Toxic effect of 1% PHMG on aquatic organisms

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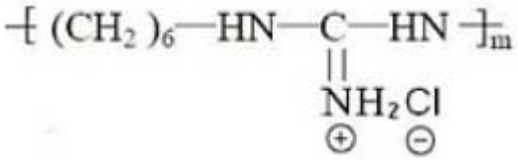
Abstract: Tests of toxicity are part of evaluation of newly developed and into practice introduced algicidal preparations. Using of model organisms, the fish species Zebra fish (*Danio rerio*) and culture of common green alga species *Desmodesmus communis* are recommended for testing the chemical substances in toxicology. Purpose of this study was to examine toxical effects of 1% PHMG (Polyhexamethylene guanidine hydrochloride) preparation on Zebra fish (*Danio rerio*) and green alga (*Desmodesmus communis*). Determination of acute toxicity on Zebra fish was realized according to ČSN EN ISO 7346-1 and test of growth inhibition of freshwater alga was realized according to methodology ČSN ISO 8692. The concentrations of 0.001; 0.005 and 0.1 $ml.l^{-1}$ for 1% PHMG for inhibitory tests with green alga (*Desmodesmus communis*) were chosen. The concentrations of 0.010; 0.025; 0.050; 0.075; 0.100; 0.125 $ml.l^{-1}$ of 1% PHMG for short-term tests of acute toxicity on Zebra fish (*Danio rerio*) were chosen. The values of LC_{50} and IC_{50} were evaluated using graphic probit analyze. For Zebra fish (*Danio rerio*) 48hLC_{50} 1% PHMG was 0.0431 $ml.l^{-1}$ and 48hIC_{50} 1% PHMG was 0.0010 $ml.l^{-1}$ for *Desmodesmus communis*.

Key words: algicide, *Desmodesmus communis,* green algae, IC_{50}, LC_{50}, test of toxicity, Zebra fish (*Danio rerio)*

Introduction

Tests of toxicity are important part of evaluation of newly developed and into practice introduced algicidal preparations [8]. The emergence and development of new algicidal products is caused by the increasingly growing pond construction and use of these products in fish farming especially for the disposal of unwanted algae and cyanobacteria. Cyanobacteria and algae produce a variety of substances that negatively affect the quality of the environment, many are also dangerous to humans [7]. The mass development of cyanobacteria is not a problem just in the Czech Republic but worldwide as well. There are no simple measures that would be effective against the development of cyanobacteria, applicable to different types of water tanks and would prevent damage to the aquatic ecosystem at the same time [4]. One of the most common options in the fight against algae and cyanobacteria includes application of algicidal preparations [6]. Widespread algicide is a blue copperas. The biggest advantage of this algaecide is low cost and rapid effect. However, its disadvantage is a copper contain in it. The effectiveness of copper is strongly influenced by the composition of the water in which is the blue copperas dosed [5].

Fig. 1 The structural formula of polyhexamethylene guanidine hydrochloride (PHMG)



Among the newly evolving algicides belongs also a polyhexamethylene guanidine hydrochloride (PHMG). It is a cationic polyelectrolyte which has unique physico-chemical biocidal effects. This polymer is colourless and odourless and is non-flammable and non-explosive. Furthermore, it is completely soluble in water and soluble in alcohol. It is not perishable at low temperatures and maintains its biocidal effect at temperatures up to 120°C. PHMG is most commonly used in limiting G^- and G^+ bacteria (eg. *Mycobacterium tuberculosis*), against viruses, fungi, including yeasts and moulds and is expected to limit cyanobacteria and algae [10].

For the testing of chemical substances in toxicology is recommended to use a model species of Zebra fish (*Danio rerio*). Recommended use of Zebra fish does not preclude the use of other species. It is

possible to use other species of freshwater, marine or brackish fish, assuming that appropriate adjustments such as dilution water quality and temperature conditions of the test are made [1].

For inhibition tests test organisms of planktonic algae, for example *Desmodesmus communis, Desmodesmus subcapitatus* or *Pseudokirchneriella subcapitata* may be used. These species are planktonic green algae belonging to the order *Chlorococcales* and in culture they are usually unicellular [2]. The algae and cyanobacteria are common testing organisms sensitive to many chemicals, and therefore they are widely used in toxicity tests [14]. The algae are key functional organisms because they are dominant primary producers and therefore they represent basic segment in aquatic food chains [9].

Material and Methods

Median lethal conentration (LC_{50}) of tested algicidal preparatus 1% PHMG for fish (*Danio rerio*) was tested in acute toxicity tests. Tested fish were exposed for 96 hours to various concentrations of the tested substance dissolved in standardly prepared dilution water. For short-term acute toxicity tests we chose six different concentrations of 1% PHMG: 0.010; 0.025; 0.050; 0.075; 0.100 and 0.125 $ml.l^{-1}$. As a control, we used an aquarium with fish filled only with the diluting water without tested preparation. Every 24 hours, mortality of fish was monitored during the test. Also temperature, pH and dissolved oxygen in water were measured using HACH HQ40D device and conductivity using Hanna combo device in 24 hours interval.

4 months old Zebra fish individuals of total body length 15-20 mm were used in the test. Individual fish were randomly selected to test from the stock tank and were not fed during the test. Each test aquarium contained 10 fish in 3000 ml of test solution without aeration. Dilution water was prepared according to ISO 6341 from stock solutions in an amount of 11.76 g of $CaCl_2 \cdot 2H_2O$, 4.93 g of $MgSO_4 \cdot 7H_2O$, 2.59 g of $NaHCO_3$ and 0.23 g of KCl [12]. Such prepared dilution water was saturated with air oxygen for 24 hours (aeration).

Subsequently, we determined the inhibitory effect (IC_{50}) of 1% PHMG on growth of green alga (*Desmodesmus communis*). Before starting the test, the growth medium according to standard ČSN EN ISO 8692 was prepared [2]. Tests were carried out for 168 hours under laboratory conditions in Erlenmeyer flasks with green alga (*Desmodesmus communis*). Concentrations for inhibition tests of 1% PHMG were chosen as follows: 0.001; 0.005 and 0.01 $ml.l^{-1}$. As the control, there were green algae incubated without any additional substances. Each variant contained 50 ml of the tested solution. Test vessels were sealed by cellucotton to prevent airy contamination and reduce evaporation, but in order to maintain gas exchange [2]. To determine the inhibitory or stimulatory effects of a tested substance, a quantitative method of counting cells in Bürker chamber using a fluorescence microscope was used. This counting was carried out for 72 hours in 24 hours intervals. The principle according to Bürker counting is based on the counting in chamber under a cover glass, where a thin layer of water with a height of 0.1 microns is applied [12]. The number of cells counted in the Bürker chamber was further recalculated using the formula for the amount of cells present in 1 ml.

Fig. 2 The formula for recalculating the cells in 1 ml

$$\frac{\text{amount of cell (individuals)}}{\text{area in mm}^3} * 1000$$

Subsequently, chlorophyll-a was determined within 168 hours based on the principle of chlorophyll extraction with hot ethanol and measuring by spectrophotometer at wavelengths ranged between 665 and 750 nm. Hydrochloric acid (HCl) and pure ethanol (C_2H_5OH) is used to determine the chlorophyll-a [3].

Results and Discussion

For acute toxicity test on fish and inhibition test on algae algicidal substance 1% PHMG was chosen. The aim of the test was to determine the median lethal concentration (LC_{50}) and the inhibitory concentration (IC_{50}) by using probit analysis. During the test, the average water temperature in all variants and control was 23.5°C. Temperature fluctuations were not observed. Oxygen saturation ranged from 53.5 to 89.5% (from 4.43 to 7.49 mg^{-1}). pH value in all aquaria showed a slightly alkaline environment which is suitable for fish farming. Conductivity of the water ranged from 39.2 to 43.0 mS. Mortality at concentrations of 0.050; 0.075; 0.100 and 0.125 $ml.l^{-1}$ after 48 hours was 100%. Concentrations up to 0.025 $ml.l^{-1}$ didn´t cause any mortality. The median lethal concentration

Table 1 Average number of cells and ±SD of *Desmodesmus communis* in 1 ml and the amount of chlorophyll-a

Hours	Control	±SD	0.001 ml.l^{-1} 1% PHMG	±SD	0.005 ml.l^{-1} 1% PHMG	±SD	0.01 ml.l^{-1} 1% PHMG	±SD
0	11 500	4 000	11 500-	4 000	11 500	4 000	11 500	4 000
24	14 468	2 165	7 523	5 367	1 763	1 418	0	0
48	8 935	10 449	17 940	9 113	0	0	0	0
72	27 199	6 993	16 204	5 729	0	0	0	0
96	23 727	4 331	24 306	6 496	0	0	0	0
	Chlorophyll a v µg.l^{-1}							
168	70.25	11.33	57.06	3.58	1.97	1.48	0.00	0.00

(48hLC$_{50}$) for 1% PHMG was calculated using probit analysis on 0.0431 ml.l^{-1}. In Table 1, the average cell counts of green alga *Desmodesmus communis* and the amount of chlorophyll-a after 168 hours is presented. For concentration of 0.01 ml.l^{-1} inhibition occurred within 24 hours, and the inhibition was 100%. At concentration of 0.005 ml.l^{-1} occurred 100% inhibition within 48 hours. At concentration of 0.001 ml.l^{-1} inhibitory effect was observed within 24 hours. After 24 hours, the inhibitory effects receded and the substance began to manifest as stimulant, when at the end of the test was found more than double amount of cells compared to the initial amount. After 168 hours from the start of the test chlorophyll-a was determined in all variants [3]. The average concentration of chlorophyll-a in control achieved the value 70.25 µg.l^{-1} which represents 100%. Tests of determination the chlorophyll-a were performed in Erlenmeyer flasks of 100 ml volume with an initial amount of 11 500 cells in 1 ml (± 4000) under the artificial light in the interval of 13 hours light - 11 hours dark. All variants were in three repetitions. In the variant with 0.001 ml.l^{-1} 1% PHMG the value reached 57.06 µg.l^{-1} (81.2%), in the variant with 0.005 ml.l^{-1} was 1.97 µg.l^{-1} (2.8%) and the latest version with 0.01 ml.l^{-1} there was no chlorophyll-a. For 1% PHMG 48hIC$_{50}$ at 0.001 ml.l^{-1} was set.

The results can be compared with Vaněk [13], who tested the same substance (1% PHMG) and achieved similar results. In our tests, 100% inhibition at 0.01 ml.l^{-1} after 24 hours was found. Vaněk [13] presents, that at the same concentration was 100% inhibition after 48 hours. In acute toxicity tests on fish Vaněk [13] used initial concentration of 0.5 ml.l^{-1}, which caused 100% mortality within 24 hours. The same results occurred in our tests, as it is shown in the Table 2.

Svobodová et al. [11] presents, that the widely used algicidal agent for limiting the mass development of cyanobacteria and algae was Kuprikol 50, containing at least 47.5% metallic copper in the form of copper oxychloride. In determination of the acute toxicity of Kuprikol 50 on aquatic organisms they determine the amount of 48hLC$_{50}$, which was for fish *Poecilia reticulata* 129 mg.l^{-1}. In comparison with Kuprikol 50, our tested preparation 1% PHMG has lesser lethal concentration.

Conclusion

Tests of acute toxicity on fishes (*Danio rerio*) and inhibition tests with selected culture of green alga *Desmodesmus communis* was carried out with 1% PHMG. The effective inhibitory concentrations for algae extermination and the median lethal concentration (LC$_{50}$) for fish were tested. We checked the effectiveness of preparation using density measurements in Bürker chamber under the microscope with fluorescence and 48hIC$_{50}$ 1% PHMG on *Desmodesmus communis* were counted. When comparing 48hLC$_{50}$ 1% PHMG, which is 0.043 ml.l^{-1} for fish, with inhibitory concentration required to limit algae 48hIC$_{50}$, which is 0.001 ml.l^{-1}, we could say that this is a sufficiently safe substance that should not have any negative effects on tested fish *Danio rerio* and vice versa should be sufficiently effective for the reduction of green algae. It is very important for algicidal agents not to cause massive mortality of biomass and prevent the releasing cellular contents into the surrounding area.

The aim is to reduce the photosynthetic assimilation, so the colonies will sink to the bottom out of the reach of photosynthetically active solar radiation and gradually began to decompose there. Therefore at the end of the test the content of chlorophyll-a of green algae *Desmodesmus communis* was determined because then it was possible to see, in which variants of the test with different concentrations of 1% PHMG were algae still

photosynthetically active and for which photosynthetic processes ended.

Table 2 Physico-chemical parameters and mortality of acute toxicity test on fish (*Danio rerio*)

Concentration 1% PHMG	Temperature [°C]					O_2 [%]				
[ml.l^{-1}]	0 h	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
Control	23.4	23.8	23.5	23.4	23.7	85.6	57.9	70.8	71.2	71.3
0.010	23.4	23.6	23.5	23.5	23.6	82.6	53.5	59.8	66.8	64.1
0.025	23.3	23.6	23.4	23.4	23.6	87.9	57.9	56.6	56.0	56.6
0.050	23.4	23.7	-	-	-	88.5	76.9	-	-	-
0.075	23.3	23.6	-	-	-	88.9	81.5	-	-	-
0.100	23.2	23.6	-	-	-	88.7	83.5	-	-	-
0.125	23.2	23.6	-	-	-	89.5	86.7	-	-	-
Concentration 1% PHMG	pH					Mortality [pcs]				
[ml.l^{-1}]	0 h	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
Control	7.43	7.30	7.41	7.50	7.46	0	0	0	0	0
0.010	7.70	7.47	7.63	7.67	7.64	0	0	0	0	0
0.025	7.73	7.44	7.53	7.56	7.57	0	0	0	0	0
0.050	7.73	7.59	-	-	-	0	10	-	-	-
0.075	7.74	7.65	-	-	-	0	10	-	-	-
0.100	7.76	7.63	-	-	-	0	10	-	-	-
0.125	7.76	7.68	-	-	-	0	10	-	-	-

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