Toxicity of *Bacillus thuringiensis israelensis* on the Nontarget Organisms *Triops cancriformis, Branchipus schaefferi, Leptestheria dahalacensis* (Crustacea: Branchiopoda: Notostraca, Anostraca, Spinicaudata)

Erich Eder* and Iris Schönbrunner

University of Vienna, Department of Evolutionary Biology, UZA-I Althanstraße 14, 1090 Wien, Austria, Europe

Abstract: In a blind and randomised study, early postlarval stages of the tadpole shrimp *Triops cancriformis*, the fairy shrimp *Branchipus schaefferi*, and the clam shrimp *Leptestheria dahalacensis* were exposed to different concentrations of a commonly available *Bti* suspension, equivalent to 0, 4.5 (recommended treatment concentration), 45, 450, and 4500 AA/l. No statistically significant correlations were found between *Bti* concentration and mortality or longevity of the examined organisms.

Keywords: Bti, toxicity, Triops, Branchipus, Leptestheria.

INTRODUCTION

The Morava and Thaya flood plains in Lower Austria are a protected area subject to the “Natura 2000” initiative (GZ 5500/5-0, 2004-08-20, Provincial Government of Lower Austria) and the Ramsar convention (BGBl. II Nr. 390/1999, Republic of Austria). Regular inundations in spring and/or summer represent the main reason for the international value of the region [1]. After high waters temporary pools and puddles represent the habitats of an endangered crustacean group, the large branchiopods (Anostraca, Notostraca, Spinicaudata, Laevicaudata) [2]. Ten of the 16 species occurring in Austria have been documented along the rivers Thaya and Morava [3], which makes the region the most important refuge of large branchiopods in Central Europe [4]. Branchiopods play a key role in the food web of standing waters, as they often occur in extremely high densities and represent a link between planktonic algae and aquatic vertebrates [5].

Ephemeral water bodies host a more common, but less popular animal group, the mosquitoes (Culicidae, species list for Austria see [6]), a fact that leads to conflicts between conservational objectives and local political interests. Since 1997, *Bti* products (*Bacillus thuringiensis israelensis*, for nomenclature see [7]) have been tested at a local level for mosquito control [8]. A more extended application along the Thaya and Morava rivers, planned for the next years, is discussed controversially.

Five proteins (Cry IVA, Cry IVB, Cry IVC, Cry IVD, CytA) are responsible for the effect of *Bti* on the insect midgut [9-13]. The assumption that only target organisms (culicid larvae) are affected, cannot be supported [14], as varying concentrations and combinations of these proteins change the effect on different insect larvae and other organisms. Boisvert & Boisvert [15] provide an extensive review of laboratory and field experiments on the effects and “side effects” of *Bti*.

There are only few published studies on the toxicity of *Bti* on European large freshwater branchiopods, with the exception of tests on adult *Eubranchipus grubii* specimens [16, 17] and a study indicating that *Bti* is well tolerated by adult *Branchipus schaefferi* [18]. Studies on non-European large branchiopods (*Triops newberryi*, *Eulimnadia texana*) and salt water species (*Artemia salina*) did not show any toxic effects of *Bti* [19-23]. Only high overdosage (180 ppm) in *Eubranchipus grubii* resulted in 100% mortality after 24 hours, control experiments at <5.4 ppm did not show any adverse effects [17].

Austrian large branchiopods are listed on local Red Lists of endangered animals [2, 25], and some species are protected by the Lower Austrian regulation for species conservation (GZ 550/7-0, 2005-08-12, Provincial Government of Lower Austria). Therefore an examination of possible toxic effects on representative large branchiopod species seemed to be indispensable. As mosquito larvae and large branchiopods both hatch early after the emergence of temporary pools, we decided to use juvenile stages for our tests. We chose three representative species, belonging to three different branchiopod orders that are easy to breed in laboratory, such as *Triops cancriformis* (Bosc, 1801), or protected by law, such as *Branchipus schaefferi* (Fischer, 1834) and *Leptestheria dahalacensis* (Rüppel, 1834).

MATERIAL AND METHODS

Sand with cysts of *T. cancriformis* (Notostraca: Triopsidae) was taken from a laboratory breed established in 1996 with organisms from a population in the Morava river flood plains (Lower Austria). Sand with cysts of *B. schaefferi* (Anostraca: Branchipodidae) from St. Valentin (Lower Austria) was provided by R. Gottwald; cysts of *L. dahalacensis* (Spinicaudata: Leptestheriidae) were taken from a collection in the Morava river flood plains [26].

The commercially available suspension “Neudomück” (3000 AA/mg) was used to prepare different *Bti* concentrations in deionised water. Dry cysts of the examined species were brought to hatching with deionised water at room temperature in 11 l plastic aquaria. A conventional aquarium

*Address correspondence to this author at the University of Vienna, Department of Evolutionary Biology, UZA-I Althanstraße 14, 1090 Wien, Austria, Europe; Tel: +43 1 4277 54496; Fax: +43 1 4277 9544; E-mail: erich.eder@univie.ac.at

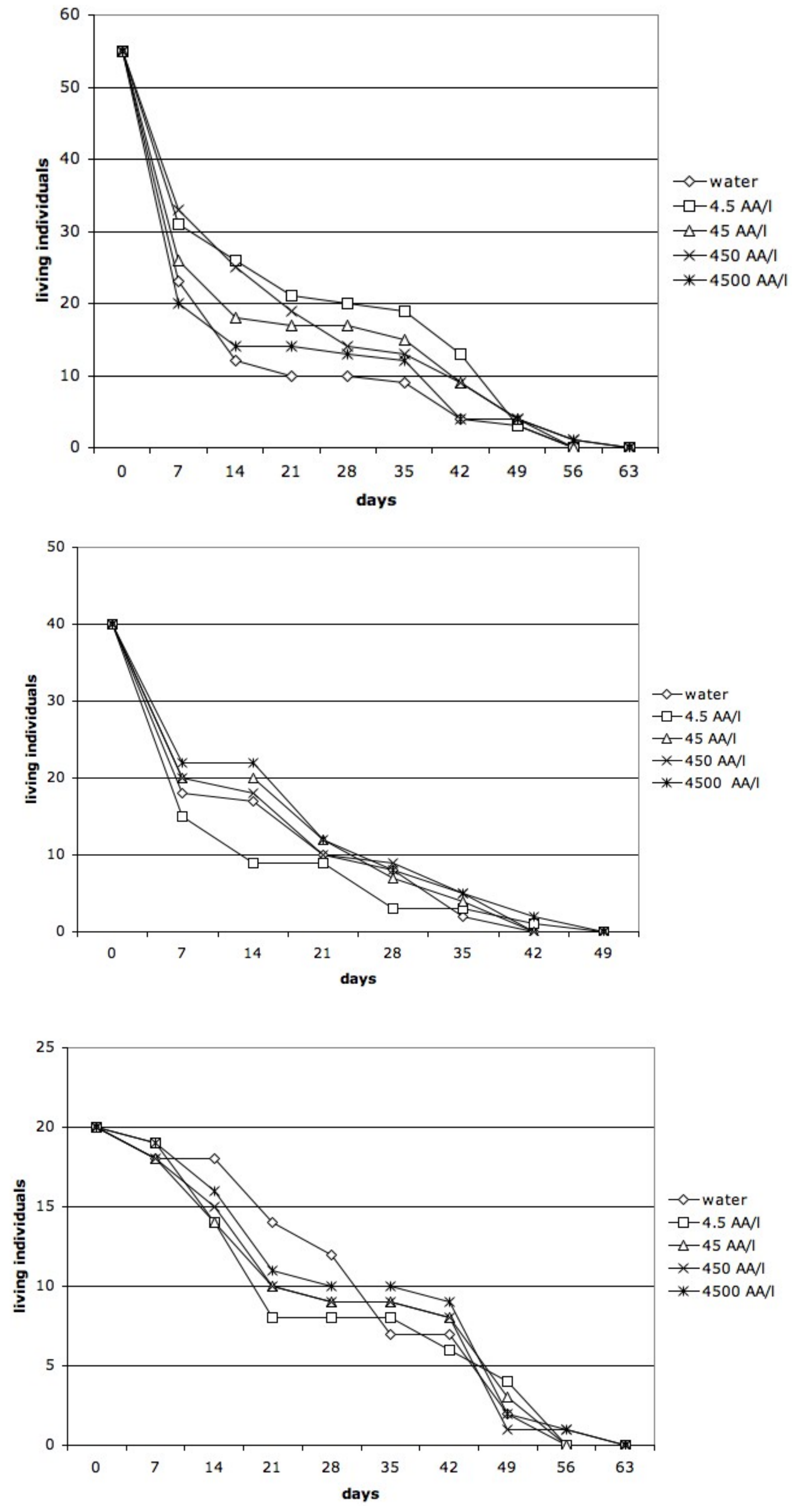

Fig. (1). Surviving individuals of *Triops cancriformis* (**a**), *Branchipus schaefferi* (**b**), and *Leptestheria dahalacensis* (**c**), summary of all performed tests.

aeration pump (75 l/min) supplied air. After approximately four days, at juvenile (postlarval) stage 1 or 2, hatchlings were transferred into the test aquaria. The bottoms of both breeding aquaria and test aquaria were covered with natural Morava river sand, in order to provide similar ionic concentrations.

The test aquaria contained:

1) Recommended concentration equivalent to *Bti* 4.5 AA/l

2) 10 times rec. concentration equivalent to *Bti* 45 AA/l

3) 100 times rec. concentration equivalent to *Bti* 450 AA/l

4) 1000 times rec. concentration equivalent to *Bti* 4500 AA/l

(each dissolved in deionised water)

5) deionised water (control group).

The test was performed randomised and blind in order to avoid any subjective influence on the results. One author prepared the test concentrations and assigned them with individual (alphabetic) codes. The other author filled the aquaria with the different test concentrations and assigned a new (numeric) code to each aquarium.

In total, 275 postlarval individuals of *T. cancriformis*, 200 of *B. schaefferi*, and 100 of *L. dahalacensis* were used for the tests. Living individuals in each aquarium were counted weekly, and, if possible, their sex was determined. After having performed the tests, the *Bti* concentration of each aquarium was unblinded following the alphabetic and numeric codes.

SPSS for Windows 11.5 [27] was used for statistical analysis and graph plotting. The nonparametric Kruskal-Wallis H-test [28] was used to test the difference between the five test groups of each species. A probability of error of 5% (p< 0,05) was considered as statistically significant.

RESULTS

No statistically significant differences between the test concentrations and the control groups could be detected for any of the three species. However, the results for *T. cancriformis* showed a much lower error probability than in the other two examined species, close to significance.

In *T. cancriformis* (Fig. **1a**, **2a**; p = 0.056), almost 52% of the test organisms died within the first week. Animals that survived the first week had an average lifespan of approximately five weeks. The two individuals with the longest life had been exposed to 450 and 4500 AA/l, respectively, and died within the 9th week. Remarkably, the lowest overall survival rate was observed in the untreated control group.

In *B. schaefferi* (Fig. **1b**, **2b**; p = 0.427), 52.5% of the individuals died within the first week. Animals that survived

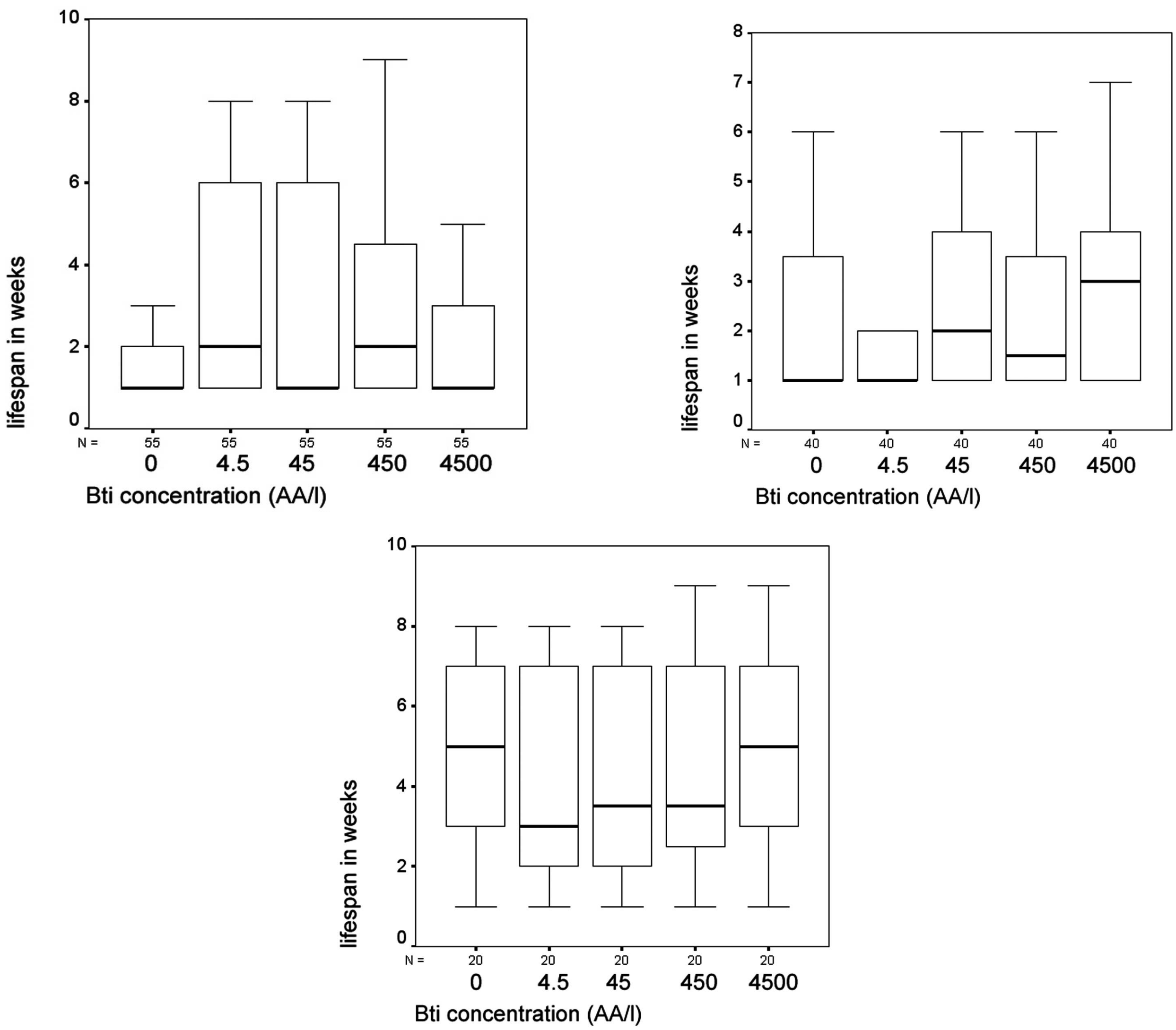

Fig. (2). Boxplots showing the lifespan of 55 *Triops* (**a**), 40 *Branchipus* (**b**), and 20 *Leptestheria* (**c**) individuals each belonging to five test groups (4 BTI groups and control group). The lowest overall lifespan was observed in the control group.

the first week had an average lifespan of approximately four weeks. The three animals with the longest life had been exposed to 4.5 and 4500 AA/l, respectively, and died within the 7th week.

In *L. dahalacensis* (Fig. **1c**, **2c**; p = 0.913), 92% of the individuals survived the first week. The animals had an average lifespan of approximately five weeks. The two individuals with the longest life had been exposed to 450 and 4500 AA/l, respectively, and died within the 9th week.

DISCUSSION

Usually, *Bti* is applied shortly after the emergence of temporary pools, in order to kill mosquito larvae before hatching of adults takes place. At this time, only larval and juvenile stages of large branchiopods are present. Therefore, studies performed with adult large branchiopod stages [16-18] do not reflect the situation predominating outdoor. Besides, adult specimens might be less sensitive to *Bti* exposition than juveniles.

The present study did not show any toxic effects of the examined *Bti* suspension on juvenile specimens of the large branchiopod species *Triops cancriformis*, *Branchipus schaefferi* and *Leptestheria dahalacensis*. Furthermore, no influence on the lifespan could be detected.

In the experiments with *T. cancriformis* and *B. schaefferi*, we observed a mortality of more than 50% within the first week. This was observed in the control group as well, where the mortality was obviously not due to *Bti*. High larval mortality is frequently observed in large branchiopods [24, 29] and might be additionally due to the transfer of the larvae from one aquarium into another. Previous observations from laboratory breeding had shown that large branchiopods are sensitive to water change (own unpublished data).

Surprisingly, in *T. cancriformis*, the groups treated with *Bti* showed a higher viability than the control group that was close to statistical significance. A possible explanation could be that filter-feeding organisms tolerating *Bti* exposition are able to use *Bti* proteins as an additional food source. In presence of the fairy shrimp *B. schaefferi*, Blaustein & Margalit [18] observed a reduced mortality of mosquito larvae, but could not prove a quantitative reduction of the *Bti* concentration.

Large branchiopod populations do not only consist of the free-swimming stages, but also of the so-called "cyst bank" [30]. For a long-term evaluation of effects on large branchiopod populations, we suggest that the reproductive success in *Bti* treated animals should be examined as well. In this study, we did neither investigate the reproductive success of individuals exposed to *Bti* nor the hatching success of cysts exposed to *Bti*. In a preliminary experiment, we could not observe a clear relation between the exposure of *T. cancriformis* individuals to *Bti* and their reproductive success (own unpublished data, data not sufficient for statistical analysis).

Although toxicity of *Bti* on the investigated species seems highly unlikely, the present data cannot entirely rule out a low toxicity. More important, our results do not exclude long-term and cumulative effects on the examined species, the species composition of their natural habitats, or effects on the food web. Lacey & Merritt [31] point out that neither the results of repeated *Bti* application nor long-term influence on the ecosystem are sufficiently known. Unpredictable effects on higher trophic levels and on the ecosystem structure should be considered [5, 15]. Therefore, a potential application of *Bti* in the Morava flood plains has to be accompanied by a long-term monitoring of diversity and population density not only of large freshwater branchiopods, but also of other co-occurring organisms [5].

ACKNOWLEDGEMENTS

This study was financed by the Regionalverband March-Thaya-Auen and the Provincial Government of Lower Austria, Dep. RU-5. We gratefully acknowledge the support in statistical analysis by Norbert Milasowszky and the scientific advice of Georg Wolfram.

REFERENCES

[1] Zulka KP, Lazowski W. Hydrologie. In: Kelemen J, Oberleitner I, Eds. Fließende Grenzen. Lebensraum March-Thaya-Auen. Federal Environment Agency, Vienna: Austria 1999; pp. 24-50.

[2] Eder E, Hödl W. Large freshwater branchiopods in Austria: diversity, threats, and conservational status. In: Escobar-Briones E, Alvarez F, Eds. Modern approaches to the study of Crustacea. Kluwer Academic/Plenum Publishers: New York 2002; pp. 281-9.

[3] Eder E, Hödl W. Catalogus Novus Faunae Austriae, No.1. Die Groß-Branchiopoden Österreichs, Crustacea: Branchiopoda excl. Cladocera. (The large branchiopods of Austria, Crustacea: Branchiopoda excl. Cladocera). Biosyst Ecol Ser 2003; 20: 1-56.

[4] Hödl W, Eder E. Die Groß-Branchiopoden der österreichischen March-Auen. Stapfia 1996; 42: 29-50.

[5] Wolfram G, Großschartner M, Eder E. Literaturstudie im Rahmen des Projektes "Gelsenregulierung March 2006/2007" des NÖ Landschaftsfonds. Regionalverband March-Thaya: Hohenau 2007; p. 37.

[6] Seidel B, Gruber E, Paulus HF. Quantitative Untersuchung einer Stechmückengesellschaft (Culicidae) im Osten Österreichs. Poster Int. Entomol. Tagung, Basel 1999; p. 1.

[7] Crickmore N, Zeigler DR, Feitelson J, *et al*. Revision of the Nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol Mol Biol R 1998; 62: 807-13.

[8] Dittami J, Seidel B. Regulation von Stechmückenplagen in Niederösterreich unter besonderer Berücksichtigung ökologischer Zusammenhänge und mit Einsatz von naturverträglichen Methoden. Unpublished project application. University of Vienna 2002; p. 35.

[9] Bechtel DB, Bulla LA Jr. Electron microscope study of sporulation and parasporal crystal formation in *Bacillus thuringiensis*. J Bacteriol 1976; 127: 1472-83.

[10] Höfte H, Whiteley HR. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol Rev 1989; 53: 242-55.

[11] KABS (Kommunale Arbeitsgemeinschaft zur Bekämpfung der Schnakenplage e.V.). Report to the Hessisches Ministerium für Landesentwicklung, Wohnen, Landwirtschaft, Forsten und Naturschutz 1991; pp. 10-29.

[12] Krieg A. *Bacillus thuringiensis*, ein mikrobielles Insektizid - Grundlagen und Anwendung. Parey Scientific Publishers: Hamburg & Berlin 1986; p. 191.

[13] Lambert B, Peferoen M. Insecticidal promise of *Bacillus thuringiensis*. BioScience 1992; 42: 112-22.

[14] Plän T, Theiss J, Wunderer HJ. Gutachterliche Stellungnahme für den BUND Hessen zum Einsatz von *Bti* und BS zur Schnakenbekämpfung in den Naturschutzgebieten "Lampertheimer Altrhein" und "Kühkopf Knoblochsaue". Institut für Naturschutzforschung e.V. (INF), Regensburg 1992; p. 34.

[15] Boisvert M, Boisvert J. Effects of *Bacillus thuringiensis* var. *israelensis* on target and nontarget organisms: a review of laboratory and field experiments. Biocontrol Sci Technol 2000; 10: 517-61.

[16] Becker N, Margalit J. Use of *Bacillus thuringiensis israelensis* against mosquitoes and blackflies. In: Entwistle P, Bailey M, Cory J, Higgs S, Eds. *Bacillus thuringiensis israelensis*, an environ-

mental biopesticide: Theory and practice. John Wiley & Sons Ltd.: Sussex, UK 1993; pp. 147-70.

[17] Morawcsik J. Untersuchungen zur Wirkung von *Bacillus thuringiensis* var. *israelensis* auf aquatische Nontarget-Organismen. Diss. Univ. Heidelberg 1983; p. 114.

[18] Blaustein L, Margalit J. Indirect effects of the fairy shrimp, *Branchipus schaefferi* and two ostracod species on *Bacillus thuringiensis* var. *israelensis*-induced mortality in mosquito larvae. Hydrobiologia 1991; 212: 67-76.

[19] Garcia R, des Rochers B, Tozer W. Studies on the toxicity of *Bacillus thuringiensis* var. *israelensis* against organisms found in association with mosquito larvae. Proc California Mosquito Vector Control Assoc 1980; 48: 33-6.

[20] Glare TR, O'Callaghan M. Environmental and health impacts of *Bacillus thuringiensis israelensis*. Report for the Ministry of Health. Biocontrol & Biodiversity. Grasslands Division. AgResearch: Lincoln 1998; p. 58.

[21] Mulla MS. Activity, field efficacy, and use of *Bacillus thuringiensis israelensis* against mosquitoes. In: de Barjac H, Sutherland DJ, Eds. Bacterial Control of Mosquitoes and Blackflies: Biochemistry, Genetics & Applications of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*. Rutgers University: New Brunswick, NJ. 1990; pp. 134-60.

[22] Stark JD. A review and update of the report "Environmental and health impacts of *Bacillus thuringiensis israelensis*" 1998 by Travis R. Glare and Maureen O'Callaghan. Report for the New Zealand Ministry of Health. Washington State, USA 2005; p. 32.

[23] Su T, Mulla MS. Toxicity and effects of microbial mosquito larvicides and larvicidal oil on the development and fecundity of the tadpole shrimp *Triops newberryi* (Packard) (Notostraca: Triopsidae). J Vector Ecol 2005; 30: 107-14.

[24] Jawahar Ali A, Dumont HJ. Larviculture of the fairy shrimp, *Streptocephalus proboscideus* (Crustacea: Anostraca): effect of food concentration and physical and chemical properties of the culture medium. Hydrobiologia 1995; 298: 159-65.

[25] Hödl W, Eder E. Urzeitkrebse (Branchiopoda: Anostraca, Notostraca, Conchostraca) - 1. Fassung 1999. In: Rote Listen ausgewählter Tiergruppen Niederösterreichs. Amt d. NÖ. Landesregierung, St. Pölten 2000; pp. 4-33.

[26] Hödl W, Eder E. Rediscovery of *Leptestheria dahalacensis* and *Eoleptestheria ticinensis* (Crustacea: Branchiopoda: Spinicaudata): an overview on presence and conservation of clam shrimps in Austria. Hydrobiologia 1996; 318: 203-6.

[27] Norušis MJ. SPSS 10.0 Guide to Data Analysis. Prentice Hall: Upper Saddle River, NJ 2000; p. 577.

[28] Kruskal WH, Wallis WA. Use of ranks on one-criterion variance analysis. J Am Stat Assoc 1952; 47: 538-621.

[29] Centeno MDF, Brendonck L, Persoone G. Acute toxicity tests with *Streptocephalus proboscideus* (Crustacea: Branchiopoda: Anostraca): influence of selected environmental conditions. Chemosphere 1993; 27: 2213-24.

[30] Brendonck L, Riddoch B, Van de Weghe V, Van Doore T. The maintenance of egg banks in very short-lived pools - a case study with anostracans (Branchiopoda). In: Brendonck L, de Meester L, Hairston N Jr, Eds. Evolutionary and ecological aspects of crustacean diapause. Adv Limnol 1998; 52: 141-61.

[31] Lacey LA, Merritt DL. The safety of bacterial microbial agents used for black fly and mosquito control in aquatic environments. In: Hokkanen HMT, Hajek AE, Eds. Environmental Impacts of Microbial Insecticides: Need and Methods for Risk Assessment. Kluwer Academic Publishers: Netherlands 2004; pp. 151-68.

Received: October 20, 2009 Revised: December 18, 2009 Accepted: March 12, 2010