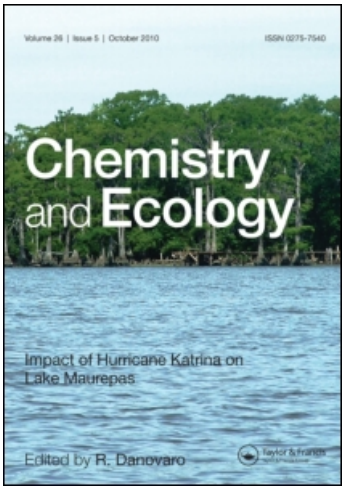

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

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To cite this Article Vesela, Sarka , Kuca, Kamil and Jun, Daniel(2006) 'Toxicity of the nerve agent tabun to *Daphnia magna*, a new experimental species in military toxicology', Chemistry and Ecology, 22: 2, 175 — 180
To link to this Article: DOI: 10.1080/02757540600579383
URL: http://dx.doi.org/10.1080/02757540600579383

Chemistry and Ecology
Vol. 22, No. 2, April 2006, 175–180

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Toxicity of the nerve agent tabun to *Daphnia magna*, a new experimental species in military toxicology

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(Received 9 November 2005; in final form 11 January 2006)

Daphnia magna, a freshwater microcrustacean, is currently tested as an alternative experimental species in research dealing with nerve agents poisonings treatment. Because of this, the toxicity of the nerve agent tabun (a cyanide-group containing organophosphate) to *Daphnia* had to be examined by estimating the EC_{50} values. The immobilization of daphnids was chosen as the end-point. It was found that *D. magna* is sensitive to small amounts of tabun, even after 15 min exposure, and tabun toxicity increases with time. The estimated EC_{50} values for 15, 30, 45, and 60 min exposure were as follows: 67.39, 38.10, 26.95, and 21.9 $\mu g\,l^{-1}$. In addition, the toxicity of media to which tabun was added 24 h before the start of experiments was examined. The results obtained indicate that daphnids can be used in experiments with nerve-agent intoxication treatment.

Keywords: Acetylcholinesterase; Chemical warfare agents; Cholinesterases; Organophosphates; Crustacean

1. Introduction

The most potent of the known chemical agents are those used in chemical warfare. Among them, nerve agents, such as sarin, tabun, soman, or VX are fluorine-, cyanide-, or thioester group-containing organophosphates similar to insecticides. They are rapidly lethal and hazardous by any route of exposure. The target sites of these substances are cholinesterases (ChE). The main toxic effect is caused by their covalent binding to the enzyme acetylcholinesterase (AChE; EC 3.1.1.7) causing irreversible phosphorylation and subsequent inhibition of enzyme's active site. This results in acetylcholine accumulation at neuroeffector junctions in the peripheral and central nervous system, and leads to a disruption of a normal nervous system function [1]. A human antidotal therapy of organophosphate intoxications is generally a combination of an anticholinergic substance (atropine mainly) together with a so-called AChE reactivator [1, 2].

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Chemistry and Ecology
ISSN 0275-7540 print/ISSN 1029-0370 online
http://www.tandf.co.uk/journals
DOI: 10.1080/02757540600579383

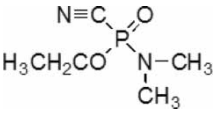

Figure 1. Chemical structure of tabun.

Daphnia magna is a freshwater microcrustacean widely used in ecotoxicological tests [3]. Beside this, intensive research has been carried out on its use as an alternative experimental species in military toxicology. It has already been shown that tests with daphnids can be employed with satisfaction to prescreen the toxicity of newly synthesized AChE reactivators [2]. As a subsequent step, the intention is to use them in experiments dealing with organophosphate poisoning treatment. To perform this, it is necessary to examine the toxicity of nerve agents to daphnids. As a first agent, tabun (GA, *O*-ethyl-*N*,*N*-dimethylamidocyanophosphate; figure 1) was chosen to be tested. This choice was made because it belongs to the chemical warfare agents that are still present in world stockpiles [4], and intensive research on its poisonings treatment has been carried out [5, 6]. Its toxicity to freshwater invertebrates as well as its toxicity after short-time decomposition in water environment has not yet been examined [7].

The objectives of this study were twofold: to characterize the acute toxicity of tabun to *D. magna*, and to examine the toxicity of media to which tabun was added 24 h before beginning of the experiment.

2. Materials and methods

Tabun was obtained from the Technical Institute, Brno (Czech Republic). Its purity was 89% measured acidimetrically. ISO medium was prepared according to EN ISO [8]. Chemicals used for its preparing ($CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $NaHCO_3$, KCl) were purchased from Lachner–Lachema (Czech Republic) in analytical grade, and the water was ultrapure (with a conductivity less than $5\,\mu S\,cm^{-1}$).

Neonates of *Daphnia magna*, clone HK (clone *a* sensu Baird *et al.* [9]), that originated from third- to sixth-brood females were used as mothers to produce test neonates. Mothers were kept in groups of 10 animals per 1000 ml of medium, at a temperature of $20 \pm 1\,^{\circ}C$, with a light regime of 16 h light : 8 h dark and a food level equivalent to 2 mg of carbon per litre of *Scenedesmus acutus* MEYEN.

For tests, female neonates from the third to sixth brood not older than 24 h were used. For each treatment, three replicates of seven animals were used. Test concentrations were subsequently prepared by adding an appropriate aliquot volume of tabun solution to the synthetic ISO medium immediately before start. Concentrations ranged from 13.10 to 187.0 μg tabun l^{-1} in the first experiment and from 20.0 to 62.5 $\mu g\,l^{-1}$ in the second experiment, and a sufficient number of treatments to obtain a good description of the whole dose–response curve were used. For more details of methodology and experimental conditions, see EN ISO [8].

The aim of the tests is to determine the median effective concentration, EC_{50}. This is defined as the concentration at which 50% of the exposed organisms are affected by measured effect [10]. In our tests, inhibition was chosen as the effect. All daphnids are considered as inhibited when as a result of the toxic action of the tested substance they are immobilized; unable to swim within 15 s after gentle shaking of the medium in the test beaker [8]. In the experiments on tabun acute toxicity, the inhibition of neonates was determined after 15, 30, 45, and 60 min. For the second experiment, the test concentrations were prepared 24 h before the start of

tests and stored at a light regime of 16 h light : 8 h dark and a temperature of 20 ± 1 °C. The inhibition of neonates was determined after 16, 24, and 48 h.

EC_{50} values and dose–response curves were calculated by nonlinear regression using Hill's equation (a four-parameter logistic equation), a standard model to fit dose–response data [11], using the computer program GraphPad PRISM, version 4.0.

3. Results and discussion

Acute toxicity of tabun to *D. magna* increased with time dependence (figure 2). The EC_{50} values found were as follows: exposure for 15 min 85.03 $\mu g\,l^{-1}$ (95% CL 79.9–90.48 $\mu g\,l^{-1}$, slope 1.39, R^2 0.9381), exposure for 30 min 38.10 $\mu g\,l^{-1}$ (95% CL 35.54–40.84 $\mu g\,l^{-1}$, slope 4.047, R^2 0.9194), exposure for 45 min 26.95 $\mu g\,l^{-1}$ (95% CL 24.91–29.17 $\mu g\,l^{-1}$, slope 4.28, R^2 0.9081) and exposure for 60 min 21.90 $\mu g\,l^{-1}$ (95% CL 19.7–24.34 $\mu g\,l^{-1}$, slope 5.86, R^2 0.8201). All calculations were based on nominal spiked concentrations, and no tabun concentrations were measured at the end of the tests. The relation between tabun concentrations at the beginning of the test and the percentage inhibition of neonates after 15, 30, 45, and 60 min is shown in figure 3.

The highest EC_{50} value (i.e. the lowest toxicity) was obtained for the shortest exposure and the lowest value for the longest exposure. There was a large difference between the EC_{50} value found for exposure 15 min and the other three EC_{50} values (exposures 30, 45, and 60 min). The first was twice as high as the second and almost four times as high as the last. This can be explained by the time the agent needed to pass through the body's barriers and to have its toxicity take effect.

It was shown that daphnids have a cholinergic nervous system [12, 13] and that after exposure to organophosphate and carbamate insecticides, the reduction in AChE activity in daphnids is followed by an increase in their immobility [14]. Because AChEs were found not just in fish but also in other aquatic invertebrate phyla, notably molluscs and annelids [15], tabun is expected to be toxic also for other aquatic species.

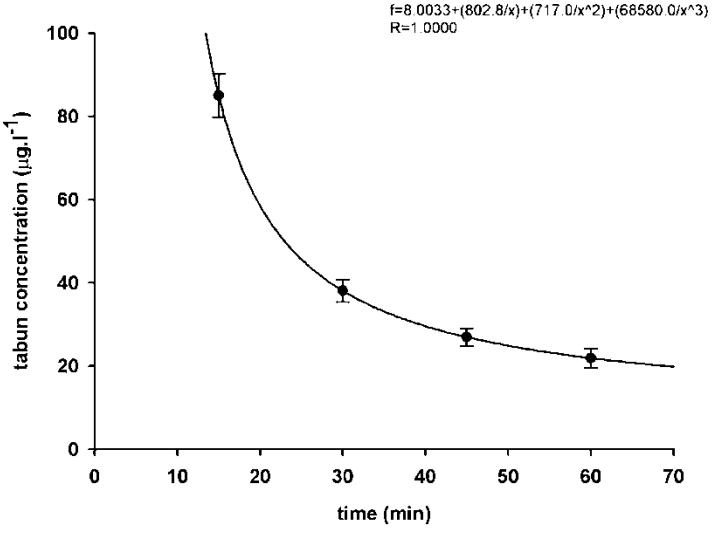

Figure 2. Toxicity of tabun represented by EC_{50} values for 15, 30, 45, and 60 min exposure of *Daphnia magna* neonates. Error bars represent 95% CL.

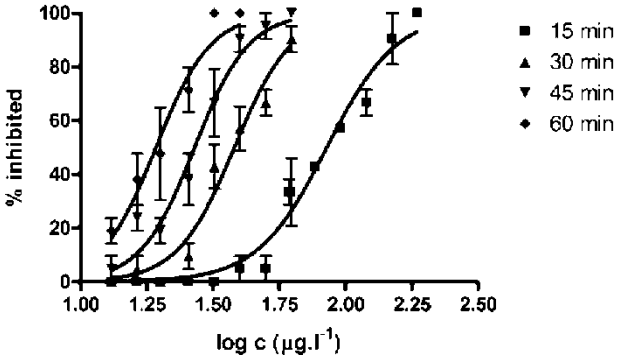

Figure 3. Relation between percentage inhibition in *Daphnia magna* neonates and tabun concentration in ISO medium after 15, 30, 45, and 60 min exposure. Error bars represent ±1 S.D.

The only data on toxicity of tabun to water organisms (LC_{50}) were found for fish species, namely for *Lepomis cyanellus*, *Pimephales promelas*, and *Carassius aureus*: 0.7, 0.6, and 1.3 mg l^{-1} for an exposure of 20 min [7]. The standard wet weight of fish used in fish acute toxicity tests with the purpose to determine the LC_{50} value is 0.1–0.5 g [3]. The wet weight of neonates of *Daphnia magna* is about 50 µg (unpublished results). Considering the data mentioned above, tabun could represent a threat mainly to small species or juveniles shortly after discharge of a large amount of tabun into water bodies.

However, it is known that tabun easily hydrolyses in aqueous solution. Its decomposition rate increases with an increasing pH, temperature and salinity [16]. If discharged to water bodies, its decomposition as well as the dilution potential in the receiving water and sequester to organic matter will decrease its toxicity in time. The half life of hydrolysis at 20 °C and pH 7.4 is approximately 8 h [7]. Based on the aforementioned facts, after 24 h of spontaneous hydrolysis at 20 ± 2 °C and pH 7.8, *i.e.* the temperature and pH of the ISO medium, it was expected that most of the tabun in the medium would be hydrolysed, and only its trace amount would still be present at the start of the tests. The results of the tests done after 24 h of tabun presence in the test medium were as follows: exposure for 16 h 43.64 µg l^{-1} (95% CL 42.42–44.91 µg l^{-1}, slope 13.02, R^2 0.9774), exposure for 24 h 38.80 µg l^{-1} (95% CL 37.09–39.40 µg l^{-1}, slope 10.16, R^2 0.9792) and exposure for 48 h µg l^{-1} (95% CL 33.06–37.10 µg l^{-1}, slope 7.89, R^2 0.9288). All values were calculated on the basis of amounts of tabun added to the ISO medium 24 h before beginning the tests, and no tabun concentrations measurements were performed during or after termination of the tests. The relationship between the tabun concentration added to the test media 24 h before the start of the test and the percentage inhibition of juveniles after exposure for 16, 24, and 48 h is shown in figure 4. The toxicity to *D. magna* again increased with time dependence, but the difference between values was not as great as in the previous experiment.

The trace amounts of tabun present at the beginning of the tests and their decrease during the experiment explain the larger difference in EC_{50} values between 16 and 24 h and the smaller difference between the 24 and 48 h values as well as the decreasing slopes of the dose–response curves in time. The principal pathway of tabun hydrolysis under neutral conditions is to hydrogen cyanide and *O*-ethyl-*N*,*N*-dimethylamidophosphoric acid, which is then hydrolysed via dimethyl phosphoramidate to phosphoric acid [17]. Of these, the most toxic product is hydrogen cyanide. The EC_{50} for *D. magna* in the 48 h test is 1.8 mg l^{-1} [18]. This value is far greater than the EC_{50} values found in the experiment, so this product plays a secondary role in the total toxicity of the media.

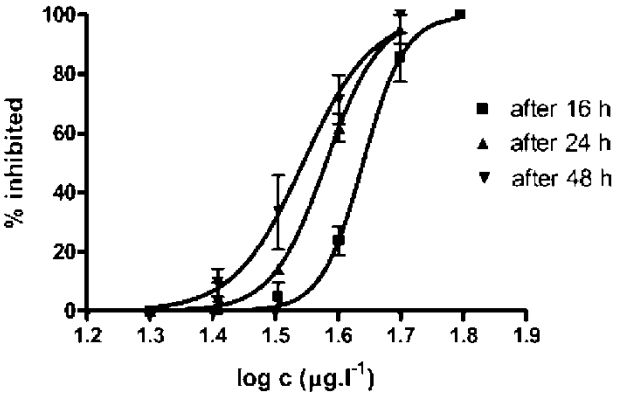

Figure 4. Relationship between percentage inhibition in *Daphnia magna* juveniles and toxicity of ISO media to which tabun was added 24 h before the start of the experiment estimated after 16, 24, and 48 h. Error bars represent ±1 S.D.

In our previous research, we focused on finding an animal model which can be used in early screen toxicity tests of newly synthesized AChE reactivators [2]. Daphnids were shown to be suitable. They are easy to breed at a low cost, they are of a convenient size, and a large number of animals of the same genotype can be used in one test. They have also proved to be sensitive to small amounts of tabun in a very short time. Because of this, it would seem promising to investigate whether this model could be beneficial also in experiments dealing with organophosphate-poisoning treatment.

Acknowledgements

We would like to thank Jiri Patocka and Koos (J.) Vijverberg for stimulating discussions and comments. The experiments described in this work were carried out in compliance with the current law of the Czech Republic. The work was supported by the grant of the Ministry of Defence, No. OBVRSVZDR200301.

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