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Published in:

Global Security: Health, Science and Policy – an Open Access Journal

Link to article, DOI:

[10.1080/23779497.2016.1219962](https://doi.org/10.1080/23779497.2016.1219962)

Publication date:

2016

Document Version

Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):

Mohammed Abdullah Christensen, I., Sanderson, H., Baatrup, E., Storgaard, M. S., Fauser, P., & Hansen, S. F. (2016). Acute toxicity of sea-dumped chemical munitions: Luminating the environmental toxicity of legacy compounds. *Global Security: Health, Science and Policy – an Open Access Journal*, 1(1), 39-50. DOI: 10.1080/23779497.2016.1219962

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To cite this article: Ilias Mohammed Abdullah Christensen, Morten Swayne Storgaard, Patrik Fauser, Steffen Foss Hansen, Erik Baatrup & Hans Sanderson (2016) Acute toxicity of sea-dumped chemical munitions: luminating the environmental toxicity of legacy compounds, *Global Security: Health, Science and Policy*, 1:1, 39-50

To link to this article: <http://dx.doi.org/10.1080/23779497.2016.1219962>



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Published online: 28 Sep 2016.



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Acute toxicity of sea-dumped chemical munitions: luminating the environmental toxicity of legacy compounds

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ABSTRACT

As a result of the disarmament of Germany after the Second World War, 65,000 tons of chemical munitions were dumped in the Baltic Sea. Approximately 13,000 tons containing chemical warfare agents (CWAs) of which 11,000 tons were dumped in the Bornholm Basin east of Bornholm. This paper addresses the ecotoxicity of compounds actually present in the Bornholm dumpsite by obtaining novel acute ecotoxicity data. EC_{50} values were successfully obtained for 12 CWAs from acute tests using *Allivibrio fischeri* (Microtox™). The three most toxic compounds were α -chloroacetophenone, 2-chlorovinylarsonic acid and 1,2,5-trithiepane having EC_{50} values of 11.20, 31.20 and 1170 $\mu\text{g L}^{-1}$, respectively. *A. fischeri* demonstrated hormesis when exposed to triphenylarsine and triphenylarsine oxide at concentrations of 100 and 50 mg L^{-1} , respectively. Four different mixtures were assessed including compounds which were dissolvable; a mixture of sulphur mustard degradation products, a mixture of the three most toxic sulphur mustard compounds, a mixture of organoarsenical degradation products and a mixture of all compounds. The mixtures deviate by a factor of 1.5–2.5 from the prediction of the concentration addition model and hence, the mixtures demonstrate no sign of synergism or antagonism. The compounds presented in this study are mainly CWA.

ARTICLE HISTORY

Received 19 May 2016
Accepted 30 July 2016

KEYWORDS

Chemical warfare agents; Baltic Sea; environmental toxicity; *Allivibrio fischeri*; legacy compounds

1. Introduction

2015 marked the 100th year of the first large-scale use of chemical weapons in the First World War. During the course of this war, over 1 million people were either killed or injured by chemical warfare agents (CWAs) (Gilchrist, 1928), despite the majority of European countries and the USA having formally committed themselves to refraining from the use of CWAs by the Hague Convention in 1899 (United Nations [UN], 1899). The development of CWAs continued throughout the Second World War. By the end of the war, reports show that up to 65,000 tons of active CWAs had been stockpiled by Germany alone (Gatsby, 1997). The chemical weapons were disposed of either by incineration, buried, stockpiled or dumped at sea. Table 1 shows the confirmed sea-dumped compounds in the Bornholm Deep (Helcom, 1994).

The presence of sea-dumped chemical munitions continues to raise environmental concerns (Brewer & Nakayama, 2008; Sanderson, Fauser, Thomsen, & Sorensen, 2007). Dumpsites are found off the US East and

West Coasts, Hawaii, Canada, Australia, off the east coast of Japan, in parts of Asia in the seas north of Russia, and in the Adriatic Sea off the coast of Italy (Brewer & Nakayama, 2008). One of the locations with the highest confirmed amount of sea-dumped CWA in relative close proximity to human settlements and at relatively low depth (<100 m) is the Bornholm Deep in the Baltic Sea. This is where the main part of the dumping of mostly German CWA and conventional munitions occurred in the Baltic Sea (Helcom, 2013), hence this location has received significant research attention over the past 25 years. Sanderson et al. (2010) reported the measured environmental concentrations of CWA residues found in the CWA dump site in the Bornholm Deep, near Denmark as part of the MERCW project (MERCW, 2007; Sanderson et al., 2010). Fifty-nine sediment samples and 61 near-bottom water samples (<0.2 m above the seafloor) were taken from 63 sampling points in February 2008 (mainly within the dumpsite) in the MERCW project. No intact CWA was found in any of the samples analysed except for one

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Research was conducted at this institution (Danish Centre for Environment and Energy, Aarhus University).

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Table 1. Dumped compounds (tonnes) in the Bornholm Deep (Helcom, 1994).

| Compound | CWA dumped in tons |
|---|--------------------|
| α -chloroacetophenone ^a | 515 |
| Sulphur mustard gas ^b | 7027 |
| Adamsite ^c | 1428 |
| Diphenylchloroarsine (Clark I) ^c | 711.5 |
| Triphenylarsine ^{c,d} | 101.5 |
| Phenyldichloroarsine ^{c,d} | 1017 |
| Trichloroarsine ^{c,d} | 101.5 |
| Other ^e | 74 |
| Monochlorobenzene ^f | 1405 |

^aRiot controlling agent.^bBlistering agent.^cOrganoarsenic blistering agent.^dArsine oil constituent.^eE.g. Blood agent.^fAdditive.

stable component in arsenic oil, (triphenylarsine in the sediment). Degradation products of sulphur mustard, Lost (German sulphur mustard), diphenylchloroarsine (Clark I), 10-Chloro-5,10-dihydrophenarsazine (Adamsite) and arsenic oil components were detected in the 59 sediment samples (Sanderson et al., 2010). In 2012 and 2014 Sanderson and colleagues reported the measured CWA residues just outside the CWA dumpsite in the Bornholm Deep along the Nord-Stream pipeline route in more than 300 samples from 2008 to 2012 (Sanderson, Fauser, Rahbek, & Larsen, 2014; Sanderson, Fauser, Thomsen, & Sørensen, 2012). Both papers concluded that parent CWA compounds are rarely found in the sediment and never in free water, but that metabolites of the compounds are found with detection frequencies up to 25% in the sediment (Sanderson et al., 2012; Sanderson et al., 2014). Similar conclusions have been reached by the CHEMSEA project (Söderström, 2014). The table below shows the compounds detected in sediment from the Baltic Sea (hereunder the Bornholm Deep), as well as their detection frequencies (DF%) and highest detected concentrations in sediment from the three projects (MERCW; Nord-Stream and CHEMSEA). Despite being dumped in the Baltic Sea after the Second World War, no organophosphorus nerve agents (e.g. tabun and soman) were found in the sediment and pore water samples from the Baltic Sea expeditions.

Recent risk assessments and environmental toxicology studies have focused on fish (Baršienė et al., 2014, 2016; Della Torre et al., 2010; Sanderson et al., 2014). However, it is clear that there is a general lack of sound toxicity data for the observed CWA residues in Table 2. Until recently it was not known which compounds were actually present in the sediment after the dumping of the CWAs, and there has not been any significant interest in exploring the environmental toxicity of these compounds according to REACH as most of them have no industrial use (Sanderson & Fauser, 2015).

To our knowledge, these compounds have only recently been discovered in the MODUM and CHEMSEA projects and hence, many of the compounds in Table 2 have never been assessed for their environmental toxicity.

This paper contributes to the assessment of the potential environmental risk of sea-dumped CWA in the Baltic Sea by assessing the toxicity of found CWA residues measured in the Bornholm Deep from more than 400 samples analysed in the past decade in the MERCW, Nord-Stream and CHEMSEA projects. We have first screened the literature for toxicity data on the compounds and then we have measured the acute toxicity of all detected compounds in Microtox™ (ISO 11348-3) using the marine bacteria *Allivibrio fischeri* to provide a comprehensive and fully comparable first-tier acute marine environmental toxicity assessment for the observed CWA residues in the Baltic Sea. Additionally, we provide mixture toxicity data on the compounds found in the Baltic Sea by a set of representative mixtures and worst-case scenarios. The concentration range of the mixtures is based on acute ecotoxicity measurements found in the single-chemical tests and by the predictive power of the concentration addition (CA) model. The difference will reveal whether the compounds act synergistic, additively or antagonistic.

2. Materials and methods

2.1. Compounds

The compounds tested are either parent compounds or degradation products of CWA found in the Baltic Sea. In total, 14 compounds are included and reflect the current environmental status. All 14 compounds could be purchased or synthesised and were therefore included in the test. These comprise two detected parent compounds (triphenylarsine and CAP), a metabolite of triphenylarsine, six sulphur mustard metabolites and organoarsenical metabolites from PDCA, Lewisite I + II, Clark I and Adamsite, respectively, see Table 2. α -chloroacetophenone, 1,4-oxathiane, 1,4-dithiane, thiodiglycolic acid, triphenylarsine, triphenylarsine oxide and 3,5-dichlorophenol (reference compound) were purchased from Sigma-Aldrich Denmark ApS. Phenylarsonic acid was purchased from abcr GmbH. The remaining compounds thiodiglycol sulfoxide, diphenylarsinic acid, phenarzasinic acid, 1,2,5-trithiepane and 1,4,5-oxadithiepane were synthesised by Envilytix GmbH. The two Lewisite degradation products, 2-chlorovinylarsonic acid and bis(2-chlorovinyl)arsinic acid were synthesised by Finnish Institute for Verification of the Chemical Weapons Convention (VERIFIN). The majority of the compounds tested were of purity $\geq 97\%$ except the two Lewisite degradation

Table 2. Detected compounds, maximum concentrations measured and associated detection frequencies from samples taken in the Baltic Sea in connection with the MERCW-, Nord-Stream- and CHEMSEA project.

| Compound | CAS# | Sediment fraction | | Pore water fraction | | Source project |
|---|-------------|--------------------------------------|--------|-------------------------------------|--------|----------------|
| | | Max. conc. ($\mu\text{g kg}^{-1}$) | DF (%) | Max. conc. ($\mu\text{g L}^{-1}$) | DF (%) | |
| Triphenylarsine ^a | 603-32-7 | 1200 | 8.7 | 68 | 2.7 | CHEMSEA |
| | | 81,250 | 32.8 | | | MERCW |
| Triphenylarsine oxide | 1153-05-5 | 590 | 4.35 | 20 | 1.35 | CHEMSEA |
| α -chloroacetophenone ^a | 532-27-4 | 7.5 | 1.1 | – | – | CHEMSEA |
| 1,4-dithiane ^b | 505-29-3 | 45 | 5.4 | – | – | CHEMSEA |
| 1,4-oxathiane ^b | 15980-15-1 | 120 | 1.6 | – | – | CHEMSEA |
| 1,4,5-oxadithiepane ^b | 3886-40-6 | 40 | 8.7 | 19 | 1.1 | CHEMSEA |
| 1,2,5-trithiepane ^b | 6576-93-8 | 35 | 8.7 | 3.4 | 0.5 | CHEMSEA |
| Thiodiglycol sulfoxide ^b | 3085-45-8 | 610 | 2.1 | – | – | CHEMSEA |
| | | 3.3 | 1.7 | – | – | MERCW |
| Thiodiglycolic acid ^b | 123-93-3 | 550 | 1.1 | – | – | CHEMSEA |
| Phenylarsonic acid ^c | 98-05-5 | 1300 | 2.2 | 4 | 2.2 | CHEMSEA |
| | | 10,833 | 81.0 | 442 | 5.2 | MERCW |
| | | 327 | 2 | – | – | Nordstream |
| Diphenylarsinic acid ^d | 4656-80-8 | 1700 | 8.2 | 940 | 2.2 | CHEMSEA |
| | | 9583 | 50 | 1,538 | 5.2 | MERCW |
| | | 140 | 19.5 | – | – | Nordstream |
| Phenarsazinic acid ^e | 4733-19-1 | 1400 | 7.1 | 17 | 1.1 | CHEMSEA |
| | | 354 | 62.1 | – | – | MERCW |
| | | 200 | 3.5 | – | – | Nordstream |
| 2-chlorovinylarsonic acid ^f | 64038-44-4 | 54.9 | 2 | – | – | Nordstream |
| Bis(2-chlorovinyl)arsinic acid ^f | 157184-21-9 | 70.3 | 2 | – | – | Nordstream |

^aParent compound.^bSulphur mustard metabolite.^cPhenyldichloroarsine (PDCA) metabolite.^dClark I metabolite.^eAdamsite metabolite.^fLewisite metabolites

products, 2-chlorovinylarsonic acid and bis(2-chlorovinyl)arsinic acid with purities of ≥ 90 and $\geq 91\%$, respectively.

Some of the compounds tested are not readily soluble in water and hence, the approach was to make a strong stock solution and then dilute. 1,4-dithiane was dissolved in ethanol and no more than 2% ethanol was used as it has previously been determined as the No Observable Effect Concentration (NOEC) in the Microtox™ test (Nalecz-Jawecki, Rudz, & Sawicki, 1997). Phenarsazinic acid was dissolved in 60% methanol, 40% water and few μLs of 5 M NaOH. When necessary, pH was adjusted with 5 M HCl. Methanol was not used in higher concentrations than 1% which is in accordance with Kwan and Dutka (1990) who established a maximum allowable concentration of 4% for methanol in Microtox™ (Kwan & Dutka, 1990). Triphenylarsine was dissolved in dichloromethane and acetonitrile in 50:50 ratios, and 1,2,5-trithiepane, as well as triphenylarsine oxide, were dissolved in dichloromethane leaving no more than 0.5% solvent in the final solutions. In Microtox™, the EC_{50} value for dichloromethane has been determined to be 3500 mg L^{-1} (Ricco, Tomei, Ramadori, & Laera, 2004), justifying the maximum 0.5% of dichloromethane present in the final solution. Acetonitrile was used to dissolve 1,4,5-oxadithiepane. Since no studies could be found regarding the ecotoxicity of acetonitrile towards *A. fischeri*, we tested its toxicity in Microtox™. Based on this test, concentration ranges were designed so that all solutions contained less than the EC_{05} for acetonitrile.

Bis(2-chlorovinyl)arsinic acid was tried dissolved in demineralised water followed by ultrasound, mixing, heating and adding acetone. However, the compound could not be dissolved. 2-chlorovinylarsonic acid was almost completely dissolved in dimethyl sulphoxide (DMSO $\geq 99.5\%$). The compound was not dissolved completely after stirring, storing at room temperature, heating to $50 \text{ }^\circ\text{C}$ and ultrasound treatment. After diluting to a stock concentration of 457.5 mg L^{-1} it was almost completely dissolved. However, we decided to test the compound with dilutions based on the stock concentration and hence, the toxicity described here can be seen as a conservative value. No more than 0.0625% DMSO was used in the highest tested concentration which is in accordance with the established NOEC for DMSO of 1% (Nalecz-Jawecki et al., 1997).

Where solvents are used and the literature did not reveal applicable solvent concentrations, a blank/control was always included containing only the solvent in the highest applied concentration (positive control). Tests were rejected if any inhibitory effect is observed.

Due to the rapidity of the tests, we assume solutions are in nominal concentrations throughout the test duration.

2.2. Microtox™

The method used to run the bioluminescence inhibition test, Microtox™, followed the ISO 11348-3 standard (International Organization for Standardization, 2007).

The test organism was freeze-dried bacteria *A. fischeri* (lot. no. 14H4122 from Modern Water). Light inhibition is represented by the EC_{50} value, which is the effect concentration at which there is a 50% luminescence inhibition compared to the control. Luminescence was measured by an M500 photometer (Modern Water) with integrated cooling keeping compound dilutions and bacteria suspension at a constant 15 °C. A dilution factor of two–three was used. The freeze-dried *A. fischeri* were activated by adding reconstitution solution (2% NaCl), stirring and leaving the suspension at 4 and 15 °C for 10 min in a refrigerator and 20 min in the cooled M500 wells, respectively. An appropriate amount of bacteria suspension (50 to 200 µL) was added to each test tube and acclimatised for an additional 10 min. Compound dilutions were similarly acclimatised at 15 °C in the M500 prior to testing and were prepared in 2% NaCl demineralised water. Luminescence was measured just after acclimatisation (before adding compound dilutions) which was the light emission at $t = 0$. The test is then started by adding compound dilutions to each test tube at a steady pace, ensuring quantifiable readings. Luminescence was then measured 5, 15 and 30 min after test start at the same speed as compound dilutions were added to the test tubes so each tube was measured at 5, 15 and 30 min. pH was measured at the end of the test in the control and highest concentration confirming a pH of 6–8.5. Luminescence measurements were captured and stored on a coupled pc using Microtox Omni v1.18.

2.3. Mixture toxicity

Four different mixtures were prepared for toxicity testing: (1) a mixture mimicking the composition of a sulphur mustard bomb by mixing all detected degradation products of sulphur mustard; (2) a mixture of the three most toxic sulphur mustard degradation products; (3) all organoarsenic compounds; and (4) all compounds (included in this study). These mixtures were chosen to assess a worst-case scenario containing all detected compounds and categorised by their structural formula, namely sulphur mustard and organoarsenicals. As sulphur mustard is the compound dumped in the largest quantity, an additional mixture of the three most toxic sulphur mustard degradation products was assessed. Triphenylarsine, L1 [ox] and L2 [ox] were excluded in these tests due to the above-mentioned solubility issues. Table 4 summarises the compounds present in the mixtures and a dilution factor of two was used. The mixtures were prepared by adding the compounds in the concentration level eliciting the EC_{50} value, in the single-chemicals test, is tested in the mixture. Available stock solutions from the single-chemicals test were used in the mixture toxicity test.

CA was used to predict the EC_{50} value of the mixtures and chosen for its excellent predictive power in Microtox™ tests with similarly acting compounds (Altenburger et al., 2000). The CA model, also called the Loewe equation, originates from the early works of Loewe (1953), below adopted by to Faust et al. (2001):

$$ECx_{mix} = \sum_{i=1}^n \left(\frac{p_i}{ECx_i} \right)^{-1} \quad (1)$$

in which p_i is the fraction $p_i = \frac{ECx_i}{\sum_{i=1}^n ECx_i}$ which is the ratio between (single) chemical and mixture.

The deviation between the observed toxicity (Microtox™ test) and from the CA predicted toxicity is expressed as the model deviation ratio:

$$MDR = \frac{EC_{50pred}}{EC_{50obs}} \quad (2)$$

2.4. Statistical analyses

The measured inhibitory effect of the respective compounds and mixtures is corrected by applying correction factors according to the ISO 11348-3 standard. EC_{50} s and 95% confidence intervals are derived assuming a logarithmic normal distribution of data using a nonlinear dose-response regression. All dose response-curves complied with a logarithmic normal distribution (Goodness of fit < 0.5). Data shown are from the 30-min recording. The model has previously been described (Christensen, Kusk, & Nyholm, 2009).

3. Results

3.1. Microtox™

Of the 14 compounds detected in MERCW, Nord Stream and CHEMSEA, 13 were tested in Microtox™. Bis(2-chlorovinyl)arsinic acid could not be dissolved. The measured EC_{50} values of the compounds are listed in Table 3 where $\log K_{OW}$ and solubility values are obtained through use of the US EPA QSAR module EPI Suite predictions (US EPA, 2016).

The three most toxic compounds are α -chloroacetophenone, 2-chlorovinylarsonic acid and 1,2,5-trithiepane with EC_{50} -values of 11.20, 31.20 and 1170 µg L⁻¹, respectively. Thiodiglycol sulphoxide and triphenylarsine did not induce an effect at the concentration level tested. Triphenylarsine was tested up to 200 mg L⁻¹ and if tested in higher concentration, the concentration of solvent would either be too high or volatilise. As almost no inhibition occurred, a dose-response curve is not shown for triphenylarsine. The values listed as EC_{50} for these two compounds (<200 and 74,250 mg L⁻¹ for triphenylarsine

Table 3. Toxicity screening of CWA residues found in the Baltic Sea. The columns listing logKow and solubility are based on Episuite findings.

| Compound | CAS# | LogKow | Water solubility [mg/L ⁻¹] | EC50 <i>A. fischeri</i> (95% CI) (mg L ⁻¹) |
|---|-------------|--------|--|--|
| Triphenylarsine ^a | 603-32-7 | 5.97 | 0.12 | >200 ^g |
| Triphenylarsine oxide | 1153-05-5 | 5.97 | 0.07 | 155 ^g (124–194) |
| α -Chloroacetophenone ^a | 532-27-4 | 1.93 | 1635 | 0.0112 (0.0086–0.0145) |
| 1,4-Dithiane ^b | 505-29-3 | 0.77 | 3,000 | 9.97 ^g (8.36–11.90) |
| 1,4-Oxathiane ^b | 15980-15-1 | 0.53 | 39,880 | 47.4 (42.1–53.3) |
| 1,4,5-Oxadithiepane ^b | 3886-40-6 | 1.49 | 16,277 | 1.70 ^g (1.46–1.99) |
| 1,2,5-Trithiepane ^b | 6576-93-8 | 2.34 | 769 | 1.17 ^g (0.92–1.50) |
| Thiodiglycol sulfoxide ^b | 3085-45-8 | -2.76 | 1,000,000 | >74,250 |
| Thiodiglycolic acid ^b | 123-93-3 | 1.16 | 400,000 | 22.5 (21.3–23.8) |
| Phenylarsonic acid ^c | 98-05-5 | 0.03 | 1,000,000 | 97.1 (91.5–103) |
| Diphenylarsinic acid ^d | 4656-80-8 | 2.8 | 1629 | 124 (118–137) |
| Phenarsazinic acid ^e | 4733-19-1 | 2.33 | 3338 | 5.33 ^g (5.02–5.67) |
| 2-Chlorovinylarsonic acid ^f | 64038-44-4 | -0.472 | 1296 \times 10 ⁵ | 0.0312 ^g (0.0284–0.0343) |
| Bis(2-chlorovinyl)arsinic acid ^f | 157184-21-9 | 1.79 | 38,204 | Not tested (insoluble) |

^aParent compound.^bSulphur mustard metabolite.^cPhenyldichloroarsine (PDCA) metabolite.^dClark I metabolite.^eAdamsite metabolite.^fLewisite metabolite.^gSolvent used.

and thiodiglycol sulfoxide, respectively) are the maximum concentration at which they were tested. The solution of 2-chlorovinylarsonic acid was clear, but contained very few crystallisation particles. Therefore, we suggest that the toxicity presented here should be seen as conservative. Figures 1 and 2 below illustrate the treatment-response curves for single chemical tests, whereas Figure 3 shows mixture treatment-response curves. As evident in Figure 2(B), triphenylarsine oxide shows hormesis at around 50 mg L⁻¹. The hormesis seen in the organoarsenicals mixture, seen in Figure 3(C), is probably also due to triphenylarsine oxide. The parent compound, triphenylarsine, demonstrated similar pattern of hormesis at 100 mg L⁻¹ (unpublished data).

3.2. Mixture toxicity

Of the different compounds detected in the Baltic Sea, four different mixtures were assessed for toxicity: One including the sulphur mustard degradation products, one including the organoarsenicals, a combination of the two and finally a combination of the three most toxic sulphur mustard compounds. The CA model was applied to each mixture to predict an EC₅₀ value in order to make suitable mixtures in the range of the predicted EC₅₀ value of the mixture. The mixture tests demonstrated lower toxicities than predicted by CA except the organoarsenical mixture as shown in Table 4. Despite the slight differences the observed toxicity and the predictive toxicity lie within a factor of 1.5–2.5 (except for the sulphur mustard mixture). The deviations will be a subject of the discussion.

4. Discussion

This study has provided ISO-standardised acute toxicity data for CWA degradation products in support of the comparability and regulatory toxicity characterisation of the compounds. Other recent ecotoxicity studies on CWAs have dealt with parent compounds and sub-lethal effects. For example, Della Torre et al. (2010, 2013) studied genotoxicity of sulphur mustard and its effect on detoxification enzyme concentrations in fish from CWA dumpsites in the Mediterranean Sea (Della Torre et al., 2010, 2013). Baršienė et al. (2014, 2016) investigated genotoxicity in fish from the Baltic Sea (Baršienė et al., 2014, 2016). The focus on long-term effects in fish is understandable due to the assumed continual exposure to CWAs in low concentrations in marine environments.

We have measured the acute toxicity of detected CWA degradation products in Microtox™ (ISO 11348-3) using the marine bacteria *A. fischeri*. The most toxic compound towards *A. fischeri* was the organochloride parent compound α -chloroacetophenone. Only one other parent compound, triphenylarsine, was detected which had a significantly lower toxicity (EC₅₀ > 200 mg L⁻¹). The 13 compounds tested were mainly a variety of organic arsenicals and sulphuric metabolites with generally low toxicity (EC₅₀ > 10 mg L⁻¹) but a substantial portion was found to be more toxic (EC₅₀ < 10 mg L⁻¹) including 1,2,5-trithiepane, 1,4,5-oxadithiepane as well as 1,4-dithiane and 2-chlorovinylarsonic acid was tested to be even very toxic (EC₅₀ < 1 mg L⁻¹) according to the Globally Harmonized System developed by the United Nations (2011). Some metabolites of sulphur mustard may be more toxic compared to their parent compound (EC₅₀ = 25 mg L⁻¹

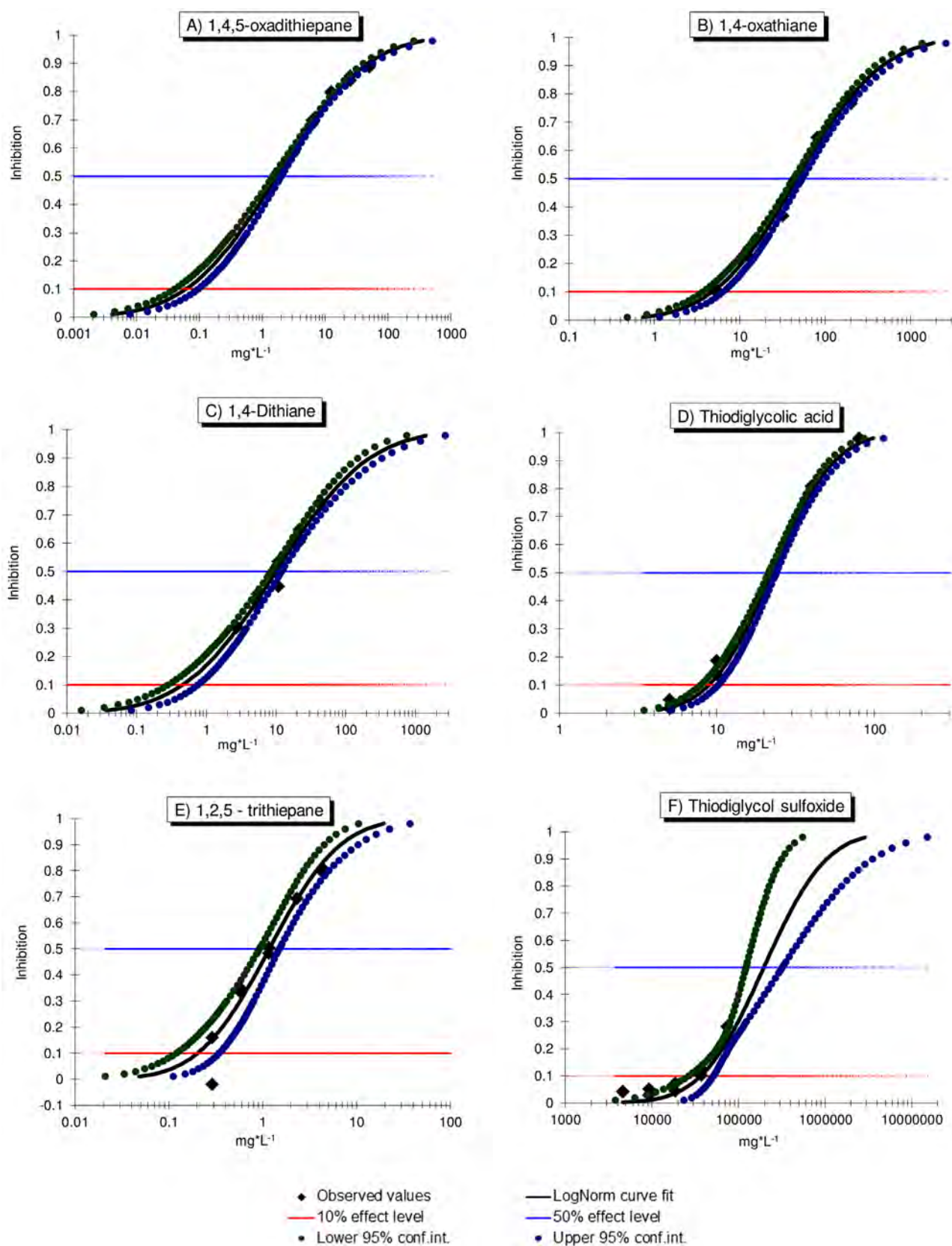


Figure 1. (A–F) Treatment–effect curves from Microtox™ tests for single-chemical tests showing fitted logarithmic curves, confidence intervals (95%), and inhibition at 10 and 50% (horizontal lines).

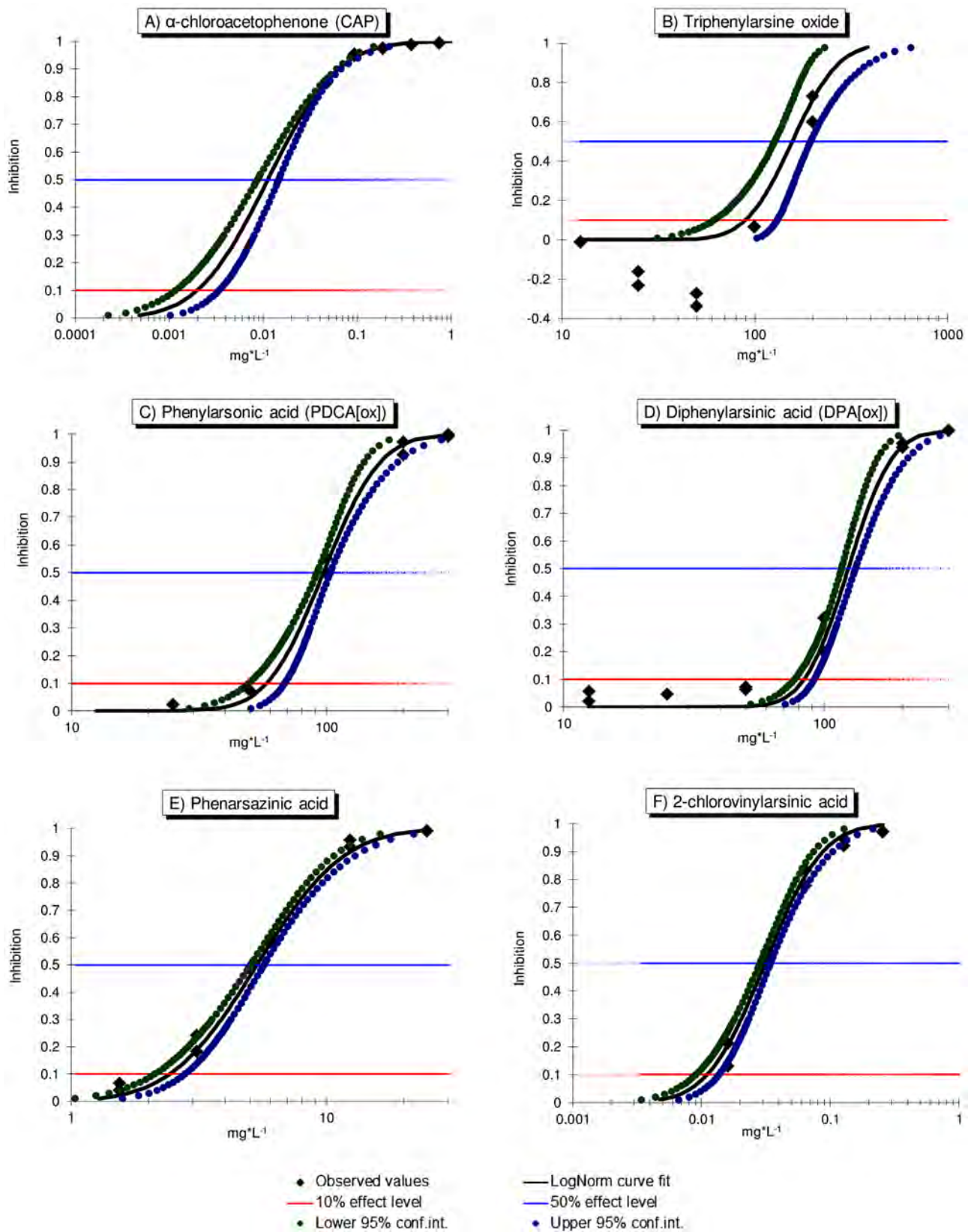


Figure 2. (A–F) Treatment–effect curves from Microtox™ tests for single-chemical tests showing fitted lognorm curves, confidence intervals (95%), and inhibition at 10 and 50% (horizontal lines). A curve could not be fitted for triphenylarsine due to an incomplete test range.

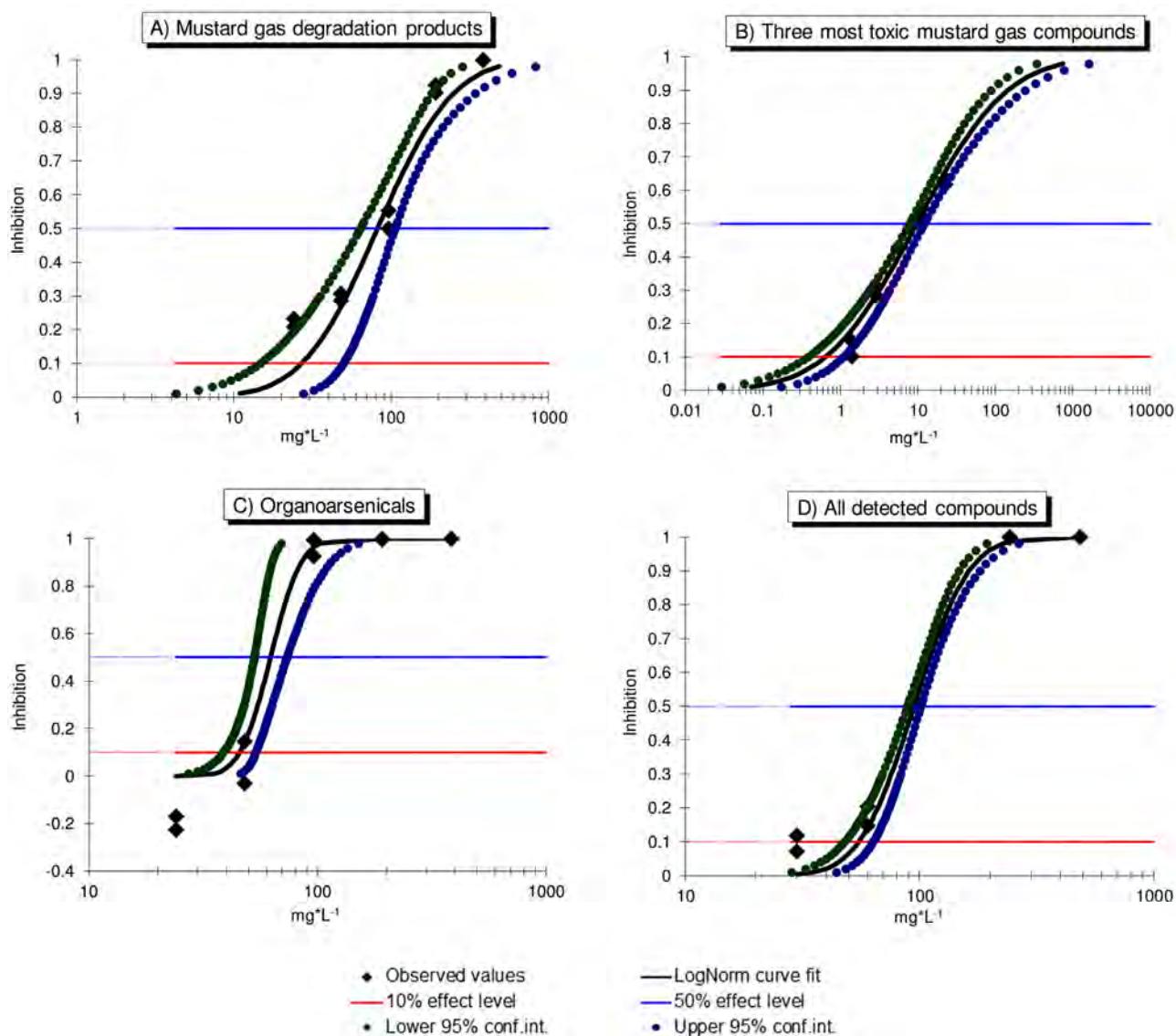


Figure 3. (A–D) Treatment–effect curves from Microtox™ tests for the mixture test showing fitted lognorm curves, confidence intervals (95%), and inhibition at 10 and 50% (horizontal lines). The X-axis shows the accumulated concentration of the compounds in the mixture.

(fish) (Sanderson et al., 2007)). This may be the case for 1,4,5-oxadithiepane and 1,2,5-trithiepane which are a factor 10 more toxic when comparing Microtox™ results to ecotoxicity data from fish experiments. Several organoarsenic metabolites were more acutely toxic than triphenylarsine ($EC_{50} > 200 \text{ mg L}^{-1}$), but only phenarsazinic acid ($EC_{50} = 5.33 \text{ mg L}^{-1}$) and 2-chlorovinylarsonic acid ($EC_{50} = 31.2 \mu\text{g L}^{-1}$) were considerably more toxic. Both, however, were only soluble using solvents. Triphenylarsine oxide (and triphenylarsine) seems to produce a hormetic effect when exposing *A. fischeri* for concentrations of 50–100 mg L^{-1} . Hormesis is defined in Calabrese and Baldwin (2002) and is a biphasic dose-response curve with a stimulatory and inhibitory part. The elevated luminescence might be ascribed to either of two hypotheses (Calabrese, 1999): direct stimulation hormesis

or overcompensation stimulation hormesis (OCSH). The latter is the compensatory biological processes following an initial disruption of homeostasis which agrees with our data when examining the temporal progression from 5 to 15 and finally 30 min (only data after 30 min shown). The OCSH has been experimentally proven (Calabrese, 1999) and, to the authors' knowledge, is the predominant hypothesis. Calabrese (1999) found an overcompensation response from peppermint plants *Mentha peperita*, exposed to phospon, a synthetic plant growth inhibitor, in doses ranging from 100 to 750 ppm for five weeks. The 100-ppm group showed a pronounced hormetic growth response after five weeks after initially being inhibited (low growth) (Calabrese, 1999). However, phospon as a specific plant growth inhibitor on plants, should be considered when in fact measuring growth. Therefore, the

Table 4. Toxicity screening of mixtures of CWA residues found in the Baltic Sea.

| Mixture/compound | CAS # | Highest concentration tested (mg L ⁻¹) | EC ₅₀ (CA-predicted) (mg L ⁻¹) | EC ₅₀ <i>A. fischeri</i> (95% CI) (mg L ⁻¹) | Model deviation ratio (MDR) |
|---|------------|--|---|--|-----------------------------|
| <i>Sulphur mustard gas bomb</i> | | | | | |
| TDG-acid | 505-29-3 | 55.0 | 16.5 | 82.0 (63.6–106) | 0.21 |
| 1,4-dithiane | 15980-15-1 | 18.9 | | | |
| 1,4-oxathiane | 3886-40-6 | 94.7 | | | |
| 1,4,5-oxadithiepane | 6576-93-8 | 3.4 | | | |
| 1,2,5-trithiepane | 3085-45-8 | 2.2 | | | |
| TDG [ox] | 123-93-3 | 211 | | | |
| <i>Three most toxic sulphur mustard gas compounds</i> | | | | | |
| 1,4-dithiane | 505-29-3 | 18.9 | 4.21 | 9.71 (7.80–12.1) | 0.43 |
| 1,4,5-oxadithiepane | 3886-40-6 | 1.70 | | | |
| 1,2,5-trithiepane | 6576-93-8 | 2.2 | | | |
| <i>Organoarsenic mixture^a</i> | | | | | |
| Phenylarsonic acid | 98-05-5 | 97.1 | 95.4 | 62.4 (52.9–73.6) | 1.53 |
| Phenarsazinic acid | 4656-80-8 | 5.33 | | | |
| Diphenylarsinic acid | 4733-19-1 | 127 | | | |
| Triphenylarsine oxide | 1153-05-5 | 155 | | | |
| <i>All tested compounds^a</i> | | | | | |
| TDG-acid | 505-29-3 | 55.0 | 61.6 | 94.8 (88.9–101) | 0.65 |
| 1,4-dithiane | 15980-15-1 | 18.9 | | | |
| 1,4-oxathiane | 3886-40-6 | 94.7 | | | |
| 1,4,5-oxadithiepane | 6576-93-8 | 3.4 | | | |
| 1,2,5-trithiepane | 3085-45-8 | 2.20 | | | |
| TDG [ox] | 123-93-3 | 211 | | | |
| Phenylarsonic acid | 98-05-5 | 97.1 | | | |
| Phenarsazinic acid | 4656-80-8 | 5.33 | | | |
| Diphenylarsinic acid | 4733-19-1 | 127 | | | |
| Triphenylarsine oxide | 1153-05-5 | 155 | | | |
| α-chloroacetophenone | 532-27-4 | 0.0224 | | | |

^aExcept for TPA and L1 [ox] as these compounds was excluded due to solubility problems.

effect seen might also be ascribed to upregulation of the production of light-producing proteins. To experimentally validate this lies beyond the scope of this study. The phenomenon of hormesis is commonly seen when using bioluminescent tests. For instance, Shao, Wu, Gao, and Wang (2012) found increased light production when exposing the freshwater *Vibrio qinghaiensis* to low concentrations of sulphides (S²⁻, H₂S, HS⁻) with pH from 5 to 10 and Fulladosa, Murat, Bollinger, and Villaescusa (2007) found hormesis effect when exposing *A. fischeri* to the two organic arsenical compounds of arsenobetaine and monomethylarsonic acid. Considering the solubility issues experienced with TPA, the term biphasic should be used with caution. No EC₅₀ value was found and the inhibitory part of the biphasic dose-response curve was not found at the concentration level tested.

Few of the CWA degradation products tested in this study had previously been tested in an ecotoxicological context. For example, for 1,4-dithiane an EC₅₀ of 24 mg L⁻¹ towards bacteria (*Photobacterium phosphoreum*) was found in the literature (Gälli, Rich, & Scholtz, 1994). A measured LC₅₀ was also found for phenylarsonic acid having a 48-h LC₅₀ of 420 mg L⁻¹ towards the medaka (*Oryzias latipes*) (Tsuji, Tonogai, Ito, & Kanoh, 1986). The corresponding EC₅₀ of 97.1 mg L⁻¹ found in this study is lower (following ISO 11348-3:2009). α-chloroacetophenone was tested on the Green Sunfish (*Lepomis cyanellus*),

by Summerfelt and Lewis in 1967, revealing an LC₅₀ of 1.1 mg L⁻¹ (Summerfelt & Lewis, 1967). However, differences in test methods should be considered. Sanderson, Fauser, Thomsen, and Sørensen (2008) derived fish community HC₅ concentrations for CAP and triphenylarsine at 500 and 0.5 µg L⁻¹, respectively. This suggests that further experimental toxicity testing is needed to determine the most accurate effect concentration to be used in risk assessments (Sanderson et al., 2008).

The present study also provides mixture toxicity assessments of four different mixtures containing the detected chemicals. Mixture toxicity has gained increasing attention as it depicts a more realistic situation as compared to single-chemical tests. The mixtures included in this study deviate slightly from the CA model, see Table 4. The sulphur mustard mixture deviates the most, but some of this deviation can be ascribed to leaving out thiodiglycol sulphoxide (EC₅₀ > 74,250 mg L⁻¹) when calculating the predicted EC₅₀. Despite the absence of an EC₅₀ value of thiodiglycol sulphoxide, it was decided that thiodiglycol sulphoxide must be a component in the mixture at a concentration level and it was present at concentration levels higher than the least toxic sulphur mustard compound with the EC₅₀ value shown in Table 4. This was done to assess the complete range of sulphur mustard degradation products. The CA model has been used for its excellent

predictive power in mixtures of similarly acting chemicals (Altenburger et al., 2000). This might not be the case with the mixtures assessed in this study, i.e. by examining the chemical structure of the compounds. For instance, the chemical structures are different within the sulphur mustard degradation products. The most toxic compounds in Microtox™ are ring-structured (e.g. 1,2,5-trithiepane and 1,4,5-oxadithiepane), whereas the least toxic compounds are acyclic compounds (e.g. Thiodiglycol sulphoxide). For mixtures of strictly dissimilar acting compounds the Independent Action Model has shown great predictive power (Backhaus et al., 2000). However, the CA predictions deviate by a factor 1.5–2.5 suggesting a successful prediction and hence, no synergistic or antagonistic behaviour. For compounds with narcotic (baseline toxicity, non-specific) mode of action, the Funnel hypothesis by Warne and Hawker is worth noting. It states that mixtures should show less deviation from additive behaviour with increasing number of components (Warne & Hawker, 1995). Besides the sulphur mustard mixture, the other mixtures only deviate within a factor of 1.5–2.5. The mixture with the fewest compounds (three most toxic sulphur mustard degradation products), shows the highest deviation from the predictive value. However, the mode of action of the compounds has not been assessed experimentally.

Due to the lack of (and need for) toxicity data on the tested compounds, *A. fischeri* was used as the test organism. It is a part of the first tier of organisms (plankton), affected by the compounds. A clear advantage of using the Microtox™ test is that toxicity data can be obtained within minutes. Higher organisms, such as fish constitute an important anthropogenic resource, might be of greater concern and relevance, and calls for attention in future risk assessments.

The data that have been generated during the last decade from more than 400 samples analysed in the MERCW, Nord-Stream and CHEMSEA projects have demonstrated that the most abundantly detected compounds related to CWA dumping are in fact CWA metabolites that have not previously been risk assessed or toxicity tested. Prior to the present study, few of the detected CWAs and metabolites have been tested for their acute ecotoxicity and, to the authors' knowledge, none of the detected CWAs and metabolites have been tested in Microtox™. Therefore, it is important that future assessments on chronic and long-term effects focus on the metabolites considered in this paper rather than on parent compounds. The observed exposure concentrations presented in Table 2, combined with the field observations of e.g. Della Torre et al. (2010, 2013) and Baršienė et al. (2014, 2016) suggest that risk appraisal could still be improved primarily by more accurate chronic toxicity testing at higher tiers of the compounds detected in the environment.

5. Conclusions

This study has provided new ecotoxicity data on 13 out of 14 different parent CWAs and their degradation products by performing the ISO-standardised Microtox™ test on the marine bioluminescent bacteria *A. fischeri*. An EC_{50} value was determined for 12 of the compounds. This study provides a first step in understanding the ecotoxicity of the CWAs and their degradation products found in the Baltic Sea. The three most toxic compounds were α -chloroacetophenone, 2-chlorovinylarsonic acid and 1,2,5-trithiepane having EC_{50} values of 11.20, 31.20 and 1170 $\mu\text{g L}^{-1}$, respectively. This study has also performed the Microtox™ test on four different mixtures. The mixtures deviate by a factor of 1.5–2.5 (MDR: 0.43–1.53) from the predictions of CA (except for the sulphur mustard mixture). However, the mixtures act according to additivity as predicted by CA. It is noteworthy that triphenylarsine oxide (and triphenylarsine) demonstrated hormesis effects at 50 and 100 mg L^{-1} , respectively. The Microtox™ test is a first-tier screening test that provides the first insight into the ecotoxicity of CWAs and their metabolites found in the Baltic Sea. However, one should interpret these data in the context of risk assessment and further testing is necessary to understand the impact of the CWAs and their degradation products on aquatic organisms.

Acknowledgements

We would like to thank K. Ole Kusk (Professor Emeritus, Technical University of Denmark) for providing us with his statistical- and graphing software. The authors would like to express our gratitude to the two anonymous reviewers who greatly improved the manuscript and the editors involved for a smooth publication process.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study is a part of Towards the Monitoring Dumped Munitions Threat (MODUM) which is a NATO Science for Peace and Security-funded project.

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