period; however, the variation in the data was large.
- The reasons for the large variation could be related to inhomogeneity of DMox and DAox in the water (i.e. the compounds stick to glass or stay at the surface).
- It should also be noticed that the samples have been frozen for several months before analysis.

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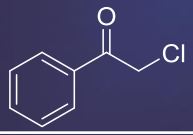
Analytes

- Analytes and analysis techniques – GC-MS/MS

#	Chemical (acronym) CAS	Structure	Description	GC-based			LC-based
				Intact	HFBI	$TiCl_3$	Intact
	CN α-chloroacetophenone 532–27–4		Dumped CW agent	*Mussels (12)*			

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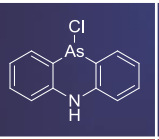
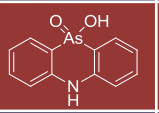
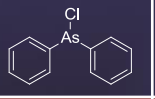
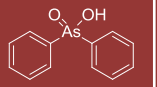
Analysis

- Analytes and analysis techniques – LC-MS/MS

#	Chemical (acronym) CAS	Structure	Description	GC-based			LC-based
				Intact	HFBI	$TiCl_3$	Intact
2	**Adamsite (DM)** **578-94-9**		Dumped CW agent	***Not analysed as such***			
2O	**5,10-Dihydrophenoarsazin-10-ol 10-oxide** **4733-19-1**		Oxidation product of **2** and all of its degradation products (either natural or with H_2O_2)				Exposed mussels (17)
3	**Clark I (DA)** **712-48-1**		Dumped CW agent. Also component in dumped arsine oil.	***Not analysed as such***			
3O	**Diphenylarsinic acid** **4656-80-8**		Oxidation product of **3** (either natural or with H_2O_2)				Exposed mussels (17)

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Sample preparation for GC-MS/MS biomarkers of α-Chloroacetophenone (CN)

- Mussel (CN)
  - **Homogenization of 1.0 g of mussel + 200 µl 1 % HCOOH**
  - **Addition of 1 % HCOOH, BuOAc and NaCl**
  - **Mixing, sonication and centrifugation**
  - **Concentration of BuOAc phase and volume adjusting to 1 ml with hexane**
  - **SPE clean-up**
  - **DCM fraction**
  - **Concentration**

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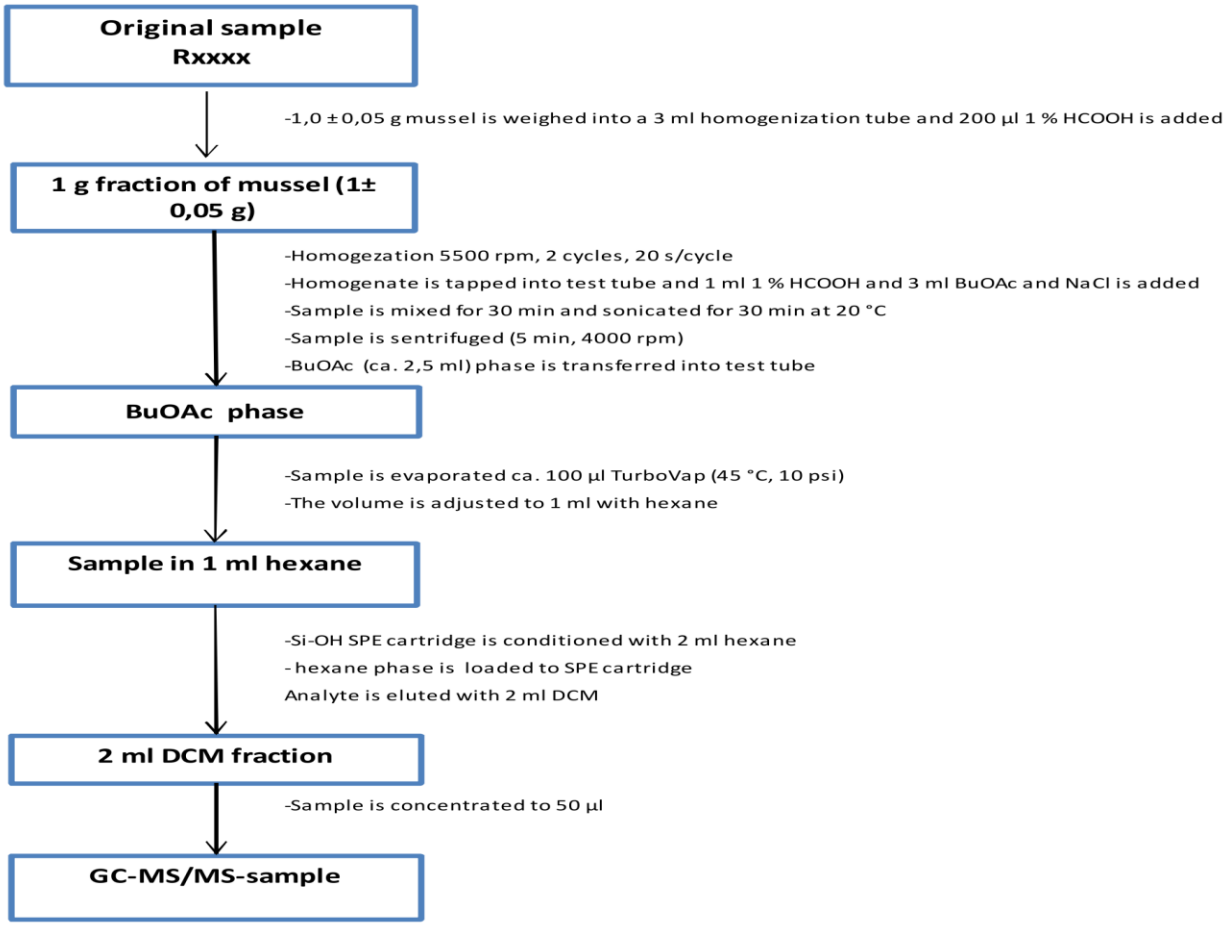
Sample preparation for GC-MS/MS biomarkers of α-Chloroacetophenone (CN)



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Sample preparation for LC-MS/MS biomarkers of Adamsite, Clark

- Mussel (DM-ox,DA-ox)
  - Homogenization of 3.0 g of cod muscle/mussel meat + 1 ml of water
  - Addition of water, ACN and buffered salts
  - Mixing and centrifugation
  - ACN layer, addition of hexane to remove excess fat
  - Shaking
  - Discarding the hexane layer and separating ACN layer
  - Concentration of the sample
  - Dilution of sample with water and filtration
  - Addition of 25 µl ISTD (DMMP)

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Analysis methods

- GC-MS/MS analysis –CN
  - Triple quadrupole mass spectrometer with GC and autosampler
  - Capillary column DB-5ms (Agilent, 30 m x 0.25 mm i.d., 0.25 µm film)
  - The column temperature programme: 40°C (isothermal time 1 min) to 320 °C, 20 °C/min
  - Transfer line temperature 325 °C
  - Splitless injection (splitless time 1 min, 1 µl)
  - Injector temperature 250°C
  - EI (70 eV)
  - Selected reaction monitoring (SRM)
  - LOQ 0.2 ng/g for CN

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Analysis methods

- LC-MS/MS analysis - DM-ox and DA-ox
  - Triple quadrupole mass spectrometer with two LC pumps and autosampler
  - Waters XBridge BEH C18 column (2.1 x 100 mm, 2.5 µm) + Pre-Column
  - Column temperature 30°C
  - Flow-rate 0.4 ml/ml
  - Injection volume 5µl
  - Eluent (A) 0.1% formic acid in water
  - Eluent (B) 0.1% formic acid in MeOH
  - APCI in positive mode by SRM
  - Mussels: LOQ 10 ng/ml for DMox and Daox

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Results of mussel samples after Adamsite *in vivo* exposure

Code	DMox (ng/g, ppb)	DM in exposure mixture (ng/ml)
Blank 1	NF	No DM added
Blank 2	NF	No DM added
Control	NF	No DM added
Solvent Control	NF	No DM added
N1	**77**	**2.5**
N2	**339**	**10**
N3	**128**	**2.5**
N4	**358**	**10**
N6	**586**	**10**
N5	**280**	**2.5**
N7	**159**	**2.5**
N8	**394**	**5.0**
N9	**366**	**10**
N10	**209**	**5**
N11	**136**	**5**
N12	**99**	**5**
N13	**141**	**5**
Quality blank	NF	-
Blank mussel	NF	-

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N11	**136**	**5**
N12	**99**	**5**
N13	**141**	**5**
-Quality blank	NF	-
-Blank mussel	NF	-

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Results of mussel samples after Clark *in vivo* exposure

Code	DAox (ng/g, ppb)	DA in exposure mixture (ng/ml)
Blank sample	NF	No DA added
Blank sample	NF	No DA added
Control	NF	No DA added
Solvent control	NF	No DA added
N1	**127**	**1.25**
N2	**62**	**1.25**
N3	**569**	**5.0**
N4	**337**	**5.0**
N6	**91**	**1.25**
N5	**90**	**1.25**
N7	**724**	**5.0**
N8	**584**	**5.0**
N9	**77**	**2.5**
N10	**98**	**2.5**
N11	**62**	**2.5**
N12	**133**	**2.5**
N13	**226**	**2.5**
Quality blank	NF	-
Blank mussel	NF	-

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Results of mussel samples after Clark *in vivo* exposure

Code	DAox (ng/g, ppb)	DA in exposure mixture (ng/ml)
Blank sample	NF	No DA added
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Solvent control	NF	No DA added
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N3	**569**	**5.0**
N4	**337**	**5.0**
N6	**91**	**1.25**
N5	**90**	**1.25**
N7	**724**	**5.0**
N8	**584**	**5.0**
N9	**77**	**2.5**
N10	**98**	**2.5**
N11	**62**	**2.5**
N12	**133**	**2.5**
N13	**226**	**2.5**
Quality blank	NF	-
Blank mussel	NF	-

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Quality blank	NF	-
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N7	**724**	**5.0**
N8	**584**	**5.0**
N9	**77**	**2.5**
N10	**98**	**2.5**
N11	**62**	**2.5**
N12	**133**	**2.5**
N13	**226**	**2.5**
Quality blank	NF	-
Blank mussel	NF	-

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Results of mussel samples after CN *in vivo* exposure

Code	CN (ng/g, ppb)	CN in exposure mixture (ng/ml)
Solvent control	NF	No CN added
N1	NF	5
N2	1.3	50
N4	1.2	5
N5	NF	50
N6	1.3	5
N7	NF	5
N8	1.6	15.8
N9	NF	15.8
N10	NF	50
N11	NF	16
N12	NF	15.8
N13	1.7	15.8
Quality blank	NF	-
Blank mussel	NF	-

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Future research

- Improve sample preparation methods for analysis
- Need for internal standards, stable isotope labelled reference chemicals appreciated
- New reference chemicals to be synthesized e.g. Glutathione conjugates
- Toxicity studies
- Metabolism in mussels

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Conclusions

- Real proof for the source of ecotoxical effects can only be found after chemical analysis of degradation products/metabolites in mussel samples
- EU identification criteria followed
- High concentrations of oxidized DM and DA were measured the soft tissue from in-vivo exposed mussels

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