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Assessment of acute and chronic toxicity of water-soluble polyguanidines towards hydrobionts

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Abstract. There were obtained water-soluble polyguanidines of various structures and molecular weights by polycondensation in the melt of aliphatic amines and guanidine hydrochloride in the presented report. There was assessed the toxicity of a number of polymeric compounds in the acute and chronic experiment on fish. In turn this allowed the vision of the link between the structure of the macromolecular chain and toxic action. There was established the average fatal concentration and the threshold level of the fatal effect, which does not exceed the range of 0.5–2.5 mg/l for different polymer structures. Taking into account the fact that such concentrations are destructive for many unicellular organisms, it is possible to predict their high efficiency in the fight against ectoparasites of fish.

1. Introduction

It is known that fish diseases are divided into infectious and invasive. In existing aquacultures, ectoparasites of fish occupy an important place among the causative agents of invasive diseases. The main methods of dealing with such parasites are reduced to the use of drugs, the active ingredients of which are highly toxic compounds, mainly triphenylmethane dyes and their derivatives [1]. The effectiveness of such compounds, in most cases, is associated with enormous harm [2], applied both to the environment and to the fish themselves. Therefore, the development of new methods for combating fish diseases with the use of drugs whose effect has a minimal negative impact on the object of treatment and the conditions of its existence is a relevant task.

In a number of works, the use of formaldehyde and inorganic salts (CuSO₄, KMnO₄, NaCl) [3, 4] has been proposed as an alternative. Their use turned out to be ineffective, and mandatory certification for such an application purposes increased their cost in several times. Antibiotic therapy is a costly method and often leads to the accumulation of active substances in the body of the fish. This can lead to occurrence a number of serious side effects on the human body (allergies, toxic shock, etc.) [5]. The known works on the use of extracts of medicinal plants (*P. corylifolia*, *Dryopteris*, *Kochia*, *Polygala*) [6-8], were also did not show satisfactory outcome. It is known the use of oxidative systems [9], which demonstrate a high biocidal activity in model experiments, however, under the conditions of real aqua farms, their efficiency does not



meet modern requirements. The one forward-looking approach is the use of photodynamic substances that are non-toxic in themselves and can be activated by light. Chlorophyll and its derivatives are used as such substances, which show a positive trend in the fight against a number of ectoparasites [10]. However, such studies are not completed yet and the aspect of the economic feasibility of their use on an industrial scale is controversial. Is of interest the use of water-soluble polyguanidines (PG), which have a high biocidal effect and are used as some broad spectrum disinfectants. It was well studied the effect of PG on bacteria and warm-blooded animals [11], however, their influence on hydrobionts has not been practically investigated yet.

2. Models and Methods

PHMGHC of low molecular weight (sample I), linear (sample II) and branched structure (sample III), as well as a linear structure copolymer obtained on the basis of two diamines (hexamethylenediamine (HMDA) and octamethylenediamine (OMDA)) and guanidine hydrochloride GHC (sample IV) were obtained by the melt polycondensation method of relevant amounts of monomers of HMDA, OMDA (for polymer 4) and guanidine within 6-8 hours at the temperature at 165-185 °C. N- of octylsubstituted product (sample V) was obtained *in situ* by adding a certain amount of alkylating agent (n-octylamin) to the equimolar amount HMDA and GGH.

The structure of all received polymers and copolymers was confirmed by IR spectroscopy and elemental analysis. Molecular weights were characterized by the method of end-groups determination and viscometry.

Care and maintenance of fish were carried out according to the standard [12]. All fish were kept in the laboratory in rectangular aquariums with a working volume of 25 liters, in drinking water ($T = 24 \pm 1$ °C, pH 6.5 ~ 8.0), with intensive aeration, with an exposure to their use in testing 12-14 days. The diet of fish consisted of standard dry food TetraMin (flakes), Germany. The experiment was carried out in accordance with the standard [13] in a static test. Control of the concentration of polymers and copolymers during the experiments was carried out daily by UV spectroscopy. The introduction of the drug increased the pH of the water slightly, but the figures did not go beyond the prescribed range for testing. There were used of 10 fish in all test groups. The fish were exposed to the test substances in aqueous solutions of various concentrations for 96 hours. Mortality was recorded on the 24th, 48th, 72nd and 96th hour. After following manipulations was determined the concentration of the substance causing the death of 50% of fish group. As a result of research was determined the average lethal concentration - LC₅₀.

The study of chronic toxicity toward fish was carried out in accordance with the standard [14]. Test preparation conditions are similar to the definition of acute toxicity.

3. Results and Discussion

It is necessary to take into account a number of factors during development approaches to the synthesis of polymers and copolymers of the guanidine series: the availability and stability of monomers during storage, ease of synthesis, no need to purify the final product (deep conversion of monomers). Mono- and polyfunctional amines (the length of the hydrocarbon fragment from C2 to C8), guanidine hydrochloride (GHC) were used as monomers. The synthesis was carried out by melt polycondensation [15]. By changing the ratio of guanidine and amines from equimolar to stoichiometric (from 1: 1 to 1: 1.5) polymers and copolymers of linear and branched structure were synthesized. Comb-shaped polymers were obtained by alkylation *in situ* in the reaction mass. Polymers with different molecular weight (M_n , M_v) are obtained by changing the synthesis conditions (duration from 5 to 8 hours, temperature from 160 to 180 °C) (Figure 1).

The following polymer samples were selected for further research:

- PGMGHC linear obtained with equimolar ratio of hexamethylenediamine (HMDA) and GGH monomers, with $M_n = 1000$ Da (Polymer 1);
- PGMGHC branched obtained by the stoichiometric ratio of monomers, with $M_v = 1500$ Da (Polymer 2);

- PGMGHC linear low molecular weight with $M_n = 550$ Da (examined to confirm the high toxicity of low molecular weight products also in relation to fish) (Polymer 3);
- linear copolymer obtained on the basis of two diamines (HMDA and octamethylenediamine (OMDA)) and GHC, with a molar ratio of 0.5: 0.5: 1, $M_n = 1150$ Da (Polymer 4);
- N-octyl-substituted PGMGHC with a degree of substitution of 50% with $M_n = 1400$ Da (Polymer 5).

All samples were obtained in high yields (>97%).

Toxicity acute was assessed on fish species selected on the basis of practical criteria, such as their availability throughout the year, ease of maintenance and handling, suitability for testing, as well as economic, biological or environmental factors. To assess the species specificity, the experiment was performed on the following fish species:

- Danio rerio (*Brachydanio rerio*);
- Guppy (*Poecilia reticulata*);
- Barbus Sumatran (*Puntius tetrazona*).

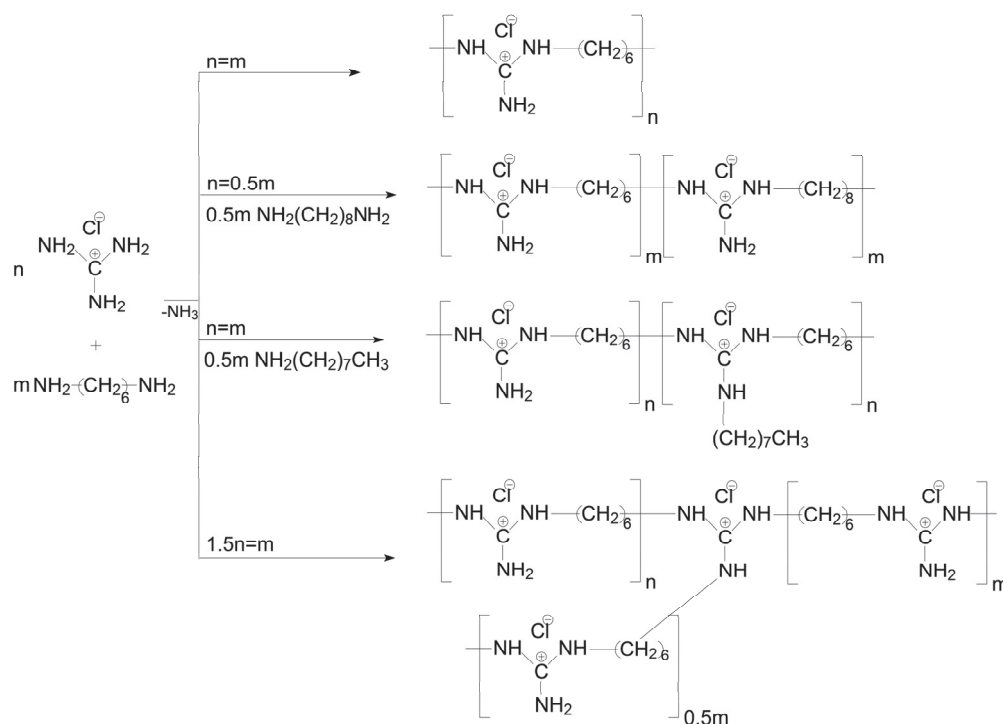


Figure 1. Polymer formation (scheme).

Fish individuals were healthy, without visible pathologies. Supplier: JSC "Aqualand", Ulan-Ude, Russia. The mortality of fish was not registered during the acclimatization period.

In the first test needed to clarify the toxic concentration for the class of the researching compounds on one species of fish (*Danio rerio*). The sample Polymer 1 was studied as the most prevalent representative. The concentration of the researched solutions was ranged from 2 to 46.9 mg/l, the requirements of GOST (at least 5 concentrations exponentially with a denominator of 2.2) and was not exceed the threshold concentration for such an experiment. During the test, the decrease in the concentration of polymer solutions did not exceed 10%. This is permissible and it is likely due to the partial sorption of the polymer on the walls of the aquarium, aquarium equipment and the fish themselves.

A short-term excitation (2-3 minutes) was observed followed by inhibition after the drug was injected and no response to external pathogens was observed and the death of all individuals was recorded for concentrations of 46.9 mg/l, 21.3 mg/l, 9.7 mg/l, 4.4 mg/l for 15, 40, 120, 240 min respectively. In some cases, the fish rose directly to the surface, which is similar to the behavior of fish in water with insufficient oxygen concentration. Also, for some individuals of fish (*Danio rerio*, Guppy) was observed a decrease in the color intensity. Concentration of 2 mg/l was not lethal. There were not observed any changes in the control group of fish (without introducing the polymer solution).

Thus, the calculated LC_{50} (calculated by approximating the geometric two concentrations) is 2.97 mg/l. On this basis, for subsequent testing, concentrations in the range from 1.5 to 3.5 mg/l were chosen (Table 1).

It was found that Guppies are the most sensitive to the action of polymers; representatives of the cyprinid fish (*Danio rerio*, Barbus Sumatran) are characterized by higher LC_{50} values. The growth of toxicity is observed in the series: Polymer 4 < Polymer 1 < Polymer 2 < Polymer 5 < Polymer 3. Low molecular weight PGMGHC has been found to be the most toxic. The relationship of structure and toxic effects on fish is not obvious. However, based on the classical concepts of the physicochemistry of high-molecular compounds, with high probability, it can be assumed that the contribution of the conformational component for polymers with similar molecular weight (all, but polymer 3), which it's determines the hydrodynamic volume of the macromolecule in solution, is noticeably larger than the change in the hydrophobic-hydrophilic balance, which it is primarily determined by the structure of the polymer.

Table 1. LC_{50} polyguanidines in relation to the tested fish species (4 days).

Fish	Polymer				
	PGMGHC lin.	PGMGHC bran.	PGMGHC lin. low mol	Copolymer OMDA:HMDA:GHC	N-octyl substituted PGMGHC
<i>Danio rerio</i>	2.74	2.23	1.22 ^a	3.24	1.73
Guppy	2.23	1.73	0.6 ^a	2.74	0.7 ^a
Barbus Sumatran	2.74	2.23	1.22 ^a	3.24	1.73

^a It was decided to reduce values of concentration to 0.5 mg/l because the lower threshold of the claimed interval leads to 100% mortality of individuals of fish.

It is impossible to confirm this assumption experimentally by measuring the viscosity characteristics of aqueous solutions of flexible-chain polymers because of the polyelectrolyte swelling effect for compounds of polycationic nature. Water-salt solutions are used to measure the characteristic viscosity of such structures. Such solutions have high ionic strength and prevent the transition of the conformation from “coil” to “core” with decreasing polymer concentration in the solution, i.e. maintain a uniform conformation for different concentrations of polymers. Such polymer molecules do not interact with each other in diluted solutions, while the low salt content, under the conditions of a real experiment, can be neglected. Therefore, as a result of osmotic processes, the conformation of macromolecules tends to be rod-like as far as the chemical structure of the chain allows (to get the maximum volume in solution).

If we compare PGMGHH linear (Polymer 1) and branched (Polymer 2) structure, then due to the compact topology of the macromolecular chain Polymer 2 will occupy a smaller volume, thus, presumably, easier to penetrate into the tissues of the body. Therefore, polymer 2 is more toxic in the experiment. For the second pair of polymers having a structure bordering the possibility of dissolving in water due to the presence of a large number of hydrophobic fragments a similar dependence is observed. So, for the polymer obtained using n-octylamine (Polymer 5), which represents a kind of comb-like copolymer with aliphatic “pendants”, the polyelectrolyte effect is leveled. Its conformation tends to be more compact due to the hydrophobic interactions of these parts in the bad solvent for them (water). For a polymer obtained using OMDA such hydrophobic interactions between the aliphatic regions are difficult because it has a linear structure (Figure 2).

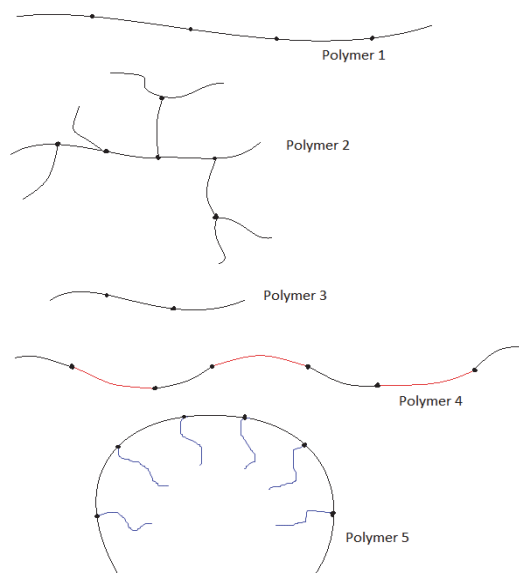


Figure 2. The probable conformation of macromolecules in aqueous solution (scheme).

Based on the results of the acute toxicity of polymers toward fish, there was carried out pre-testing acute toxicological experiment with a clonal culture of protist available from Papanin Institute for Biology of Inland Waters RAS culture collection of free-living protists. Heterotrophic flagellate *Neobodo borokensis* Tikhonenkov, Janouskovec, Keeling, Mylnikov, 2016 related to the ectoparasites causing protozoan diseases of fish was used. The fatal concentration of the polymer at which all cells (100%) of the protist die (LC_{100}) was determined in experiment. Cultivation of flagellates was carried out according to the methods described earlier [16]. The experiment was carried out with 3-day old cultures of protist in Falcon® 24-well plates, diameter of a well is 16 mm. The viability of protist cells was evaluated with a microscope after 3 hours and after 1 day after the addition of the polymer solutions. It was established that LC_{100} for polymer 3 and polymer 4 is 1.5 mg/l, for polymer 1 it is 3 mg/l.

Thus, the greatest difference in the lethal concentrations of LC_{100} in the experiment with protists and LC_{50} in the experiment with fish is observed for Polymer 4. Therefore, it is a greatest interest for further toxicological studies.

Chronic toxicity assessment was carried out on *Danio rerio* fish species. *Danio rerio* is a representative of the cyprinid family and one of the most accessible relatives of the representatives of commercial fish species bred in aquatic farms.

Feeding the fish was carried out daily 1 time. Semi-static tests were conducted with the update of the concentration of the studied solutions once every three days. The first chronic test was carried out at concentration 3 mg/l. This value did not cause a lethal effect at fish according to the results of the evaluation of acute toxicity. On the 8th day of the experiment, the death of fish was established in the amount of 3 individuals, on the following day – the death of one more individual. Thus, at the time of completion of testing, the death rate was 40%.

The concentration of the studied polymer was reduced to establish the threshold level of the lethal effect. It has been established that a concentration of 2.5 mg/l does not cause some lethal outcomes in the experiment.

The noteworthy feature here is the fact that while introducing the test solution on the first day of the experiment for all individuals of fish a low activity and inhibited reaction to external stimuli (bright light, the experimenter's approach to the aquarium) were observed with the predominant location near the surface (similar in determining of acute toxicity). Later, the behavior of fish returned to normal. However, subjectively, the behavior of fish in the control group was more active throughout the experiment.

4. Conclusion

- Water-soluble polymers and copolymers of guanidine series based on aliphatic amines and guanidine salts with different polymer chain architecture (linear, branched, comb-like) and molecular weight are synthesized.

- An assessment of the toxicity for the polymers and copolymers obtained is given in relation to fish under the conditions of an acute experiment. It was found that Guppies are the most sensitive to the action of polymers, representatives of the cyprinids (*Danio rerio*, Sumatran Barbus) are characterized by higher LC₅₀ values. It is established that low molecular weight PGMGH is the most toxic.

- The minimum inactive concentration of Polymer 4 (2.5 mg/l) was established under the conditions of the chronic experiment on *Danio rerio*. This polymer is regarded as the most attractive from the standpoint of real use.

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