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Acute aquatic toxicity of arsenic-based chemical warfare agents to *Daphnia magna*

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ABSTRACT

Sea dumping of chemical warfare (CW) took place worldwide during the 20th century. Submerged CW included metal bombs and casings that have been exposed for 50–100 years of corrosion and are now known to be leaking. Therefore, the arsenic-based chemical warfare agents (CWAs), pose a potential threat to the marine ecosystems. The aim of this research was to support a need for real-data measurements for accurate risk assessments and categorization of threats originating from submerged CWAs. This has been achieved by providing a broad insight into arsenic-based CWAs acute toxicity in aquatic ecosystems.

Standard tests were performed to provide a solid foundation for acute aquatic toxicity threshold estimations of CWA: Lewisite, Adamsite, Clark I, phenyldichloroarsine (PDCA), CWA-related compounds: TPA, arsenic trichloride and four arsenic-based CWA degradation products.

Despite their low solubility, during the 48 h exposure, all CWA caused highly negative effects on *Daphnia magna*. PDCA was very toxic with 48 h *D. magna* LC50 at 0.36 μg × L^{-1} and Lewisite with EC50 at 3.2 μg × L^{-1}. Concentrations at which no immobilization effects were observed were slightly above the analytical Limits of Detection (LOD) and Quantification (LOQ). More water-soluble CWA degradation products showed no effects at concentrations up to 100 mg × L^{-1}.

1. Introduction

Worldwide stocks of chemical warfare agents (CWAs), right after the end of the World War II in 1945, reached about 500,000 metric tons, of which arsenic-based CWAs accounted for over 12 % (SIPRI, 1971). Between 1939 and 1945 about 13,000 metric tons of Clark I (DA), Clark II, Adamsite (DM) and the arsine oil (a mixture of phenyldichloroarsine (PDCA), DA, triphenylarsine (TPA) and inorganic arsenic trichloride

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($AsCl_3$)), were produced by Nazi Germany (Makles and Śliwakowski, 1997), accounting for about 20 % of its total production at that time. Between 1922 and 1945 about 21,000 metric tons of Lewisite (L) were produced by US, which accounted for about 14 % of the total production in this period (Krauze and Nowak, 1984; Smart, 1997). Significantly smaller amounts of arsenic-based CWAs were found in the post-war stocks of Japan, Italy and Great Britain (Makles and Tyszkiewicz., 1992; Zanders, 1997). Problematic in safe storage and disposal (Pitten et al., 1999) the chemical warfare (CW) posed a high risk for human health on land. It is estimated that up to 60 countries possessed chemical munitions, out of which 40 had chosen sea dumping as the quickest and efficient method of their disposal at that time (DEPA, 2010). Sea dumping of Lewisite by U.S. forces in the Atlantic Ocean "somewhere" between England and the United States in 1918 was the earliest recorded offshore disposal of CWAs or chemical munitions (Bearden, 2001). In general, among other types of weaponized compounds, the unused loads of arsenic-based CWAs were dumped worldwide into the seas, continental shelves and ocean deeps, with several reported operations in rivers and lakes (Smart, 1997; Long, 2009; Radke et al., 2014). Globally, there are 127 documented chemical munitions dumpsites, however, it is estimated that their number exceeds 300 (James Martin Center for Nonproliferation Studies, 2019).

Since 1920, up to 35,000 metric tons of CW including arsenic-based CWAs from WWI and WWII were sea dumped close to the Belgian coast (Francken and Hafez, 2009). A significant part of it was dumped in a sandy shallow shoal called Paardenmarkt and Zeebrugge (Missiaen and Henriet., 2002), while several minor Belgian operations took place from 1954 to 1980 in the Bay of Biscay (Zanders, 1997). France dumped about 1700 barrels with CWAs in the Bay of Biscay and approx. 24,000 metric tons of chemical munitions in the Mediterranean Sea. It is also suspected that due to their operations, there are several sunken vessels filled with chemical munitions in the Skagerrak (Konopski, 2009). Post WWII sea-dumping operations of the German chemical arsenal were performed under the guidance of the Potsdam Conference, by US (Bay of Biscay), Great Britain (Skagerrak) and by the Soviet Navy between 1945 and 1948 in the Baltic Sea. At least 3761 tons of Lewisite, Adamsite and Arsine Oil were sunk mainly in the Bornholm Deep and in the Gotland Deep (Makles and Śliwakowski, 1997). Less than a ton of Adamsite, has been additionally disposed in Bornholm Basin between 1952 and 1965 by East Germany (Knobloch et al., 2013). In addition to this, the unofficial dumping site in Gdańsk Deep was confirmed (Szarejko and Namieśnik, 2009; Bełdowski et al., 2016). The dumping operations of Russian stocks were carried out in the areas of the White Sea (in the 1950s), around the Nova Zemlya, Okhotsk, Kara and Barents Seas (between 1945–1982), the Japanese Sea (1960–1961) and the Black Sea (in 1942 and 1990), unfortunately, the quantitative data is not known (Blackwood, 1999). Between 1945 and 1948 Great Britain dumped about 175,000 tons of own chemical weapons in the Atlantic Ocean on the western coast of Ireland, in Bay of Biscay, in Skagerrak, in Beaufort's Dyke and also during 1955–1959 in Internal Hybrids (Missiaen and Henriet., 2002; Carton and Jagusiewicz, 2009). Between 1918 and 1970 the USA dumped about 350,000 metric tons of unused chemical munitions into the seas and oceans (Carton and Jagusiewicz, 2009). In 1946, 1948 and 1955, the US have disposed their own stocks, including significant amounts of post-reaction mixture from the destruction of Lewisite and pure Lewisite in the Gulf of Mexico (Blackwood, 1999). Overall, more than 9900 metric tons of Lewisite and 505 metric tons of $AsCl_3$ were also dumped in at least 11 sites in the Atlantic Ocean (Bull, 2005; DEPA, 2010). More than 3000 metric tons of Lewisite were dumped in the Pacific Ocean, at Alaskan and Hawaiian coasts (DEPA, 2010; Edwards et al., 2016). Canada destroyed post-war CWAs, dumping it into rivers, lakes and oceans. In 1946, in the Atlantic, southeast of Halifax, in Nova Scotia, and also in the Pacific, west of Vancouver Island in British Columbia, Canada dumped over 27,000 tons CWAs, however, mainly sulfur mustard (Konopski, 2009). In the same time, Australia dumped CWAs, mainly Yperite and arsenic-based CWAs near the coast of New South Wales (Konopski, 2009). It is estimated that Australia dumped over 21,000 metric tons of CWAs on their east coasts (Walker, 2012). From 1945–1948 Japan had dumped over 6600 metric tons of CWAs, mainly bombs filled with sulfur mustard, Lewisite and hydrogen cyanide (Walker, 2012). As the key feature for each of the CWAs was a recognized toxicity for humans and terrestrial organisms (Munro et al., 1999), therefore the sea-dumped chemical munitions became a potential threat to the aquatic ecosystems.

During over a decade of pioneering research on sea dumped CW, first foundations have been laid to understand the impact of CWAs on aquatic ecosystems (Sanderson et al., 2010; Greenberg et al., 2016; Bełdowski et al., 2018; Brzeziński et al., 2020; Czub et al., 2020). Submerged CW included metal bombs and casings that have been exposed for 50–100 years of corrosion and started leaking (Tørnes et al., 2006; Bełdowski et al., 2016). At several Baltic Sea dumpsites, the presence of the arsenic-based CWAs and their degradation products in sediments and pore water has already been confirmed (CHEMSEA, 2014; Vanninen et al., 2020). Arsenic was believed to be a possible proxy for CWA leakage to the environment, however, studies in the Baltic Sea did not indicate observations of its elevated levels in the dumpsites (Czub et al., 2018). Yet, the variation of arsenic concentration coming from anthropogenic inputs from other sources is too big to determine the magnitude of pollution generated by munition dumpsites (Sanderson et al., 2013). Results obtained during the EU Interreg Baltic Sea Region project DAIMON ("Decision Aid for Marine Munitions"), including reports by Niemikoski et al. (2017), indicate that degradation products of arsenic-based CWA are entering the Baltic Sea food webs. Latest findings report negative effects on the subcellular and functional levels caused by the exposure of blue mussel - *Mytilus trossulus* to DA and DM (Höher et al., 2019). Unfortunately, there are still gaps in the general knowledge about the environmental toxicity of arsenic-based CWAs. This affects the ability for scientific categorization and prediction of threats in all ongoing risk assessments of their possible impact on marine and freshwater ecosystems.

The negative impact of arsenic compounds on humans and all sorts of biota has been known for centuries (National Research Council, 1977; Eisler, 1988; Shaw et al., 2007; Ravenscroft et al., 2009; Li et al., 2018). The aquatic conditions are, however, dramatically different from terrestrial in a way that could possibly alter the effects of CWAs on the aquatic biota (HELCOM, 1994). Solubility, hydrolysis and oxidation are among various factors that shape the fate and bioavailability of a chemical compound in aquatic ecosystem. Water-solubility of many arsenic-based CWAs is considered to be low, thus, without any targeted studies, their toxic properties for the aquatic fauna could be considered as non-exceptional among other arsenic compounds. In general, the toxicity of arsenic compounds follows a given rank: inorganic As^{III} > organic As^{III} > organic As^{V} (Flora, 2015). As for now the only available aquatic toxicity thresholds of selected arsenic-based CWAs have been modelled for *Daphnia magna* in the Ecological Structure Activity Relationships (ECOSAR) predictive aquatic toxicity model, based solely on physical and chemical parameters of each compound (Sanderson et al., 2007). More recent research focused on toxicity thresholds estimation for $DPA_{[ox]}$ showing potentially harmful/ non-toxic effects for *D. magna* (Storgaard et al., 2018) and several arsenic-based CWA degradation products using the Microtox™ bioluminescent bacterial test (Christensen et al., 2016).

The aim of the current study was to provide a broaden insight into aquatic acute toxicity of arsenic-based CWAs and their degradation products by using the *D. magna* as the test organism. *D. magna* is a model aquatic species in multiple ecological and ecotoxicological studies. The lack of basic toxicity data for arsenic containing CWAs, based on standard tests can be a critical gap in risk assessment of chemical munition dumpsites (Fauser et al., 2018). This paper aims to provide the laboratory-test-derived acute toxicity thresholds for those substances, which could be utilized in improvement of risk assessment derivations and further analyses of chronic toxicity and sublethal effects of CWAs on

Table 1
List of tested compounds, including their detections (+ / -) by CHEMSEA, MODUM and DAIMON projects (CHEMSEA, 2014; Nawała et al., 2016) and published analytical measurements (Soderstrom et al., 2018- [1]; Missiaen et al., 2010 – [2]; Popiel et al., 2014 – [3]).

	NATO	CAS#	Formula	Type / Source	Physico-chemical parameters		Detection in the Baltic Sea		
					Solubility in water	*Vapour Pressure*		*Sediments (dry weight)*	*Pore Water*
Adamsite 10-Chloro-510 dihydrophenarsazinine	DM	578–94-9		CWA / Dumped as such	0.064 g × L^{-1}		Adamsite – related	Bornholm Deep: ≤ 500 $\mu g \times kg^{-1}$ [1]	–
Phenarsazinic acid	DM[ox]	4733–19-1		Degradation product of DM					+
Arsenic trichloride	$AsCl_3$	7784–34-1		CWA-related compound; CWA-precursor / Component of arsine oil	Soluble and reacts (hydrolysis)	11.65 mmHg (25 °C)		NA	NA
Clark I Diphenylchloroarsine	DA	712–48-1		CWA / Component of arsine oil	0.2 g × L^{-1}		Clark I – related	Bornholm Deep: 1 – 16,400 $\mu g \times kg^{-1}$ [1], [2] Gdańsk Deep: Below LOQ [1], [2]	–
Diphenylarsinic acid	DPA[ox]	4656–80-8		Degradation product of DA and Clark II					+
Lewisite 2-chloroethenyl dichloroarsine	L	541–25-3		CWA / Dumped as such	0.5 g × L^{-1}	0.58 mmHg (25 °C)		degradation products were detected	degradation products were detected
Phenyldichloroarsine	PDCA	696–28-6		CWA / Component of arsine oil	Practically insoluble, reacts	0.033 mmHg	PDCA – related	Bornholm Deep: 50 – 6000 $\mu g \times kg^{-1}$ [1], [2]	–
Phenylarsonic acid	PDCA[ox]	98–05-5		Degradation product of PDCA	Low				+
Triphenylarsine	TPA	603–32-7		CWA-related compound / Component of arsine oil	Low	8.7 mmHg (220 °C)		Bornholm Deep: 4.2–38,800 $\mu g \times kg^{-1}$ [1], [2] Gdańsk Deep: 6.2 – 201.8 $\mu g \times kg^{-1}$ [3] Gotland Deep: 1.3 – 3.1 $\mu g \times kg^{-1}$ [3] Słupsk Farrow: 19.5 $\mu g \times kg^{-1}$ [3]	Bornholm Deep: 3 – 112 $\mu g \times L^{-1}$ [3] Gdańsk Deep: 12 – 202 $\mu g \times kg^{-1}$ [3]
Triphenylarsine oxide	TPAO	1153–05-5		Degradation product of TPA				+	+

NA – Not Analyzed; **Below LOQ** – below Limits of Quantification.

the aquatic species.

2. Methods

2.1. Test design

The Organisation for Economic Co-operation and Development (OECD) Test No. 202: "*Daphnia sp.* Acute Immobilisation Test" (OECD, 2012) was applied to test the toxicity effects of 10 substances, most of them previously detected in sediment samples from the Baltic Sea CWA dumpsites (Table 1). The list includes four CWA intact compounds: L, DM, DA (also known as Clark I), PDCA (also known as "Pfiffikus"), two CWA-related compounds: TPA, $AsCl_3$ and four CWA degradation products: phenarsazinic acid (DM[ox]), diphenylarsinic acid (DPA[ox]), phenylarsonic acid (PDCA[ox]) and triphenylarsine oxide (TPAO). All tests were performed using either the third, fourth or fifth clutch of *D. magna* individuals (aged less than 24 h) of a clone (DMB) originating from Grosser Binnensee (Germany), a coastal lake with temporal intrusions of saltwater from the Baltic Sea. The *D. magna* clone is maintained in permanent stock culture of the Department of Hydrobiology at University of Warsaw. To fit the requirements of the OECD Test, a range from five to nine tested concentrations per substance was arranged to fit a geometric series with a separation factor of 2.0. Batch-culture design of experiment was applied. The experiments were conducted in beakers filled with 0.1 L of either tested solutions, medium (control) or solvent control. Each treatment consisted of four replicates (beakers). An equal number of unfed animals (5–8) were kept in each replicate for 48 h. Borosilicate glassware was used to minimize adhesion of tested substances. After spatial randomization the beakers were covered using a filtering paper to prevent contamination by dust.

To comply with the test validation criteria, all biotests were run in a water bath at a constant temperature of 21.0 ± 0.5 °C. The tests were performed under 16:8 L:D cycle. Light intensity was measured using a Li-Cor Biosciences® (USA), 189 quantum sensor measuring radiance and was constant during all the performed tests (0.27 – 0.36 µmol × m^{-2} × s^{-1}). Dissolved oxygen concentrations were measured using an YSI ProODO® (USA) hand-held dissolved oxygen meter, while pH was measured using Mettler Toledo® (Switzerland) F20-Standard FiveEasy Benchtop F20 pH/mV Standard Kit with LE438 sensor. Both variables were measured in all control beakers and in all the highest concentrations at the beginning and at the end of each experiment. In order to validate the overall data acquisition and to account for possible time effects, each of the tested CWAs was tested twice, which provided two independent dose-response curves for each substance. Basic chemical and physical parameters of water used for the preparation of experimental media (pH, conductivity, temperature and salinity) were monitored using YSI EXO1 (USA) probe. Additional analysis of the total organic carbon (TOC) of the medium were performed using Multi N/C® 3100 analyser from Analytik Jena AG (Germany).

2.2. Preparation of solutions

TPA (99 %) and TPAO (98.5 %) were purchased from Sigma Aldrich (St. Louis, USA), $AsCl_3$ (98 %) was purchased from Alfa Aesar (Ward Hill, USA). L, DM, DA, PDCA, DM[ox], DPA[ox] and PDCA[ox] were synthesized at Military University of Technology in Warsaw in small amounts, with a purity of 98 %. Stock solutions were prepared by dissolving known amounts of tested compounds (in few cases using sonification to ensure that dissolving was complete) in medium that was filtered through Whatmann (UK) GF/C glass fiber filters and aerated for at least 48 h before use. The medium was the same conditioned lake water that is being used for *D. magna* stock cultivation and reproduction in facilities of the Department of Hydrobiology at University of Warsaw. Medium was filtered through Whatmann (UK) GF/C fibreglass filters

Table 2
Validation of selected parameters of GC–MS/MS and LC–MS/MS methods used for analysis of tested chemicals.

	Analysis method	Parent ion [m/z]	Transition precursor ion/ product ion	CCE [eV]	LOD [µg × L^{-1}]	LOQ [µg × L^{-1}]	Recovery [%]
DM	LC-MS/MS	242	242/167	36	0.2	0.6	100
			242/77	48			
			242/139	68			
DM[ox]	LC-MS/MS	276	276/77	80	1.2	3.6	100
			276/51	136			
			276/127	56			
$AsCl_3$	GC-MS/MS after thioestrification	300	300/225	10	1.7	5.1	92
			300/183	8			
			300/117	5			
DA	GC-MS/MS after thioestrification	261	261/183	13	0.8	2.4	91
			261/107	10			
			74/46	17			
DPA[ox]	LC-MS/MS	263	263/91	80	2.7	8.1	100
			263/151	48			
			263/245	12			
			263/116	56			
L	GC-MS/MS after thioestrification	286	286/176	10	0.2	0.6	89
			286/43	15			
			286/165	10			
PDCA	GC-MS/MS after thioestrification	227	227/107	13	0.3	0.9	93
			302/259	5			
			227/185	9			
PDCA[ox]	LC-MS/MS	203	203/77	24	2.5	7.5	100
			203/50	192			
			203/91	24			
TPA	GC-MS/MS	152	152/77	30	0.1	0.3	95
			306/152	5			
			152/51	35			
TPAO	LC-MS/MS	323	323/152	60	0.3	0.9	100
			232/227	36			
			232/126	148			

CCE - Collision cell energy; **LOD** – Limit of Detection; **LOQ** – Limit of Quantification.

and aerated for at least 48 h before its use. Because of low solubility of TPA in water, in one repetition it had been dissolved in acetone (Chempur, Poland, 99.5 %) prior to the preparation of experimental media. Constant volume of acetonic solution of TPA was used to prepare medium for each concentration (100 µL per 0.1 L of water) and for solvent control treatment (100 µL of acetone free of TPA per 0.1 L of water). The resulting acetone concentration in experimental media was below the *D. magna* 48 h LC_{50} threshold concentration of 9218 mg × L^{-1} and NOEC at 403 mg × L^{-1} according to Cowgill and Milazzo (1991).

2.3. Chemical analyses

Several series of gas chromatography-tandem mass spectrometry (GC–MS/MS) and liquid chromatography-tandem mass spectrometry (LC–MS/MS) analyses were performed to provide real-data concentrations for the most accurate estimation of the toxicity thresholds (Nawała et al., 2016). Analysis were performed in two ways: intact analyses by GC–MS/MS and intact analyses by LC–MS/MS. The SRM (selected ion monitoring) transition were shown in Table 2. Analytical values of all tested concentrations of were determined by either GC–MS/MS or LC–MS/MS at the beginning and at the end of all experiments in order to determine time related variations at each concentration. All measurements were repeated at least five times and the results were standardized according to Q-Dixon test.

Analysis were performed in three ways: analyses of intact chemicals by GC–MS/MS, analyses of degradation products by LC–MS/MS and GC–MS/MS analyses after derivatization (Table 2). GC–MS analyses were performed with Agilent Technologies 7890A gas chromatograph coupled with Agilent Technologies 7000 GC–MS Triple Quad tandem mass spectrometer (MS/MS). The mass spectrometer was operated in the electron impact (EI) mode. The quantitative analyses of analytes by GC–MS/MS were performed using multiple reaction monitoring (MRM) mode. The analysis was completed using a BP-5 capillary column (30 m × 0.25 mm × 0.25 µm) from SGE Analytical Science (Mulgrave, Australia), and was carried out using the following temperature program: the column was heated from 70 to 250 °C at a rate of 20 °C × min^{-1} and the final temperature was maintained for 1 min. Helium was used as the carrier gas and the gas flow rate was 1 mL × min^{-1}. The injector temperature was 250 °C. The temperatures of the transfer line, ion source and quadrupoles were 250, 230 and 150 °C, respectively. During the analysis, the injector was in the splitless mode and the assigned solvent delay was 2.5 min. The Agilent Mass Hunter software was used to obtain chromatograms and to collect quantitative data. Agilent Mass Hunter Quantitive Analysis was used for chromatograms processing. In case of intact compound analysis of L, DA, PDCA and TPA, the liquid-liquid extraction was performed. Samples (6 mL) were transferred into a falcon tube where 2 mL of dichloromethane (DCM) were added. After 30 min of shaking at 2000 rpm, the organic layer was transferred to a different falcon tube and was dried with anhydrous magnesium sulfate ($MgSO_4$). In the following step, the samples were analysed by GC-MS/MS. The recoveries of the compounds were between 75–90 %. Due to the high values of the limit of detection (LOD) and the limit of quantification (LOQ) of DA, PDCA and L in their intact form a propanothiol derivatization was applied, for the additional analyses. The same method was applied for $AsCl_3$, using liquid-liquid extraction combined with a simultaneous derivatization as a method of quantification. Samples (6 mL) were transferred into a falcon tube where 2 mL of hexane and 0.5 mL of 1 M hydrochloric acid (HCl) were added. After 60 min of shaking at 2000 rpm, the organic layer was transferred to a different falcon tube and was dried with anhydrous $MgSO_4$. In the following step, the sample was analysed by GC–MS/MS. The recoveries of the compounds were between 80–90 %.

LC–MS/MS analysis were performed with the Agilent Technologies 1260 Infinity liquid chromatograph equipped with a tandem mass spectrometer Agilent Technologies 6460 Triple Quad LC/MS using the MRM mode. Reversed-phase gradient separation was achieved on an SB-C18 column (2.1 × 100 mm, 1.8 µm). The mobile phase was 0.1 % formic acid in water (A) and 0.1 % formic acid in methanol (B). The following multigradient steps were used: 5% B, increased to 100 % after 5 min and held for 5 min, and returned to 5% B. The column was kept at 30 °C and the injection volume was 10 µL. MS detection was carried out using atmospheric pressure chemical ionization (APCI) in the positive ion mode. The following operating conditions were used: drying gas temperature 350 °C, vaporizer temperature 400 °C, drying gas flow 5 mL × min^{-1}, corona current 5 µA, capillary voltage 3.5 kV, nebulizer pressure 35 psi and fragmentor voltage 80 V (Table 2). Datasets were collected and handled *via* Muss Hunter Acquisition and Quantitative Analysis (Agilent Technologies). DM, DM[ox], DA[ox], PDCA[ox] and TPAO samples were filtered through the syringe filter with 0.15 µm pore size and no additional sample preparation was required.

2.4. LC_x, EC_x and NOEC estimation

Immobilization and mortality of *D. magna* were recorded after 24 and 48 h by visual inspection of each beaker. Distinction between dead and immobilized animals was on the basis of behaviour (moving of filtering appendages) and post-mortem features of *D. magna* (*e.g.* whitening, unnatural body position). LC_{50} values were estimated based on the observed mortality while EC_{50} values were estimated based on a summarized observed mortality and immobilization among organisms per tested concentration. NOEC is represented by the highest analytical value of concentration at which no immobilization effects were observed. Toxicity thresholds estimations were based on results from all the successfully performed biotests. Analytical values of initial concentrations of the test compounds in the test media were selected for further processing. Dose-response curves were created using the *drc* and *ggplot2* packages in the R Studios software (Ritz et al., 2015). LC_{50}, EC_{50}, EC_{10} and EC_5 values and their significance levels were automatically calculated by the software. A significance level of $\alpha < 0.05$ was applied to the statistical analyses.

3. Results

The values of nominal and analytical concentrations correspond to each other and more or less fit the geometric series factor of 2.0, as the errors of all spectrometry-based measurements for each chemical ranged between 2.1–6.0 % (Table 3). Dissolved oxygen concentrations never dropped below 8 mg × L^{-1} either at the beginning or at the end of all of the 21 tests, thus significantly exceeding minimal requirements of the test validation (3 mg × L^{-1}). Reported pH values varied from 7.5 to 8.5 but remained stable during the test and were corresponding to the properties and concentrations of the tested compounds. TOC values of the medium water were 1.61 ± 0.03 mg × L^{-1}.

With no observed mortality nor immobilization in any of the control nor solvent control treatments, all tests met the OECD validation criteria. To validate the overall data acquisition, tests were repeated in order to create a doublet of results (Fig. 1). The majority of the tests provided statistically significant ($p < 0.001$) dose-response curves (Table 3). Except for TPA, the estimates of each toxicity threshold matched between each repetition (Table 3). A 100 % mortality was noted for all four CWA compounds, and one CWA-related compound: $AsCl_3$, however, all of them, including TPA, resulted in 100 % immobilization of *D. magna* at the highest concentrations (Fig. 1). Therefore, values from the tests where the lowest concentrations in which lethal and immobilization effects had been observed or had a lower *p* value of the toxicity threshold were considered as meaningful (Table 3), and subsequently were used for comparison with existing data from different biotests (Fig. 2).

PDCA turned out to be the most toxic among all the tested substances with the acute toxicity threshold in a concentration as low as 0.355 ± 0.03 µg × L^{-1} (EC_{50}). Thresholds of L, DA and DM are approx. × 10 to ×200 times higher, however, still at the very toxic level

Table 3
Results from OECD 202: Daphnia magna Immobilization Tests performed on 10 arsenic compounds at MUT laboratories. Numbers in *Conc* column represent the amount of tested concentrations during each test; *Indv./Tot.* - number of organisms per repetition and the total number of *D. magna* individuals used in the test, while Roman letters in the upper index represent the generation of the cohort. **O_2 –** mean Oxygen concentration and mean **pH** measurement in vessels with a highest tested concentration (A) at the beginning (0 h) and at the end (48 h) of the experiment. **Concentration (A)** - values of the highest analysed concentration (*0 h* - initial *48 h* – final); Δ represents time-related difference; *Error* indicates the standard error of performed chemical analyses. Bolded CWA LC_{50} and EC_{50} values are the most trustworthy with either effects in lower concentrations or dose-response curve characterized by stronger statistics [a] – $p < 0.001$ while [b] – $p > 0.1$. **EC_{10}** and **EC_5** are estimated values at which tested compound had negative effects on either 10 % or 5% of population. **NOEC** is a highest concentration in which no observable effects were reported.

	OECD 202: *Daphnia magna* Immobilization Test															
	Date	*Conc*	*Indv./ Tot.*	O_2 [mg × L^{-1}]		pH		Concentration (A) [μg × L^{-1}]				Toxicity thresholds [μg × L^{-1}]				
				0 h	*48 h*	*0 h*	*48 h*	*0 h*	*48 h*	*Δ*	*Error*	LC_{50}	EC_{50}	EC_{10}	EC_5	NOEC	
Adamsite	31.01.18	5	8 / 192 [IV]	9.1	8.9	8.5	8.5	324.3	315.6	97.3 %	4.9 %	74.63 ± 5.63 [a]	71.53 ± 6.88 [a]	21.54 ± 3.37	17.10 ± 3.26		Very toxic
	29.06.18	5	8 / 192 [IV]									55.60 ± 3.97 [a]	44.42 ± 3.54 [a]				
Phenarsazinic acid	14.12.18	9	8 / 320 [III]	9.5	9.3	8.4	8.5	99 227.2	97 782.9	98.5 %	6.0 %					99 227 ± 5 953	Non-toxic
	15.12.18	5	8 / 192 [IV]														
Arsenic trichloride	23.11.17	5	5 / 100 [V]	9.1	8.3	7.9	8.5	48 320.0	47 130.0	97.5 %	2.1 %	7 915 ± 586 [b]	6 086 ± 4 336 [b]	9 355 ± 478	8 875 ± 555	6 240 ± 370	Harmful
	09.10.18	5	8 / 192 [V]									11 625 ± 172 [a]	10 921 ± 290 [a]				
Clark I	25.10.17	9	8 / 320 [III]	8.9	8.8	8.5	8.5	5 536.4	5 378.3	97.1 %	4.1 %	37.02 ± 5.10 [a]	37.02 ± 5.10 [a]	29.20 ± 10.69	23.75 ± 3.74	20.42 ± 1.45	Very toxic
	29.06.18	5	8 / 192 [III]									38.20 ± 0.20 [a]	38.20 ± 0.20 [a]				
Diphenylarsinic acid	05.12.18	8	8 / 288 [III]	9.0	8.5	7.7	7.5	99 063.9	97 577.1	98.9 %	2.9 %					99 064 ± 3 170	Non-toxic
	08.12.18	5	8 / 192 [IV]														
Lewisite	27.01.18	6	8 / 224 [III]	10.4	8.7	8.5	8.6	934.7	912.4	97.6 %	3.5 %	11.88 ± 1.02 [a]	10.21 ± 0.68 [a]	1.28 ± 0.17	1.20 ± 0.17	1.17 ± 0.05	Very toxic
	27.06.18	5	8 / 192 [III]									3.20 ± 0.30 [a]	2.51 ± 0.20 [a]				
Phenyldichloroarsine	31.01.18	5	8 / 192 [III]	9.2	8.6	8.5	8.4	8.12	8.01	98.6 %	4.1 %	0.36 ± 0.00 [a]	0.36 ± 0.00 [a]	0.14 ± 0.06	0.10 ± 0.05	0.09 ± 0.01	Very toxic
	29.06.18	5	8 / 192 [IV]									0.21 ± 0.10 [a]	0.17 ± 0.20 [b]				
Phenylarsonic acid	08.12.18	9	8 / 320 [IV]	9.4	9.2	7.7	8.1	99 546.7	98 451.7	98.9 %	4.0 %					99 547 ± 3 938	Non-toxic
	10.12.18	5	8 / 192 [III]														
Triphenylarsine *in water*	21.06.18	5	8 / 192 [IV]	8.9	8.8	7.9	7.9	3 982.1	3 956.9	99.4 %	3.3 %	3 808 ± 346 [a]	1 992 ± 50 [a]	1758 ± 1220	1684 ± 1569	1 002 ± 61	Toxic
Triphenylarsine *in acetone*	06.04.20	5	8 / 192 [IV]	8.4	8.0	7.9	8.0	799.3	781.2	97.7%	5.5 %	220.75 ± 15 [a]	152.91 ± 16.34 [a]	138.89 ± 17.14	118.65 ± 18.10	81.12 ± 6.32	Very Toxic
Triphenylarsine oxide	28.11.17	5	8 / 192 [III]	9.0	8.8	8.1	8.0	101 821.1	100 742.3	98.6 %	3.7 %					101 821 ± 3 767	Non-toxic
	21.06.18	5	8 / 192 [III]														

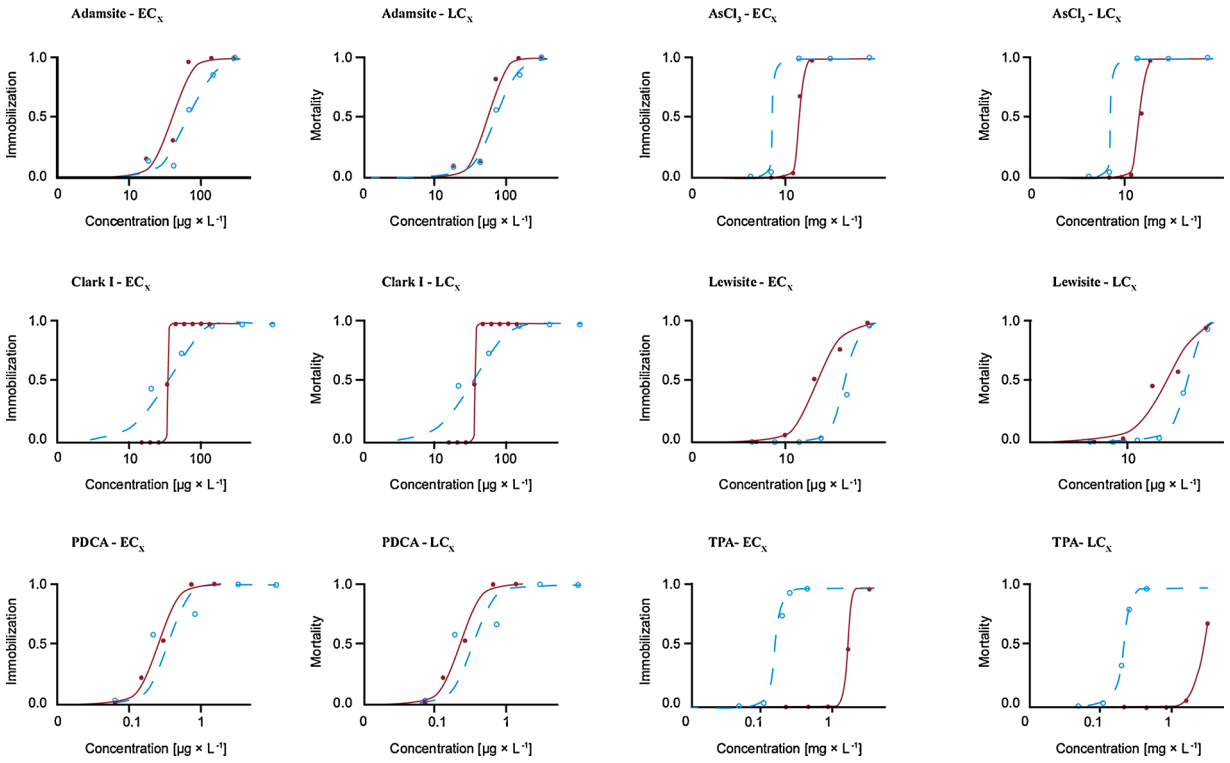

Fig. 1. Diagrammatic representation of the dose-response curves from two test repetitions of four analysed arsenic-based CWAs and two CWA-related compounds that showed immobilizing or lethal effects on *D. magna* during the 48 h of exposure. No CWA degradation products caused any observable effects; therefore, they are not represented in this figure. Results that served as a basis for EC and LC calculations are visualized as solid lines and points, while the repetitions as dashed lines and circles. Dose-response curve for TPA that was dissolved in water is visualized as solid line and points, while dose-response curve for TPA that was dissolved in acetone is visualised as dashed line and circles.

($EC_{50} < 1$ mg × L^{-1}), which places them all among very hazardous aquatic xenobiotics. TPA is toxic ($EC_{50} < 10$ mg × L^{-1}) while the inorganic $AsCl_3$ is only potentially harmful ($EC_{50} < 100$ mg × L^{-1}). The majority of the tested compounds had a low solubility in water, but it was only TPA_{water} that resulted in *D. magna* LC_{50} value at 3 808 ± 346 µg × L^{-1}, almost at its maximum solubility (Fig. 1). When diluted in acetone, the $TPA_{acetone}$ LC_{50} and $TPA_{acetone}$ EC_{50} were respectively approx. × 20 and ×10 lower than the values from the tests without solvent, thus TPA should be classified as very toxic with LC50 and EC50 values below 1 mg × L^{-1}.

4. Discussion

Values of all measured environmental variables met the validation criteria of the test (OECD, 2012), therefore the obtained results provide a solid foundation for the acute aquatic toxicity threshold estimations of all investigated compounds. Contrary to earlier assumptions, we have found that all tested arsenic-based CWAs induced stronger toxic effects on *D. magna* than sulfur mustard (Czub et al., 2020). Four tested trivalent organic arsenic compounds (PDCA, L, DA and DM) exhibited extremely high levels of toxicity and the inorganic trivalent $AsCl_3$ was potentially harmful. However, all tested pentavalent CWA degradation products exhibited no toxic effects (Table 3). Our approach aimed to test the toxicity of arsenic-based CWAs to *D. magna* without the use of solvents, to match the concentrations that can be a result of solubility and mechanical mixing in natural sediments and pore-water. Only in one case the acetone was used in a TPA repetition test, due to difficulties in obtaining homogenous solutions by sonification (especially at the maximum solubility of approx. 4 mg × L^{-1}). It seems that without solvent, at least a fraction of TPA was getting suspended rather than dissolved.

Our results provide an extended experimental verification of the predictions regarding toxicity of some arsenic-based CWAs made by Sanderson et al. (2007) using ECOSAR modelling targeting *D. magna* (Fig. 2). Mathematical modelling that utilizes structural similarities of a tested compound with available *D. magna* toxicity thresholds and physicochemical properties of other organic chemicals has drastically underestimated the potential effects of DA and L while slightly overestimating the TPA (values matched when diluted in acetone) and TPAO on *D. magna* (Sanderson et al., 2007). Comparative Microtox™ data were available only for four tested phenylarsenic degradation products: Christensen et al. (2016) report that PDCA[ox] was the only arsenic-based compound that had negative effects on the test bacterium *Allivibrio fischeri*. Toxicity threshold estimate for *A. fischeri* exposed to TPA exceeded values obtained in our study for *D. magna* (Fig. 2). It requires further testing whether this may indicate that aquatic prokaryotes are less susceptible to toxic effects driven by TPA than crustaceans. Nevertheless, our results prove that risk assessment based solely on Microtox™ may underestimate threat posed by these compounds to aquatic biota.

Knowledge about potential for exposure to CWA from continuous release from corroded containers into marine ecosystems and sediments and potential for where indication of the aquatic toxicity of the CWAs and their continuous release from corroded containers into marine ecosystems and sediments are likely to influence the management of CW dump sites. In many cases multiple types of CW containing various CWAs were dumped in relatively small areas, thus the potential effects of CWA can be considered as a multifactor threat. At the Belgian dumpsite in the North Sea, the analysed benthic communities do not differ significantly from those of the surrounding area with comparable habitat and their structural characteristics do not show any effect related to the presence of munition (Van Tomme et al., 2007). In general, the benthic communities at the wider dumpsite area are quite poor, with a total absence of macrobenthos in multiple areas, due to eutrophication

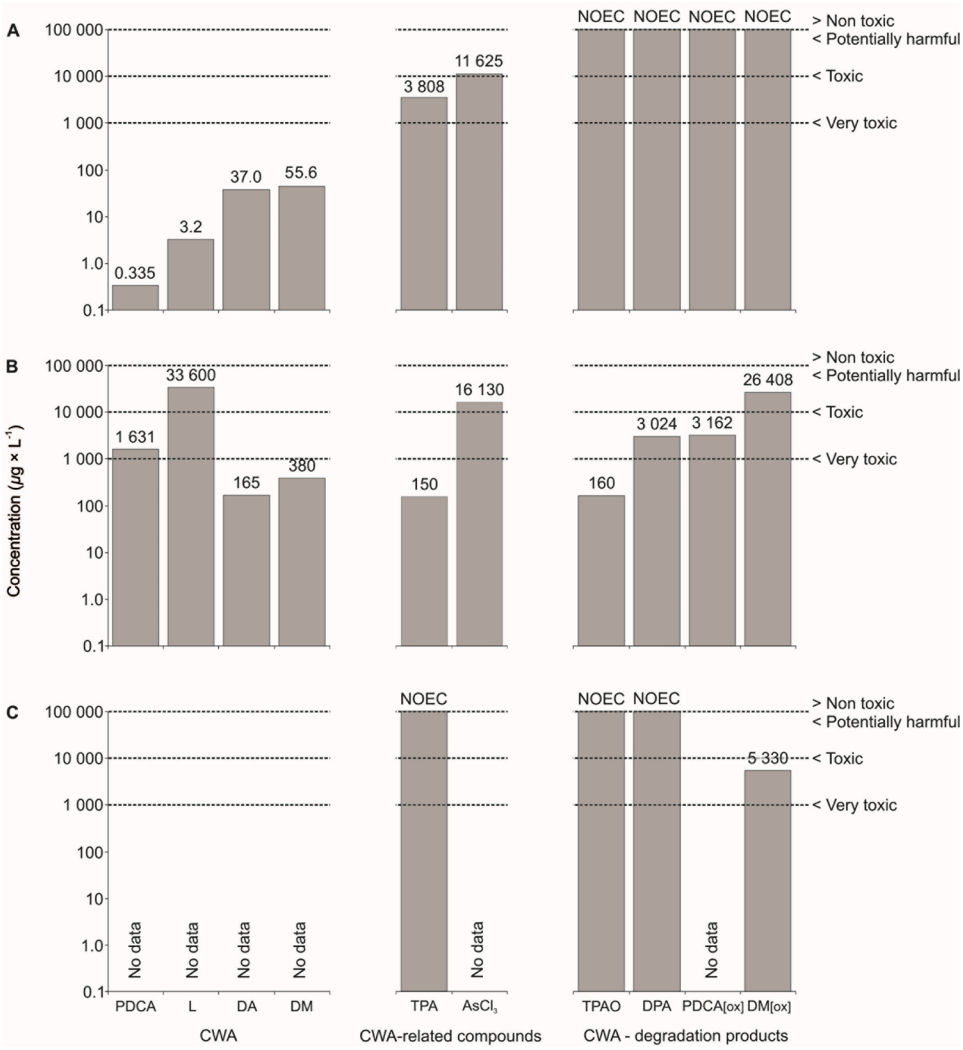

Fig. 2. Comparison of toxicity thresholds of four arsenic-based CWAs, two CWA-related compounds and four CWA degradation products based on results from three different methods: A) Organization for Economic Co-operation and Development (OECD) Test No. 202: Daphnia sp. Acute Immobilization Test; B) Ecological Structure Activity Relationships (ECOSAR) (after Sanderson et al., 2007) and C) Microtox™ (after Christensen et al., 2016). NOEC stands for No Observable Effects Concentration, meaning the compound is non-toxic for *D. magna* as there were no observable effects in highest tested concentrations (approx.100-mg × L^{-1}) during the 48 h of exposure.

and the supply of fine-grained (mud-rich) sediments from the Westerschelde estuary (Govaere et al., 1980; Vincx and Herman, 1989). Investigations by Van Tomme et al. (2007) found a slightly higher diversity in macrofauna at the dumpsite compared to reference samples in the surrounding area. A possible explanation for this could be the absence of bottom disturbing activities, since fishing and trawling is prohibited at the dumpsite. In the so-called "Primary Dumpsite" in the Bornholm Deep area of the Baltic Sea, sea-dumped loads of sulfur mustard accounted for up to 80 % of 50,000 tons of CW with organophosphorus or organoarsenic compounds making up the remaining 20 % (Knobloch et al., 2013). Furthermore, the arsine oil can enhance the potential ecological effects of sulfur mustard exposure, as it was an essential additive to the "Winterlost" mixture used as a filling in German winter-type mustard gas bombs (Konopski, 2009). Unfortunately, it is almost impossible to perform ecological *in situ* exposure studies in that area, since it is severely affected by eutrophication causing temporary or permanent anoxic/ hypoxic conditions at the sea bottom (Fig. 3) and the extension of "benthic deserts" (Feistel et al., 2008; Cartensen et al., 2014) which are limiting the occurrence of macrofauna (Czub et al., 2018). Moreover, under such reductive conditions, pentavalent arsenic species can be unstable and undergo reduction into trivalent compounds (Andreae and Froelich, 1984; Yu et al., 2016). Despite anoxic conditions, a poor presence of meiofauna was confirmed in some of the locations where chemical munitions were present (Grzelak and Kotwicki, 2016; Kotwicki et al., 2016; Czub et al., 2018), nevertheless it was not possible to distinguish *in situ* the effects of CWAs from the effects of anoxia.

In general, the unused and non-disposed loads of arsenic-based CWAs were dumped worldwide into the seas, continental shelfs and ocean deeps, with several reported operations in rivers and lakes (Smart, 1997; Long, 2009; Radke et al., 2014). The total amounts of arsenic-based CWAs dumped into the sea were not as high as those of the infamous sulfur mustard, commonly known as mustard gas, and they were believed to be less dangerous in handling than organophosphorus compounds including sarin, tabun and VX. Hence, their importance as a possible threat for aquatic environments may have been overlooked and underestimated. Our findings indicate that NOEC values of PDCA and Lewisite (Table 3) are almost at the levels of detection (LOD) and quantification (LOQ) of methods and equipment that are used in CWA contamination detection in Baltic Sea sediments (Table 2). TPA toxicity (Fig. 3) with thresholds values almost overlaps with the highest TPA detections (Table 1), that were reported by Popiel et al. (2014) in pore-water samples from CW dumpsites at Gdańsk Deep (12 - 202 µg × L^{-1}) and Bornholm Deep (3 – 112 µg × L^{-1}). The calculated *D. magna* acute toxicity thresholds of Adamsite, PDCA, Clark I and TPA (Table 3) also fall within the ranges of concentrations that are detected in the Baltic Sea sediments (Table 1). However, due to low solubility and adhesive effects of minerals and organic matter their bioavailability for the free-swimming organisms may be limited, thus reducing the risk of exposure. On the other hand, they may still pose high risk for benthic detritivores and deposit feeders, as CWA can enter their digestive systems while feeding.

The *D. magna* clone used in this study originates from the Grosser

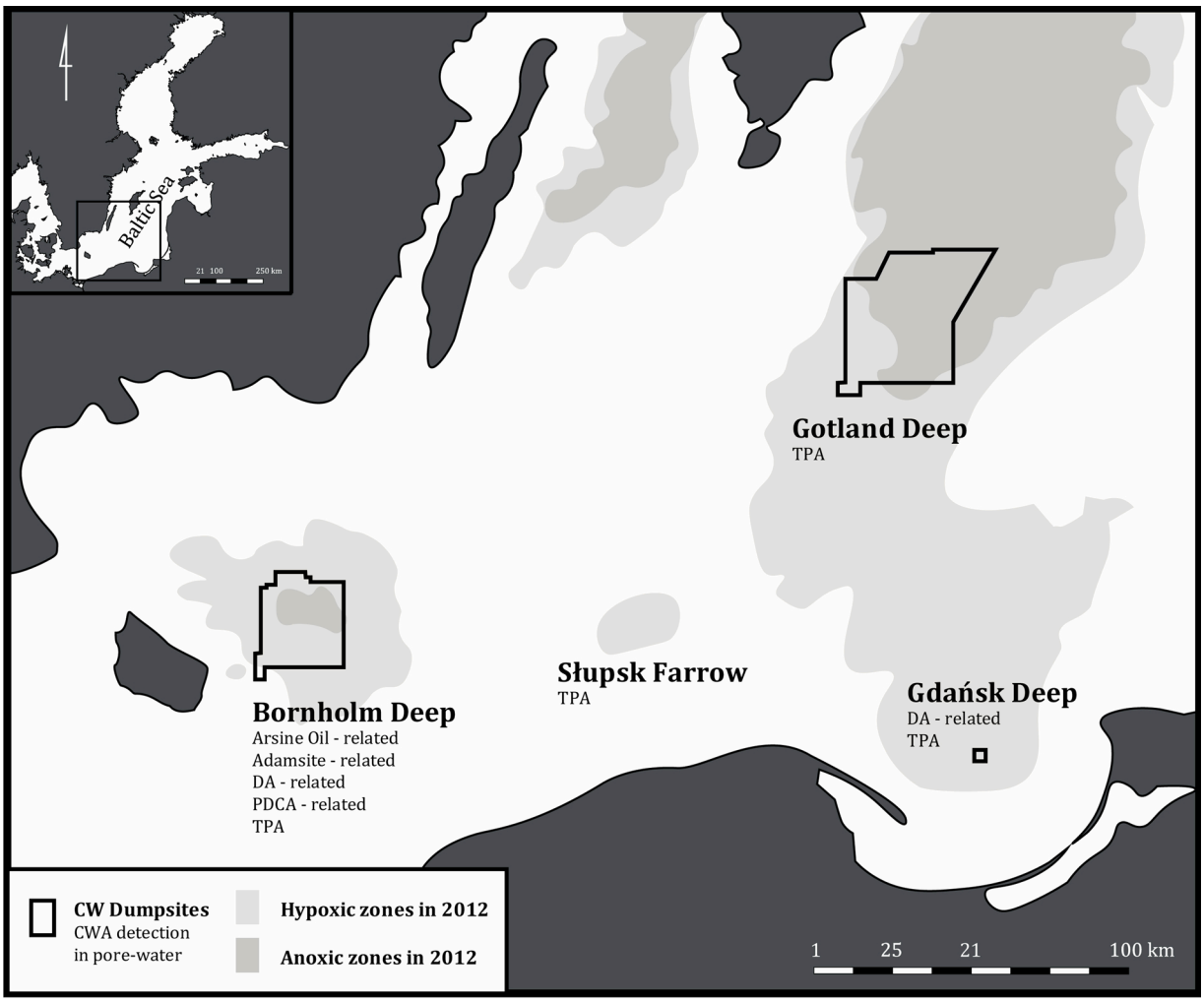

Fig. 3. Locations of deep-sea Chemical Warfare (CW) dumpsites in Baltic Sea (Czub et al. 2017) with listed arsine-based Chemical Warfare Agents (CWAs) that were detected in pore-water in the sediment samples collected within their borders. Anoxic and hypoxic zones in 2012 are redrawn from Carstensen et al. (2014).

Binnensee, the German coastal lake which is temporarily affected by seawater intrusions from the Baltic Sea, thus it can be considered as representative of both freshwater and brackish biota. If our estimates of toxicity apply to benthic invertebrates this would indicate that such levels of contamination may potentially affect benthic infauna. Already in 2016, Kotwicki et al. have reported the observed statistically significant differences between meiofaunal communities in the Baltic Sea CW dumpsites. Furthermore, detection of low concentrations of phenylarsenic compounds in fish and crustaceans from the Skagerrak and the Bornholm CWA dumpsite (Niemikoski et al., 2017, 2020) provides the first evidence for a bioaccumulation of CWA by marine organisms. On the other hand, Lang et al. (2018) did not find significant changes in health status of cod (*Gadus morhua*) from the Bornholm CWA dumpsite compared to reference sites considered free of munitions.

Until now, not much information was available in the scientific literature to explain the molecular and physiologic mechanism of the extremely high toxicity of most of the tested arsenic-based CWAs. Lewisite has a recognized mode of action which up-regulates unfolded protein response signalling, inflammatory response and apoptosis (Li et al., 2016), and is a suicide inhibitor of the E3 component of pyruvate dehydrogenase involved in the conversion of pyruvate to acetyl-CoA. Arsenic-based CWAs may also impact aquatic organisms not only by increased mortality due to acute effects, but also due to chronic toxicity and changes in life history parameters, that are crucial for fitness and reproduction. It has been found that in case of Clark I concentrations 8-fold lower than *D. magna* 48 h LC_{50} induces detectable changes in isotopic composition of tissues, fecundity, somatic growth rate and population growth rates of *D. magna*, which may be of ecological significance (Brzeziński et al., 2020). Additionally, after uptake some of the arsenic-based chemicals will probably undergo metabolism reactions, *e.g.* methylation. Report by Noguchi et al. (2006) indicates that the toxic properties of DPA[ox] increased 1000 times when it was further metabolised. Recent study from Niemikoski et al. (2020) demonstrates that CWA-related phenylarsenic chemicals undergo biotransformation reactions *in vitro*, while the conjugation with glutathione (GSH) is the major metabolic pathway. Conjugation with GSH might increase the reactivity of phenylarsenic CWAs and therefore might be responsible for the toxic properties of these chemicals.

Therefore, continuation of sea-dumped CWA research and monitoring campaigns addressing potential ecological and human food safety aspects is required, especially, since there are indications of the ongoing trawling activities in the CW dumpsite areas which may contribute to spreading of CWAs from dumping sites (Sanderson et al., 2009; Bełdowski et al., 2018; Czub et al., 2018).

5. Conclusions

Results of the presented research provide novel information on the acute toxicity of arsenic-based CWA to *Daphnia magna* that will greatly improve site-specific risk-assessments. Despite their low water solubility, PDCA, Lewisite, Clark I, Adamsite and TPA exhibit very toxic and toxic effects to *D. magna* at low concentrations. Moreover, the NOEC

values for PDCA and Lewisite are almost at the levels of compound detection. In contrast, the pentavalent arsine-based CWA degradation products did not cause any observable effects during the 48 h of exposure on the tested species. Reported toxicity values fall within the ranges of arsenic-based CWAs detections in sediments collected in the Baltic Sea CW dumpsites. Together with first reports on CWA bioaccumulation in the Baltic Sea food web, the obtained toxicity thresholds are the actual warning signals to recognize these compounds as a potentially serious hazard for the aquatic environments. Therefore, further studies of chronic toxicity and modes of action of arsenic-based CWAs as well as development of more accurate *in situ* detection methods supported by frequent monitoring campaigns of sea-dumped CW are warranted.

CRediT authorship contribution statement

Michał Czub: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Jakub Nawała:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing - original draft. **Stanisław Popiel:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft. **Tomasz Brzeziński:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition. **Piotr Maszczyk:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **Hans Sanderson:** Methodology, Data curation, Writing - original draft, Writing - review & editing. **Edmund Maser:** Conceptualization, Methodology, Writing - original draft. **Diana Gordon:** Formal analysis, Investigation, Data curation, Writing - original draft. **Daniel Dziedzic:** Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Barbara Dawidziuk:** Formal analysis, Investigation, Data curation, Writing - original draft. **Joanna Pijanowska:** Conceptualization, Methodology, Resources, Data curation, Writing - original draft. **Jacek Fabisiak:** Investigation, Writing - original draft. **Marta Szubska:** Investigation, Writing - original draft. **Thomas Lang:** Conceptualization, Writing - original draft. **Paula Vanninen:** Conceptualization, Data curation, Writing - original draft. **Hanna Niemikoski:** Conceptualization, Data curation, Writing - original draft. **Tine Missiaen:** Investigation, Writing - original draft. **Kari K. Lehtonen:** Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Supervision. **Jacek Bełdowski:** Conceptualization, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Lech Kotwicki:** Conceptualization, Methodology, Validation, Writing - original draft, Writing - review & editing, Visualization, Supervision.

Declaration of Competing Interest

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.

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