Toxicity of metal chelates mixture in aquatic environment at Danio rerio

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Abstract. In this paper, the toxic effect of a complex chelate mixture containing microelements (Fe, Mn, Zn, Se, I, Cu) on Danio rerio was investigated. Chelated compounds are used to detoxify elements in the aquatic environment, as well as feed additives for various types of farm animals. The effect of chelate complexes was studied according to the following parameters: LC50, survival in chronic experience (30 days), embryotoxicity and genotoxicity by the micronucleus test method. The established LC50 value was 2.73 mg/l, the maximum allowable concentration that does not have a negative effect on adults fish and eggs was 0.5 mg/l. The genotoxic effect on erythrocytes of fish blood was not found in the entire range of sublethal concentrations. Comparison of the data obtained on the toxicity in this study allows asserting that the toxicity of chelates is lower than that of the ionic forms of the elements, subject to their complex effect. To accurately determine the safe level of exposure to chelates, additional studies on other organisms of the hydrobiocenosis are required.

1. Introduction

Chelated metal compounds are a common additive to feed for farm animals, including fish [1, 2]. Chelates can be presented in various forms of organometallic compounds (including the necessary trace elements), for example: amino polycarboxylic chelates, hydroxyxycarboxylic chelates, organophosphates, and others [3]. The organic form of the compounds allows them to better enter in the metabolism of aquatic animals, distinguishing them against the background of traditionally used mineral salts [4].

Most organomineral supplements contain trace elements, which, in ionic form at high concentrations, are toxic [5]. In the aquatic environment, the salts formed by the metal precipitate over time. In turn, organomineral compounds, due to their chemical structure, will be in a soluble form for a longer period [6]. For these reasons, the absorption of chelate compounds from the aquatic environment will be active, and their effect on the aquatic organisms may differ significantly. The toxicity of chelates is likely to differ significantly from the standard values for metal ions.

Studies of the complex toxicity of metals are practically not conducted, since they have antagonistic properties and properties in relation to each other, which complicate the determination of toxicity. This study of the effect of chelates on the organism of aquatic animals was carried out in the context of their use as feed additives [7]. This is one of the most likely pathways for chelates to enter the environment. Chelating agents can be specially introduced into the aquatic environment and reduce the concentration...
of highly toxic ions [8]. Also, chelate complexes of metals can be formed as a result of natural processes of decomposition of organic matter, as well as with the participation of humic acids.

*Danio rerio* is a convenient experimental object that is used to study the processes of growth, metabolism, toxicity, and genotoxicity of various compounds [9]. The use of this model organism makes it possible to standardize the results obtained and assess the effect of the toxicant at all stages of fish development. For these reasons, it seems possible to use *D. rerio* for the initial assessment of the toxicity of a mixture of metal chelated compounds.

Specially developed supplements for feeding various fish species have their own trace element composition. This study examined the effects of a chelated complex organomineral supplement on survival, hematology, and genotoxicity.

### 2. Materials and methods

**Fish Acclimation and Maintenance.** *Danio rerio* were kept in aquariums with a volume of 20 liters at a temperature of 20-24 °C and Ph 7.2-7.4 in compliance with natural conditions of illumination (12 h cycle), 20 individuals of the same sex. Кормление осуществлялось The fish were fed commercially pelleted food according to the protocols for keeping the model objects [10].

The study was complied with the guidelines of the Local Ethics Commission of the Institutional Review Board of Moscow State University of Technology and Management (No. 4, January 21, 2021).

**Chelate mixture.** As an organomineral chelate additive, were used a preparation developed by a research group and manufactured based on a technological production (Jupiter LLC, Russia). The chelating agent for all the microelements included in the complexes was ethyldiaminedisuccinic acid (EDDA). The composition of the stock solution of the mixture is presented in table 1.

| Concentration (g/l) | Fe | Mn | Zn | Se | I | Cu |
|---------------------|----|----|----|----|---|----|
| 10                  | 10 | 15 | 35 | 0.3| 1.1| 3  |

The chelate solutions were renewed every five days to prevent loss of trace element composition.

**Treatment schedule.** For the experiment selected males of size (3 ± 0.3 cm) and age (6 months), without visible damage Fish were placed in the 20 l tank in which the concentration of added chelating investigated. The experiment was performed in the three-time repetition (n = 3). Duration of the experiment was 30 days, which made it possible to evaluate the chronic effects.

The following concentrations were chosen as the initial for the toxicity assessment: 1, 5, 10, and 20 ml/l. At these concentrations, the LC50 was established, and after that, the concentrations were adjusted for chronic experiments.

The concentration spectrum for the 30-day experiment was: 0.1, 0.5, 1, and 2.5 ml / L. The exact composition of trace elements in these solutions are shown in table 2.

| Concentration (ml/l) | Fe   | Mn   | Zn   | Se  | I    | Cu |
|----------------------|------|------|------|-----|------|----|
| 0.1                  | 0.05 | 0.075| 0.175| 0.003| 0.011| 0.03|
| 0.5                  | 0.5  | 0.75 | 1.75 | 0.015| 0.055| 0.015|
| 1                    | 1    | 1.5  | 3.5  | 0.03 | 0.11 | 0.3 |
| 2.5                  | 2    | 3    | 7    | 0.06 | 0.22 | 0.6 |
| 5                    | 5    | 7.5  | 17.5 | 0.3  | 1.1  | 3  |
| 10                   | 10   | 15   | 35   | 0.6  | 2.2  | 6  |
After 30 days of exposure, the survival rate of fish was determined and material was selected for hematological studies.

**Hematological examination.** After the completion of the experiment, three fish were selected from each experimental group for hematological examination. Blood was taken from the posterior vena cava after preliminary anesthesia of the fish in MS-222 solution (10 mg/L) by cutting off the caudal peduncle. A drop of blood was smeared on glass and dried in air. Fixation was carried out in an ethereal alcohol mixture (1:1), the preparations were stained according to Ramonovsky-Giemsa according to the standard method.

The finished blood devices were viewed under an Olympus BX53 light microscope (Olympus Corporation, Japan) with an eyepiece attachment Carl Zeiss ERc 5s (Zeiss, Germany) and ZEN lite software (Zeiss, Germany).

Blood cells were counted using the ImageJ software (National Institutes of Health, USA), open source. On each preparation, 5000 cells were observed. The micronucleus test conducted according to the methodological guidelines [10, 11].

2.1. **Embryotoxicity**
D. rerio eggs were obtained from two groups of producers and incubated in plates for 72 hours at a temperature of 24 °C at concentrations determined based on the LC50 for adult fish in duplicate (n = 2), 96 eggs each 24 hours.

2.2. **Statistical analysis**
A one-way ANOVA followed by Tukey's post-haw test was used to compare numerical data that fit a normal distribution. The level of confidence was chosen P <0.05 and the results are presented as mean ± SD (standard deviation).

3. **Results**

3.1. **LC50**
Acute toxicity test of chelate compounds showed that the LC50, determined by calculation, is 2.73 mg /L. Fish survival data are presented in table 3 and figure 1.

| Survival rate (%) | Control | 0.1 | 0.5 | 1 | 2.5 | 5 | 10 |
|-------------------|---------|-----|-----|---|-----|---|----|
| 24                | 100     | 100 | 100 | 100 | 70  | 51.66 | 0 |
| 48                | 100     | 100 | 96.6 | 85  | 53.3 | 0   | 0 |

**Figure 1.** Graph of determination of LC50 for a mixture of chelates on Danio rerio.
3.2. Chronic examination

Based on the obtained data, the following concentrations were taken for a chronic experiment to determine the toxicity of solutions of chelate compounds: 0.1, 0.5, 1.5, 2 mg / L.

| Concentration (ml/l) | Day 1 | Day 6 | Day 12 | Day 18 | Day 24 | Day 30 |
|----------------------|-------|-------|--------|--------|--------|--------|
| K                    | 100   | 100   | 100    | 95     | 95     | 95     |
| 0.1                  | 100   | 100   | 98.33  | 98.33  | 98.33  | 98.33  |
| 0.5                  | 100   | 100   | 95     | 91.66* | 91.66* | 88.33* |
| 1.5                  | 100   | 81.66**| 76.66**| 73.33**| 71.66**| 58.33**|
| 2                    | 95    | 91.66 | 73.33**| 66.66**| 46.66**| 0      |

As a result of the analysis of the data of a chronic experiment, it can be argued that the maximum effective concentration of a mixture of metal chelates is 0.5 mg/l, and 0.1 mg/l. It can be taken as the maximum permissible concentration that is bladeless for D. rerio.

3.3. Embryotoxicity

It is known that the early embryonic development stages of fish are more sensitive to toxicants than adults. The embryotoxicity of chelate solutions is shown in Table 5.

| Concentration (ml/l) | Stages         | Incubation time (hours) |
|----------------------|----------------|-------------------------|
| 2                    | egg prelarvae  | 10 24 36 48 72         |
| 1.5                  | egg prelarvae  | 90±4.7 75±3.1 63±5.1 50±5.2 42±1.6 |
| 1                    | egg prelarvae  | 93±4.1 90±3.7 73±2.4 42±4.3 0 |
| 0.5                  | egg prelarvae  | 96±3.9 90±4.7 88±7.1 31±2.8 - |
| 0.1                  | egg prelarvae  | 93±2.6 93±4.7 90±6.8 36±5.4 - |
| Control              | egg prelarvae  | 93±3.8 93±5.9 93±4.5 33±6.7 - |

As a result of the experiment, it was found that the toxicity of chelates for zebrafish embryos remains at the same level as for adult fish. At the same time, it was noted that the processes of embryogenesis proceeded without deviations and developmental disturbances were not established. The maximum allowable concentration can be taken as 0.5, and the maximum allowable concentration is 0.1 mg / l.

3.4. Micronucleus test

Evaluation of genotoxicity was performed using micronucleus test of peripheral blood erythrocytes D. rerio. At all investigated concentrations, it was not possible to establish statistically reliable data on the
increase in the number of micronuclei (figure 2). Consequently, chelated metal compounds at experimental concentrations do not have a genotoxic effect.

Figure 2. The number of micronuclei in D. rerio blood erythrocytes in the studied concentrations in an acute experiment.

Figure 3. Micronuclei of erythrocytes in the peripheral blood of D. rerio fish exposed to a mixture of chelate compounds. (a) –0.1; (b) –0.5; (c) –1; (d) – 2. Scale bar – 20 µm.

Figure 3 shows the micronuclei at different investigated concentrations of chelates. No other nuclear abnormalities were found under study, including two nuclear cells. All detected micronuclei were from
1/5 to 1/10 of the area of the main nucleus, which indicates that micronuclei were in accordance with standard values [12]. All studied preparations contained lymphocytes with a normal morphology.

4. Discussion
Chelating agents are used to detoxify metals in animals, aquatic environments, and soil [13]. It has been shown that chelation of metal ions in an aquatic environment significantly reduces their toxicity and allows metals to remain dissolved for a longer time [14]. Thus, it can be assumed that the direct addition of metal chelates to water will have a toxic effect only at high concentrations. In natural environment, due to the greater bioavailability, metal chelates will be rapidly consumed by phytoplankton, being included in the cycle of biogenic elements [15].

Table 6. Summary Data on substances ions toxicity comprising the chelate treatment.

| Substance ion | LC50 (mg/l) | Species           | Reference   |
|---------------|-------------|-------------------|-------------|
| Fe            | 71          | Ictalurus punctatus | [16]        |
|               | 117.18      | Oncorhynchus mykiss | [17]        |
|               |             | Poecilia reticulata | [18]        |
| Mn            | 28          | Pimephales promelas | [19]        |
| Zn            | 2.8         | Oncorhynchus mykiss | [20]        |
| Se            | 12.8        | Heteropneustes fossilis | [21]    |
|               | 21.8        | Oncorhynchus kisutch | [22]        |
| I             | 1           | Oryzias latipes    | [23]        |
| Cu            | 1.56        | Ictalurus punctatus | [24]        |
|               | 0.4         | Danio rerio       | [25]        |
|               |             | Oncorhynchus mykiss | [26]        |

From table 6 it can be seen that the concentrations of elements differ significantly in the degree of toxic effects, depending on the object of study. The least toxic component is iron and most aquatic organisms are resistant to its ions. The most toxic components are copper and iodine. Therefore, when included in the study drug, they should have demonstrated similar toxicity indicators.

This study have shown that a mixture of metal chelate compounds has a lower toxicity compared to ionic form. The LC50 value established in this work on adult fish D. rerio demonstrates that the concentration of the mixture of chelates causes the death of fish at higher concentrations than when using inorganic metal salts separately (table 6).

The LC50 is close to the concentrations that inhibited the development of green algae, although lower values accelerated the development of the culture [27]. The toxicity of chelate compounds for other aquatic organisms has not been studied enough, but it can be assumed that it decreases in comparison with the corresponding metal ions.

Studies of the embryotoxicity of metal chelate complexes showed a low sensitivity of D. rerio eggs and prelarvae to these pollutants. The unaffected concentration obtained from the experiment was 0.5 ml / L. The same concentration was determine in chronic experiments on adult fish.

The low sensitivity of eggs to the compounds under study is a consequence of the low permeability of the shell for chelate complexes. The main exchange with the external environment in eggs occurs due to the diffusion of ions [28]. No developmental abnormalities were identified, which indicates the absence of teratogenic effects.

The metal ions that are part of the investigated chelate drug do not have a genotoxic effect, which is confirmed by a number of studies [25]. Micronucleus test results obtained in this study also demonstrated the lack of genotoxicity, with all the concentrations studied. A slight increase in the frequency of occurrence of micronuclei were not reliable and were within the normal range for this fish species [12]. An increase in the number of micronuclei on day 3 of the experiment is possibly a consequence of the action of zinc. Thus, some authors point to the acceleration of hemocytopenesis when zinc is added to the diet of Cyprinus carpio [29].
5. Conclusions
Chelated metal compounds entering the aquatic environment can have an ichthyotoxic effect, the level of which is somewhat lower than that of metal ions. It is likely that their danger is due to the presence of copper and iodine, which show the greatest toxicity in the ionic form. Due to the high bioavailability of chelate compounds, it can be expected that in natural water bodies these compounds are quickly included in the natural biogenic cycle and will not pose a threat to biota.

To establish the threshold limit value, an additional study of their effect on all links of the hydrobiocenosis is necessary, since there is not enough data on ichthyotoxicity. The results of this study showed that a safe concentration for fish at all stages of ontogenesis can be considered 0.5 mg/l.

The results of the micronucleus test showed the absence of the effect of genotoxicity of chelate complexes in all sublethal concentrations and practically did not change the composition of the peripheral blood of fish compared to the control.

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