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GUANICID AND PHMG TOXICITY TESTS ON AQUATIC ORGANISMS

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Abstract

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The emergence and development of new algicidal products is caused by the ever increasing popularity of garden ponds as well as the use of these products in the fisheries sector, especially for disposal of cyanobacteria and algae. Most frequent means of combating cyanobacteria and algae are applications of algicidal substances. Newly developed algaecides include Guanicid and polyhexamethylene guanidine hydrochloride (PHMG). The aim of the study was to identify toxic effects of Guanicid and PHMG on zebrafish (*Danio rerio*) and green algae (*Desmodesmus communis*). We determined the acute toxicity in fish according to ČSN EN ISO 7346-1, and conducted the freshwater algae growth inhibition test according to ČSN ISO 8692 methodology. For inhibition tests with green algae we chose Guanicid and PHMG concentrations of 0.001, 0.005, and 0.010 ml/L. For fish short-term acute toxicity tests we chose Guanicid concentrations of 0.010, 0.050, 0.150, 0.200, 0.250, and 0.300 ml/L and PHMG concentrations of 0.010, 0.025, 0.050, 0.075, 0.100, and 0.125 ml/L. In case of zebrafish (*Danio rerio*), the LC50 value for Guanicid is 0.086 ml/L, while the LC50 value for PHMG is 0.043 ml/L. Effects of Guanicid on inhibition of green algae (*Desmodesmus communis*) appear highly significant ($p < 0.010$) at a concentration of 0.010 ml/L. For PHMG, these effects are highly significant ($p < 0.001$) at concentrations of 0.005 and 0.010 ml/L in 48 hours.

Keywords: algaecide, concentration, *Desmodesmus communis*, green algae, LC50, toxicology test, zebrafish (*Danio rerio*)

INTRODUCTION

The essential tool in ecotoxicological work are toxicity tests used to identify or estimate potential toxic effects of tested compounds on living organisms (Kočí, 2006). Introduction of man-made chemicals into the environment can pose a serious risk to the environment and human health. Current legislation of European and other industrialized countries requires risk assessment data for the registration of chemicals, pesticides, biocides, and pharmaceuticals (Scholz *et al*., 2008). Aquatic organisms are an indispensable part of basic ecotoxicity tests (Lammer, 2009). For toxicological testing of chemicals it is recommended to use a model fish species, the zebrafish (*Danio rerio*). It is possible to use other types of freshwater, marine or brackish fish provided that appropriate adjustments are made, such as the adjustment of quality of the dilution water and test temperature conditions (ČNI, 1999b). Test organisms of planktonic algae, for example *Desmodesmus communis*, *Desmodesmus subcapitatus* or *Pseudokirchneriella subcapitata* can be used for inhibition assays. These algae species belong to the order *Chlorococcales* and are usually unicellular in culture (ČNI, 2005). Algae are common test organisms susceptible to many toxic substances, and thus are widely used in toxicity tests (Zhang, 2012).

The emergence and development of new algicidal products is caused by the ever increasing popularity of garden ponds as well as the use of these products in the fisheries sector, especially for disposal of cyanobacteria and algae. Mass development of cyanobacteria is not just a Czech issue, but a global problem. There is no simple measure that would be effective against the development of cyanobacteria, applicable to different types of reservoirs, and would

cause no harm to the aquatic ecosystem (Drábková *et al*., 2004). Most frequent means of combating cyanobacteria and algae are applications of algicidal substances (Jančula *et al*., 2008).

Herbicides are often applied directly to ponds to control unwanted growth of algae (colloidal, fibrous, and unicellular). Mortalities may occur in fish after the application of herbicides either directly through poisoning or by an indirect effect of the herbicide to reduce the oxygen level during decomposition processes of plants in the water, which can lead to fish suffocation (Farid *et al*., 2015).

MATERIALS AND METHODS

Characteristics of the Test Substances

Guanicid and pHMG are not primarily intended as algaecide preparations and therefore are not from manufacturers established the recommended dose for liquidation of algae.

Guanicid is a blue odourless liquid having a boiling point of 100 °C, which is used for disinfection of swimming pools. It is a mixture of ammonium compounds (quaternary ammonium salt and iminourea derivative). Structurally, it contains less than 0.2 per cent of n-alkyl (C12-C16) (benzyl) dimethyl ammonium chloride and less than 0.9 per cent of polyhexamethylene guanidine chloride (Míča, 2009).

PHMG (polyhexamethylene guanidine hydrochloride) is a cationic polyelectrolyte, which has unique physical, chemical, and biocidal properties. This polymer is colourless, odourless, non-flammable, and non-explosive. It dissolves in water and alcohol, is not subject to deterioration at low temperatures, and keeps its biocidal properties up to 120 °C. PHMG is most commonly used for reducing the growth of Gram-positive and Gram-negative bacteria (e.g. *Mycobacterium tuberculosis*), against viruses, fungi, including moulds and yeasts, and it is assumed to be used for reducing the propagation of cyanobacteria and algae (NOZA, s. r. o., 2014).

Fish Acute Toxicity Test

Acute toxicity tests were carried out on the aquarium fish species, the zebrafish *Danio rerio* (aged 4 months, the total body length of 20 ± 5 mm). Fishes were 7 days before testing acclimatized to the medium in which the test was carried out. Testing was performed according to ČSN EN ISO 7346-1 using a static method. Fish were exposed to various concentrations of Guanicid and PHMG for 96 hours. The temperature during the laboratory testing was constant (24 °C) with a controlled lighting regime – 13 h light, 11 h dark. We chose concentrations of 0.010, 0.050, 0.150, 0.200, 0.250, and 0.300 ml/L for Guanicid and 0.010, 0.025, 0.050, 0.075, 0.100, and 0.125 ml/L for PHMG. The concentrations were chosen based on previous tests and for substance PHMG we wanted to test a narrow range of concentrations. Tests were carried out in glass tanks without aeration, in 3000 ml of diluting water with 10 fish for each concentration and the control group in three replications. Fish were not fed during the test. Fish mortality was determined every 24 h of test period. The pH, temperature and dissolved oxygen content in the water were measured using a HACH HQ40d portable meter. Conductivity was measured using a Hanna combo meter.

Freshwater Algae Growth Inhibition Test

The freshwater green algae growth inhibition test was carried out on *Desmodesmus communis*. Testing was performed according to ČSN EN ISO 8692 using a static method. The test was conducted in laboratory conditions at a constant temperature of 24 °C with continuous lighting regime – 6000 lux, 4500 K, without supply of CO_2. Growth medium was prepared according to ČSN EN ISO 8692. Initial concentration of algae was 115×102 cells/ml. Acclimatization was 3 days prior to the test. The concentrations of 0.001, 0.005, and 0.010 ml/L were chosen for inhibition tests with Guanicid and PHMG. Testing was carried out in Erlenmeyer flasks placed on shakers in triplicate for each concentration and for the control sample. Each Erlenmeyer flask contained 50 ml of medium with a target concentration of tested solution. We determined the inhibitory or stimulatory effects of the test substance through a quantitative approach of counting cells in Bürker chamber using optical microscope with fluorescence. Samples for counting of algae cells were collected with a sterile pipette. The counting was carried out every 24 hours over a period of 72 hours. The number of cells counted in Bürker chamber was recalculated using the formula to the amount of cells in 1 ml. After 72 hours the number of algae cells ceased to be counted, but the samples were incubated under the same conditions up to 168 hours. After 168 hours the amount of chorlofyl-a was determined according to ČSN ISO 10260, due to confirm or deny the long-term effect of the test substance.

Data from the acute toxicity test for *Danio rerio* were processed using probit analysis to calculate the LC50 values for Guanicid and PHMG. A single-factor ANOVA – Scheffé's test in STATISTICA 12 program was used to relate the effects of Guanicid and PHMG concentrations to the number of algae cells in 1 ml.

RESULTS

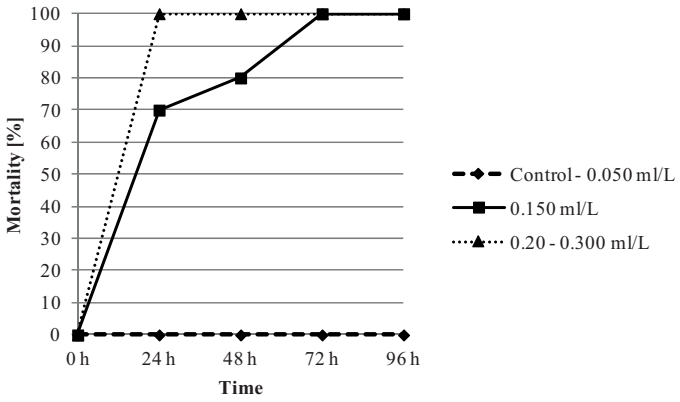
Fish Acute Toxicity Test

During the acute toxicity test of Guanicid, the temperature and conductivity were stable without significant fluctuations throughout the monitoring period. The pH level in all tanks showed a slightly alkaline environment (7.44 to 7.71). The oxygen saturation level in test solutions and control sample did not fall below 60% saturation throughout the test.

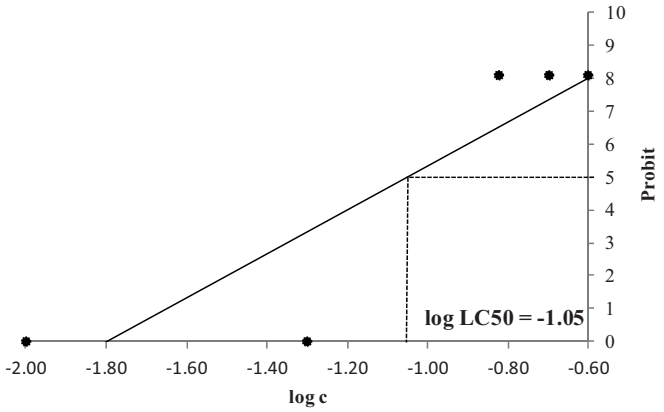
Mortality within 24 h was 100% at concentrations from 0.200 to 0.300 ml/L. There was no mortality in the control group. The median lethal concentration (LC50) value was calculated by probit analysis (see Fig. 2).

During the acute toxicity test of PHMG the water temperature was stable without fluctuations throughout the monitoring period. Water conductivity ranged from 39.2 to 43.0 $mS.m^{-1}$. The pH level showed a slightly alkaline environment in all tanks with different concentrations of PHMG. The oxygen saturation level in test solutions and control sample did not fall below 60% saturation throughout the test. Mortality within 48 h was 100% at concentrations of 0.050, 0.075, 0.100, and 0.125 ml/L. There was no mortality in the control group. The median lethal concentration (LC50) value was calculated by probit analysis (see Fig. 4).

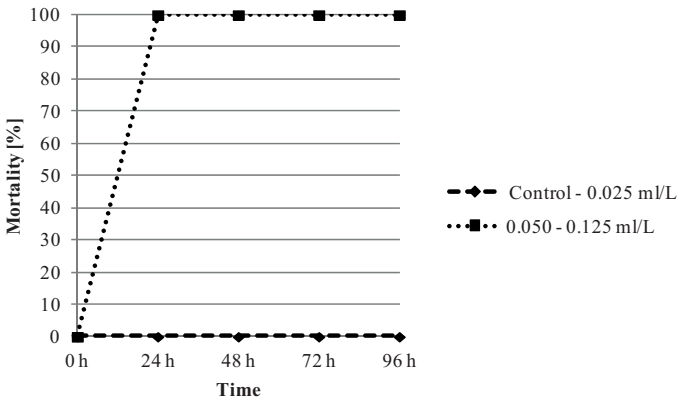
Physical and chemical parameters, which were measured during the test of acute toxicity on fish,



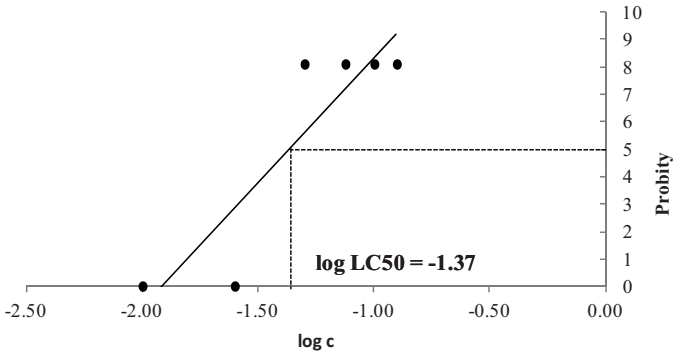
1: *Mortality of zebrafish (Danio rerio) at certain Guanicid concentrations during the acute toxicity test*



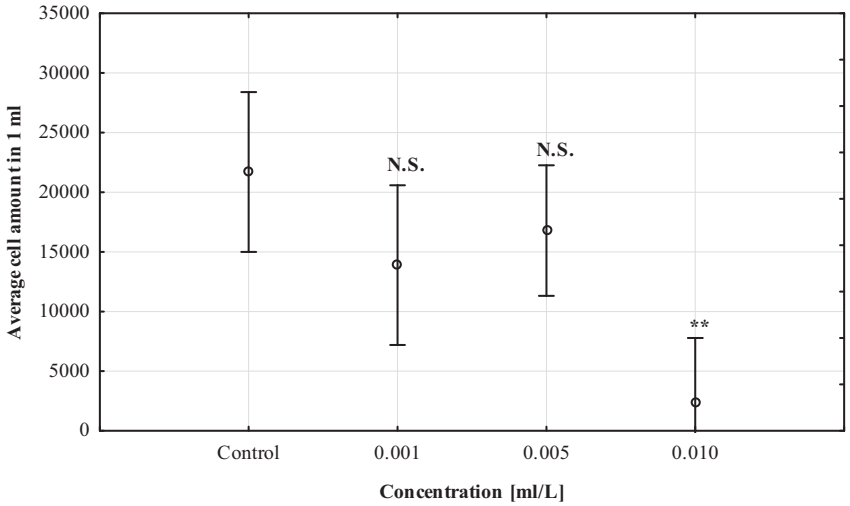
2: *Probit analysis log LC50 = –0.0639 (LC50 = 0.086 ml/L) – Guanicid*



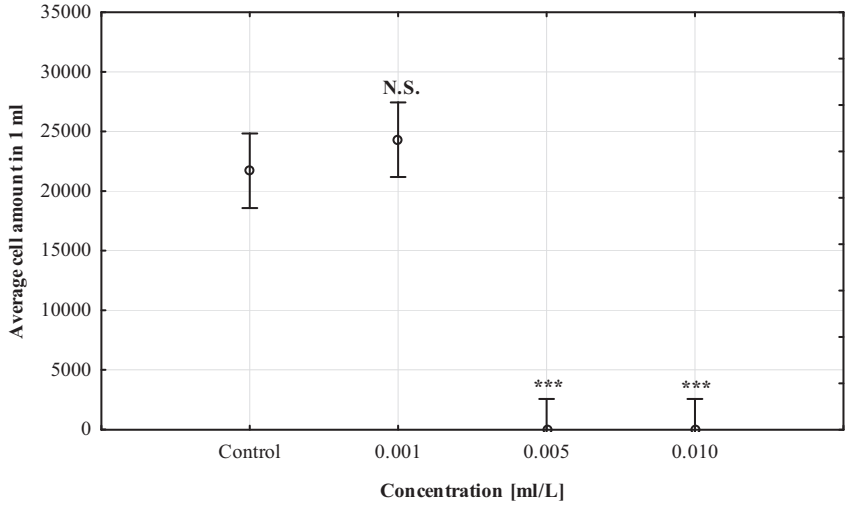
3: *Mortality of zebrafish (Danio rerio) at certain PHMG concentrations during the acute toxicity test*



4: *Probit analysis log LC50 = –1.37 (LC50 = 0.043 ml/L) – PHMG*



5: *Relation of effects of different concentrations of Guanicid to the number of green algae cells (Desmodesmus communis) in 1 ml after 48 hours (N.S. not significant; ** p < 0.010)*



6: *Relation of effects of different concentrations of PHMG to the number of green algae (Desmodesmus communis) cells in 1 ml after 48 hours (N.S. not significant, ** p < 0.001)*

were monitored in order to maintain the conditions of the test according to ČSN ISO 7346-1 and were statistically processed.

Freshwater Algae Growth Inhibition Test

Fig. 5 shows the effect of different concentrations of Guanicid on the number of green algae cells (*Desmodesmus communis*) in 48 hours. The graph

I: *Value of chlorophyll-a in green algae (Desmodesmus communis) growth inhibition test after 168 hours*

	Chlorophyll-a [µg/L]	
Control sample	704.880 ± 139.220	704.880 ± 139.220
ml/L	**Guanicid**	**PHMG**
0.001	576.020 ± 88.180	572.540 ± 43.930
0.005	426.100 ± 36.570	19.800 ± 8.150
0.010	0.000	0.000

shows a statistically highly significant difference in the concentration of 0.010 ml/L compared to control sample ($p < 0.01$). Tab. I shows the average amount of chlorophyll-a after 168 hours of test duration.

Inhibitory concentration (IC50) in 72 hours was counted using probit analysis – IC50 72 h 0.0049 ml/L.

Fig. 6 shows the effect of different concentrations of PHMG on the number of green algae (*Desmodesmus communis*) cells in 48 hours. The graph shows that 100% inhibition occurred at concentrations of 0.005 and 0.010 ml/L, and this inhibition is highly statistically significant ($p < 0.001$). Tab. I shows the average amount of chlorophyll-a after 168 hours of test duration. Inhibitory concentration (IC50) in 72 hours was counted using probit analysis – IC50 72 h 0.0011 ml/L.

In Tab. I there is indicated average amount of chlorophyll-a after 168 hours in Guanicid and PHMG. The table shows, that in concentration of 0.010 ml/L the inhibition was 100% in both tested substances. Both substances demonstrate inhibition with increasing concentration.

DISCUSSION

The best known and most commonly used algaecides are copper sulphate pentahydrate, also known as bluestone, chlorine, hydrogen peroxide, and silver nitrate. The most common reasons for using bluestone are its favourable price and high efficiency. The ability to accumulate copper in sediments may have a negative effect on benthic organisms (Hanson *et al*., 1984). Toxic concentrations on non-target organisms often overlap with those which are necessary to diminish the growth of algal communities. De Oliveira *et al*. (2004) reports EC50 to be 344 µg/L for *Pseudokirchneriella subcapitata* and 94 µg/L for *Danio rerio*. The advantage of algae growth inhibition with hydrogen peroxide is that unlike most inorganic materials it does not introduce any metals into the ecosystem and reacts very quickly to form non-toxic products (oxygen and water). Limiting factors are again the effects on non-target organisms, especially zooplankton (*Daphnia magna*) and bacteria (Xenopoulos *et al*., 1997). Silver nitrate is among the substances that restrict the growth of autotrophs, but also adversely affects non-target aquatic organisms and is highly toxic to freshwater fish. Toxicity of silver nitrate is attributed to the presence of Ag^+ (LeBlanc *et al*., 1984). Doležalová *et al*. (2008) reports LC50-96h value to be 15 ± 0.52 µg/L for *Danio rerio* and 17.14 ± 5.43 µg/L for *Poecilia reticulata*. Svobodová *et al*. (1985) reports that one of the most widely used algaecides for diminishing mass growth of cyanobacteria was Kuprikol 50 containing at least 47.5% of copper in the form of copper oxychloride. When determining the acute toxicity of Kuprikol 50 to aquatic organisms, the LC50-48h value was found to be 129 mg/L for *Poecilia reticulata*.

In comparison with our tests, the LC50 value for Guanicid was determined to be 0.086 ml/L for *Danio rerio,* and the growth inhibition of green algae *Desmodesmus communis* occurred already at a concentration of 0.01 ml/L. For PHMG, the LC50 value was determined to be 0.043 ml/L, and 100% inhibition of green algae *Desmodesmus communis* occurred at a concentration of 0.005 ml/L; at lower concentrations the effect was stimulating. The results can be compared with Vaněk (2012), who tested the same substances, but unlike us he used the *Poecilia reticulate* as test species of fish. He arrived at different results in both fish and algae. For PHMG, Vaněk (2012) indicates 100% inhibition at a concentration of 0.001 ml/L. In our tests, the statistically highly significant inhibition occurred at 0.005 ml/L, and stimulation was reported at 0.001 ml/L.

When testing algae, Vaněk used Guanicid in combination with 30% and 12% hydrogen peroxide. At a concentration of 0.010 mol/L, when we found a statistically highly significant inhibition ($p < 0.010$), Vaněk (2012) reported that the inhibition occurred in the variant with 30% peroxide, while stimulatory effects were detected in the variant with 12% peroxide.

CONCLUSION

Tests of acute toxicity to fish (*Danio rerio*) and the growth inhibition tests on green algae (*Desmodesmus communis*) were carried out with Guanicid and PHMG.

The observed lethal concentrations in fish were compared with the concentrations necessary for inhibition of green algae. Guanicid could be described as a sufficiently safe substance that should not have negative effects on the tested zebrafish (*Danio rerio*), and conversely, should be sufficiently effective for reducing green algae. The same can be argued for PHMG substance, when the LC50 value was found to be 0.043 ml/L and the statistically highly significant inhibition of green algae occurred at a concentration of 0.005 ml/L. For algicidal substances it is very important to avoid massive mortality of biomass. The aim is to reduce the photosynthetic assimilation. Therefore, we determined the content of chlorophyll-a of the green algae at the end of testing, when it was possible to see in which variant of

the test with different concentrations of algaecides the green algae were still photosynthetically active, and in which variant the photosynthetic processes stopped.

Acknowledgement

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