VNU Journal of Science: Earth and Environmental Sciences

Journal homepage: https://js.vnu.edu.vn/EES

Original Article

Toxicity of Military Chemical Sulfur Mustard on the Growth of *Daphnia magna*

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Received 07 August 2022
Revised 03 February 2022; Accepted 10 March 2023

Abstract: This study was conducted to assess the toxicity of the sulfur mustard military chemical to the growth and development of zooplankton *Daphnia magna*. *D. magna* is a group of crustaceans which have unique features such as virgin reproduction/ parthenogenesis form in a short time, easy to identify and control toxic substances so they are usually used as a standard model organism for toxicity testing in aquatic environments. *D. magna is* exposed to a sulfur mustard stimulant at 0, 0.001, 0.005, 0.01, 0.05, 0.1, and 0.5 ppm. The results showed that sulfur mustard agent affected the growth and development of *D. magna* during the 24 and 48 h exposure. The highest toxicity was observed at the concentrations of 0.1 and 0.5 ppm with 100% mortality after both 24 and 48 h of exposure. At 0.001 to 0.05 ppm, the mortality rate changed from 6.7 to 30% at 24 h and this ratio increased to 10 and 100% at the concentration of 0.001 and 0.05 ppm after 48h, respectively. The LC50 values recorded at 24 h and 48 h were 0.020 and 0.018 ppm, respectively. The results indicated that sulfur mustard can be toxic to the aquatic ecosystem and we need to take this into account when using this chemical group.

Keyword: D. magna, Sulfur mustard, Military toxicant, Mortality rate, Toxicity.

1. Introduction

Sulfur mustard (Yperite, H, HD) is a group of the chemical commonly produced and used in the history of chemical warfare (CW) and had been weaponized since 1913 [1, 2]. It can cause skin ulcers, exists as a colorless liquid or amber color, and has a recognizable distinctive

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https://doi.org/10.25073/2588-1094/vnuees.4894

odor (garlic-like odor), and is active at room temperatures [2]. Sulfur mustard is used as an agent to delay enemy action in battle, the impact time depends on the vehicle used and the weather. Although sulfur mustard is heavier than water, small droplets can float on the surface of the water and become dangerous to humans and ecosystems. Complete combustion of sulfur mustard releases the toxic products that contain oxides of sulfur and chlorine [2]. According to Konopski et al., (2009) [3] the total global production of CW up to 1945 is estimated to be around 500,000 tonnes, in which sulfur mustard is reported to be 206,000 - 257,000 tonnes [3, 4]. At the end of World War II there were at least over 1000 tons of mustard agent left in bombs and weapons stockpiling in warehouses of Germany, USSR, Japan and USA [3]. The most common method to dispose the mustard agent is sea-dumping. Residues of these chemicals have been detected in groundwater in high exposure areas [5, 6]. Furthermore, to increase lethality and improve the physical properties, sulfur mustard is often produced in mixtures (mixed with arsine oil or with Lewisite) which is now recognized as toxic for the aquatic ecological system [1, 7].

Compared with other neurotoxins, sulfur mustard has lower toxicity but still has toxic potential to organisms and ecosystems [5, 8, 9]. The effects of sulfur mustard may be delayed by more than 4-6 h, but the toxic potential time may be up to 24 h. Initially, mustard agent acts as a cell stimulant and eventually poisons the tissues. Initial symptoms include inflammation of the eyes, inflammation of the nose/throat, bronchi, trachea, lung tissue, skin redness, blistering or ulceration of the skin may be occurring when a person is exposed to this chemical [5, 8]. The eyes are sensitive to mustard even at low concentrations, whereas skin lesions require higher concentrations. Sulfur mustard breaks down very slowly and the accumulation of sulfur mustard in the body occurs with repeated exposure. The mean lethal concentration in which mortality is 50 (LC50) (via respiratory) is 1,500 mg.min/m^3, skin contact is 10,000 mg.min/m^3. The mean lethal dose in which mortality is 50% (LD50) is quite large up to 100 mg/kg [1, 2, 8, 9].

Freshwater crustacean *D. magna* belongs to the family *Cladocera* with varied species such as *Daphnia longispina*, *Daphnia pulex*, also known as the water louse and water bugs. They are distributed in many places in the ecosystem, and can eat varied types of food including mainly fresh unicellular algae, bacteria, yeast,... *D. magna* is a group of crustaceans that an reproduce by parthenogenesis (mothers only give birth to female offspring). They can grow rapidly in 7 to 8 days at optimum temperature of 21±1 ^oC. Their bodies are oval shaped with indistinct segments, and covered with crustacean [10, 11]. *D. magna* is very sensitive to environmental factors, for example: their body will change immediately when exposed to toxins even at low concentrations (eggs will turn black color in the incubator bag and hatch into males). This species is easy to observe or be manipulated. That is why *Daphnia* crustaceans are often used as a model organism for testing the toxicity in the aquatic environment [10, 11].

Currently, the tons of bombs/bullets in Vietnam containing military toxins still exist in the ground and have not been treated yet, potential risks to groundwater quality, soil quality and impacts on humans and living organisms in the environment if the handling measures do not take timely [2]. Although mustard was considered a chemical weapon about 75 years ago, understanding of its biological interaction mechanism is still unclear. Therefore, until now there is no specific antidote for mustard poisoning. The interaction mechanism between mustard and tissues are well known but the correlations between these interactions and lesions have not been established [2, 4, 8, 9]. In the past few decades, the scientists had found out many of the biological interaction mechanisms of mustard agent and put forward many important hypotheses but studying and evaluating mustard's toxicity to aquatic organisms is still limited. Therefore, this study was conducted to

evaluate the toxicity, toxic ability, and toxic level of skin ulcer toxicant sulfur mustard agent on the growth and development of zooplankton *D. magna*.

2. Methodology

2.1. Material

Chemical sulfur mustard with a purity of 99.63% was collected and refined by the Military Institute of Science and Technology for antidotal research purposes.

The *D. magna* crustaceans in this study were sourced from the Department of Ecotoxicology - University of Lige (Belgium). *Daphnia* is growing in M4 medium (ISO 6341:2012) and feeding with green algae *Chlorella vulgaris*, at 21 ± 2 ^{o}C, the light-dark cycle is 16:8 hours with intensity lighting from 500-800 lux [12]. Change the culture medium and feed every two days during 1 month until there is enough *D. magna* for the toxicity test (210 individuals). The organisms (≥ 1 day old) were not fed for 24 h before being collected for testing. DO, pH parameters in this study were measured by TOA-DKK WQC 24 (Japan) and the light intensity was measured using PEC-999 (UK).

2.2. Evaluate the Toxicity of Chemical Sulfur Mustard on Growth and Development of Crustacean D. magna

To evaluate the toxicity of chemical sulfur mustard on the growth and development of crustacean *D. magna*, the experimental setup was performed according to the steps in the OECD (Organization for Economic Cooperation and Development) 2012 protocol [13] as follows: *D. magna* was random selected for each toxicity test and cultured individually in SPL 6-well plates (Korea) containing sulfur mustard chemical and the control sample (absent sulfur mustard). Then, *D. magna* (≥ 1 day old) was exposed to the chemical Sulfur mustard at concentrations 0; 0.001; 0.005; 0.01; 0.05; 0.1 and 0.5 ppm. The toxicity of chemical sulfur mustard was calculated by survival/mortality ratio after 24 and 48 h. All samples were repeated 3 times.

2.3. Evaluate the Impact of Chemical Sulfur Mustard on the Morphology of Crustaceans D. magna

To evaluate the impact of chemical sulfur mustard on *D. magna* morphology, the experiment was conducted as follows: *D. magna* (≥ 1 day old) exposed to sulfur mustard chemical at concentration of 0.1 ppm after 24 h. Samples were collected and observed under an Olympus electron microscope at 40x magnification. The change in the morphological structure of *D. magna* was compared with the organism in the control sample (without chemical exposure).

2.4. Statistics

LC50 values at 24 and 48 h were calculated by Probit method (Finney, 1971) with statistically significant results ($\rho < 0.05$) [14]. The survival/mortality ratio fluctuations were calculated and drawn using GraphPad PRISM 6 software.

3. Results and Discussion

3.1. Effects of Chemical Sulfur Mustard on Growth and Development of D. magna

The experimental results about the influence of chemicals causing skin ulcers sulfur mustard on the growth and development of *D. magna* shown in Figure 1. The results showed that the different concentrations of chemicals have different effects on the growth of *Daphnia* and at different expos ing times, the influence is also different. Almost all the *Daphnia* crustaceans were affected with low survival after 24 and 48h in the experimental samples (0.001; 0.005; 0.01; 0.05; 0.1 and 0.5 ppm sulfur mustard) when compared with the control sample (0 ppm and the survival rate of 100%). The strongest toxicity was observed at concentrations of 0.1 and 0.5 ppm with 100%

mortality after both 24 and 48 h. At 48 h, the sulfur mustard chemical killed 100% of the experimental organisms with just 0.05 ppm concentration. With chemical addition rates varying from 0.001 to 0.05 ppm, the number of mortality individuals varied from 6.7 to 30% after 24 h, respectively. The mortality rate increased to 10% and 100% at concentrations of 0.001 and 0.05 ppm after 48 h, respectively. The experimental results about the influence of sulfur mustard on the morphology of *Daphnia* showed that the *Daphnia* crustaceans exposing with sulfur mustard were seriously affected (Figure 2b) when compared with the control sample (Figure 2a). After exposing with chemicals, the internal organs in their body were decomposed and deformed, the limbs fell off, the eggs turned black color and the *Daphnia* was no longer able to reproduce, and even died. The *Daphnia* crustacean is food for a number of aquatic organisms and fish, therefore these species' exposure to chemicals and toxicants will potentially pose a threat to the food chain in the ecosystem.

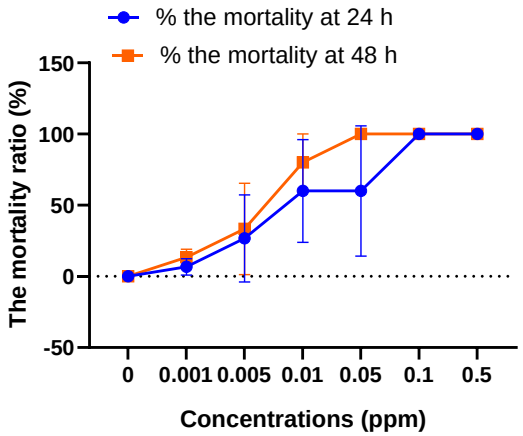

Figure 1. The mortality variation of *D. magna* crustaceans after 24 and 48 h exposure to 0; 0.001; 0.005; 0.01; 0.05; 0.1 and 0.5 ppm sulfur mustard chemical; values are reported as the mean of three replicates ± SD (n = 3).

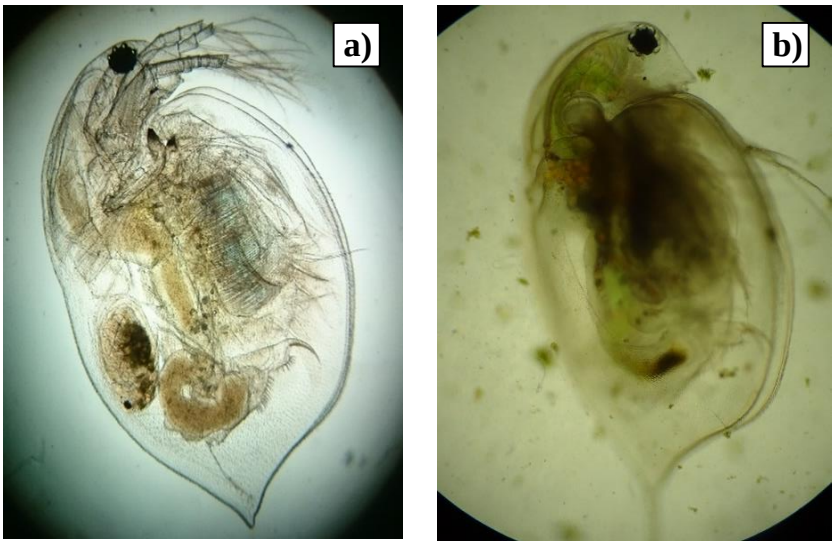

Figure 2. The morphological changes of *D. magna* crustacean before (a) and after (b) when exposed to sulfur mustard chemical at 0.1 ppm after 24 h (Bars, 20µm, Magnification 40×).

Almost general chemicals and chemicals used in the military have been shown to cause toxicity to both animals, plants and humans [1-3, 5, 8, 9]. HD is one of a group of military chemicals to be destroyed. It causes melting in several arsenals. HD is including a variety of compounds in the form of sulfur tear gas (bis-(2-chloroethyl) sulfide), agents H, HD and HT and

a small amount of organic arsine (dichloro(2-chlorovinyl) arsine) [2, 3]. The toxicity of these substances has been studied in a number of mammals, however, the studying on *D. magna* crustacean is absent or mentioned very little. Our experiments are consistent with previous results [1, 5, 11, 15]. *D. magna* exposure to mustard gas and its degradation products include: thiodiglycol sulfoxide (TDGO); thiodiglycolic acid (TDGA); 1,4-dithiane; 1,4-oxathian; 1,4,5-oxadithiepane and 1,2,5-trithiepane showed that diluted sulfur mustard is more toxic than parent sulfur mustard and sulfur mustard isomers are also more toxic [1]. Czub et al., (2020) also indicated that sulfur mustard, 1,4,5-oxadithiepane and 1,2,5-trithiepane caused 100% mortality at the highest tested concentrations, the remaining isomers have mortality rate ranged from 84 to 91% after 48 h [1]. Acute aquatic toxicity threshold LC50 was recorded of 224 ± 12 $\mu g.L^{-1}$ value for 1,2,5-trithiepane as one of the most toxic CW degradation products have been investigated so far. Another study by Chmielińska et al., (2019) showed that degradation products of mustard leaked into the groundwater of Munster city (Germany) [5]. The results of the ecotoxicity experiments with the green algae *Raphidocelis subcapitata* and *D. magna* showed that HD is a strong carcinogen, so their genotoxicity and carcinogenicity have a strong impact on the morphology of experimental organisms. *D. magna* is more sensitive than algae and also more sensitive than zebrafish, leading mutations in the p53 gene within exons 5-8 [5].

According to Chmielińska et al., (2019) [5] hydrolysis is an important degradation process of sulfur mustard gas. This process follows a first-order kinetic rate equation with a half-life in minutes [5]. Due to the low water solubility and the formation of polymers on the surface of mustard intermediates, they can remain undispersed under water for a long time and are toxic to the ecological environment [15]. Mustard-related compounds commonly detected in the environment are cyclic thioethers that can be considered as i) A by-product formed during the synthesis of mustard; ii) Degradation products formed during the decontamination process; and iii) Environmental degradation products of HD are formed. The toxicity of sulfur mustard can also follow a specific degradation pathway, leading to the formation of toxic sulphonium ions and death of the exposed organism [5]. Tran et al., (2021) also showed that the internal organs of *D. magna* crustacean was seriously affected when they exposed to tear gas CS (O-chlorobenzylidene malononitrile) using in the military, the organelles were destroyed, the reproductive capacity is lose,... these problems leading to the experimental organisms died and the mortality rate is high when the chemical dose and exposure time is prolonged [11]. *D. magna* is a crustaceans group reproducing in the form of parthenogenesis, the mother only gives birth to offspring, so the body variations have a great influence on their growth and reproduction. Plant protection products, such as POPs or military chemicals, reduce the reproductive capacity, the survival rate and increase the number of abnormal juveniles, reducing the average survival time of the mother [10, 11].

3.2. The Toxicity Evaluation of Sulfur Mustard Chemical on the Growth of D. magna Crustacean

To determine the lethal concentration level of chemicals causing skin ulcers sulfur mustard to *Daphnia*, the estimated LC50 values at 24 and 48 h exposure time were calculated by Probit method and GraphPad PRISM6 software. The results in Table 1 and Figure 3 showed that the toxicity of chemicals increases according to exposure time. The LC50 values at 24 and 48 h were recorded of 0.020 and 0.018 ppm, respectively. This result indicated that when the accumulation of chemicals in the body increases over time leading to the toxic effect in the body *D. magna* also increases. The experimental result of this study is similar to some previously published results. The chemicals such as pesticides [16], POPs [17], heavy metals [18], plastics [19] or other CWs [20],... had shown to be risk potential to plants, animals, humans, and

the environment. The different chemicals will cause different effects on the growth and development of *D. magna*. However, the other military chemical is the CS stimulant showing a lower toxicity level than the skin ulcer HD chemical used in this study. Tran et al., (2021) calculated that the LC50 values of *D. magna* after 24 and 48 h exposure to CS chemical were 0.044 and 0.034 ppm compared with the LC50 of HD in this study of 0.020 and 0.018 ppm, respectively [11]. Distance and exposure time are also factors that affect the toxicity capacity of military chemicals [21]. According to Donald et al., (2008), the smaller the distance and the longer the exposure time, the higher the mortality rate of *D. magna* [21]. After 60 min of exposure with a contact distance of 5 m, the mortality rate of the experimental crustacean *D. magna* increased from 15% to 65% after 24 and 48 h, respectively [21]. Furthermore, the effect of the toxin is strong or weak depending on the number of days of age, the weight and size of the experimental organism. Individuals with small body size often have strong respiration and high metabolic rate leading to the concentration of toxic substances entering the body faster than large individuals [11]. The F0 parent individuals exposed with chemicals and toxicants will reproductive F1 offspring more sensitive, weaker than control individuals even at low levels of toxic concentrations [11].

Table 1. Estimated LC50 values at 24 and 48 h of *D. magna* exposure to HD chemical

Mortality rate	Concentration of HD chemical (ppm)	
	24h	48h
LC_1	0.054	0.353
LC_{10}	0.033	0.097
LC_{20}	0.027	0.057
LC_{30}	0.024	0.038
LC_{40}	0.021	0.027
LC_{50}	0.020	0.018

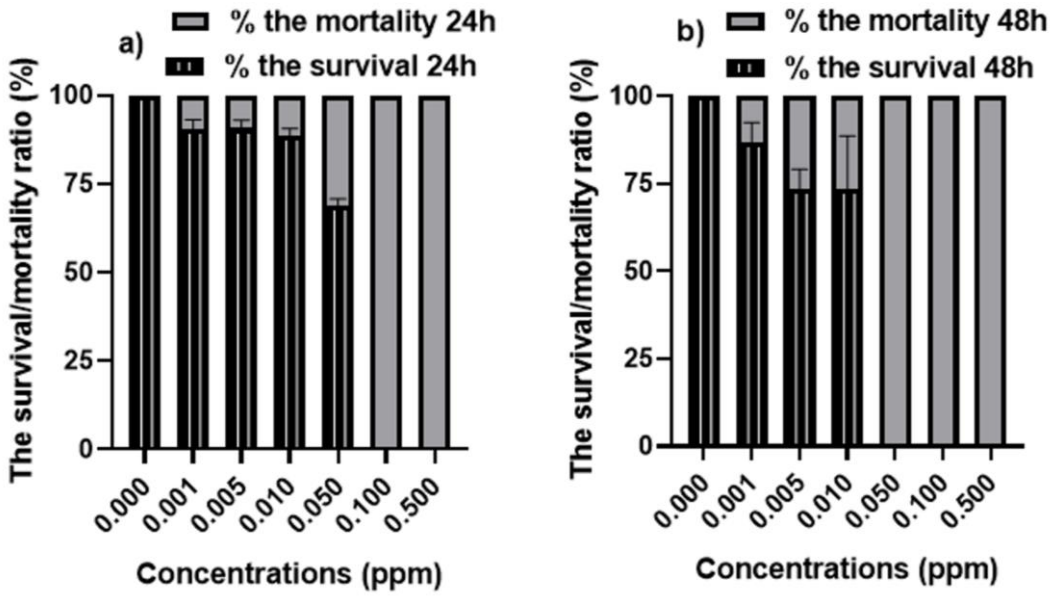

Figure 3. The mortality variation of *D. magna* crustaceans after 24 (a) and 48 h (b) exposure to sulfur mustard military chemical; values are reported as the mean of three replicates ± SD (n = 3).

According to Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous or Other Gases, and of Bacteriological Methods of Warfare (Geneva Protocol), 1925, Bureau of International Security and Nonproliferation, these military chemicals banned for use. In the

plan to destroy chemical agents used in war, the US Department of Defense enacted legislation the Deadly Stockpile Destruction Defense Act 1986 and required that bombs, bullets, and unique chemicals must be disposed before September 30, 1994. However, this goal was only achieved in 1999 and one of the toxic chemicals such as tear gas was used again to prevent riots from 2020 [9, 11], therefore, these substances always have the potential causing acute and chronic toxicity in the ecological environment. In addition, the half-life and hydrolysis of HD are longer than other active substances in the same group, the half-life of hydrolysis is 5 minutes at 37 ^{o}C. The half-life is 8.5 and 60 minutes at 25 °C in distilled water and salt water, respectively. The hydrolysis processing will produce thioglycol and hydrochloric acid which inhibits cell activity [2].

Sulfur mustard toxicant (HD) transforms to a highly chemically active sulfonium ion which serves as an electrophile agent through the intramolecular cyclization closing processing. This active electrophile can react with any nucleophile site present in the macromolecule of cells. Nucleophiles exist in peptides, proteins, ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and cell membrane components. Therefore, studying the action mechanism of the molecular biological reactions leading to the injury by mustard performed in the mammals such as mice, goats, sheep, microorganisms, and horses [9, 20-22]. The studying results showed that the LD50 values of HD is higher than the LD50 values of the other toxins, example the LC50 value for H/HD of 100 mg/kg compared with an organophosphate neurotoxin of only 0.04 mg/kg [9, 20-22]. However, the actual battlefield concentrations may be exceeded 1500 mg-min/m [9, 20-22]. Because Mustard attacks so many sites in the cell, it is extremely difficult to find a specific site to make an antidote. In fact, the treatment of Mustard poisoning also faces many obstacles and takes a long time, it is difficult to fully recover in a short time [2].

Czub et al., (2020) showed that nitrogen mustard caused more acute toxicity than sulfur mustard with an LC50 value of 2.5 mg.L^{-1} for *D. magna* after exposing with 30s; 15, 30 and 60 minutes [1]. This result also suggested that diluted HD or some degradation products of HD are more toxic than parent HD, and its isomers are also more toxic with a reported acute toxicity threshold of ">1 mg. L^{-1} and <10 mg. L^{-1}" [1]. Moreover, HD acts as a neurotoxic agent rather than a blistering drug to crustaceans leading to an increased locomotor response after initial immobilization (possibly reflecting possible burn shock). The organisms always tend to find a way out of environments that are contaminated or contain toxins, this indicates that when the environment is mechanically disturbed, sulfur mustard can pose a significant threat to benthic organisms. Environmental threats associated with HD occur only through direct contact with large lumps of sulfur mustard lying on top of the sediment [23]. On the other hand, *D. magna* is a favorite food of fish and some aquatic organisms, if fish eat individuals exposed to toxicants, they will be indirectly affected and cause adverse effects to human food safety. Therefore, it is necessary to carefully consider these substances safety when using chemicals in general and sulfur mustard in particular in order to minimize the adverse effects on the growth of organisms and the aquatic ecological environment. In addition, the arsenals also need to carefully manage and have a methodical pollution treatment plan in order to avoid the leakage of these chemicals into the soil, water and aquatic ecosystems.

4. Conclusion

Sulfur mustard is released into the environment and converted into isomers as well as different degradation products so sulfur mustard always has the potential to cause toxicity to the human, environment as well as the ecosystem. Our results showed that this chemical has seriously affected the growth and development of the zooplankton *D. magna*. Compared with the control sample with the survival rate of 100%, the experimental samples

have a low survival rate after 24 and 48 h and almost all crustaceans are affected. The strongest toxicity was observed at concentrations of 0.1 and 0.5 ppm with 100% mortality after both 24 and 48 h. The LC50 values of sulfur mustard chemical at 24 and 48 h were recorded as 0.020 and 0.018 ppm, respectively. The results showed that sulfur mustard adversely affected the growth and development of *D. magna* and has potential hazards to aquatic ecosystems. Therefore, it is necessary to consider comprehensively when using these chemicals to limit the impact on organisms as well as their living environment.

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