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Chronic Toxicity Testing with *Daphnia magna* in Three Generations

Anna Olkova*
Vyatka State University, Krasnoarmeyskaya Str. 36, Kirov, 610001, Kirov region, Russia

***Corresponding author:** morgan-abend@mail.ru

This work shows the informativeness and predicted value of multigenerational testing on *Daphnia magna* Straus on exposure of 75 days in comparison with standard bioassays for chronic toxicity on exposure of 21–24 days. *D. magna* were exposed in water polluted with strontium chloride (1 and 2 mg Sr/L). No acute toxicity (death within 96 hours) and chronic toxicity (mortality and fertility within 24 days) were observed. In 50 days of the experiment, mortality of crustaceans was more than 30%, and fertility decreased by 2 times ($P < 0.05$). In 75 days of the experiment, mortality of the experimental daphnia was above 90%, and most of the individuals did not reproduce. The effects intensified in subsequent generations F2 and F3. In the third generation, the number of juveniles in 24 days of exposure (2 mg Sr/L) was only 9% of the control value ($P = 0.03$); then the juveniles did not appear. We also noted an increase in the number of abortive eggs and dead juveniles in generations F2 and F3 compared with F1. The totality of the observed effects testifies the delayed toxic effect of strontium. Delayed mortality and reduced fertility of the remaining individuals in a series of generations threaten the existence of the population of organisms. Therefore, chronic toxicity testing using *D. magna* is a method for detecting and predicting loss of biodiversity in the environment.

Keywords: bioassay, multigenerational testing*, Daphnia magna,* chronic toxicity, delayed toxicity, strontium.

Introduction

The effects of toxicants on the offspring of organisms are difficult to predict, but they are of prime importance for assessing the risk of declining biomass or significant loss of biodiversity in real ecosystems (Ellis et al., 2021. Therefore, bioassays that assess potential reproductive toxicity are becoming relevant (Zhao et al., 2021). The environmental and social significance of such research increases with the start of the use of nanomaterials, the latest generation of pesticides, and new medicinal drugs (Ismail et al., 2018; Li and Liu, 2019).

Bioassay methods are very varied today. They allow taking into account the biochemical, physiological, morphological and behavioural responses of test-organisms (Reifferscheid and Buchinger, 2017; Olkova, 2018). To assess chronic effects, scientists use bacteria (Zhang et al., 2020), frogs (Zhao et al., 2021), freshwater and marine crustaceans, molluscs (Jonczyk and Gilron, 2005; Saili et al., 2021), fish (Dionne et al., 2021), rats and mice (Matsumoto et al., 2021).

Among Cladocerans that are used for bioassay, *Daphnia magna* Straus is the most widespread. This type of daphnia is recommended for assessment of pre-lethal, acute and chronic effects of aquatic environments and toxicants by many international and national methods (EPS, 1996; OECD, 2004; FR, 2007; ISO, 2012). *D. magna* has the following benefits for chronic multi-generational bioassay (Olkova, 2017):

- convenience and relative ease of cultivation, including keeping the culture in clean water, daily separation of juveniles from adult females, feeding;
- the use of genetically homogeneous juveniles in bioassays, which is ensured by parthenogenetic reproduction and maintenance of a synchronized model population, which is considered to be a group of individuals at the same stage of development;
- quick maturation of crustaceans: at optimal temperature (+20 ± 2°C) and good nutrition the first juveniles appear on days 5–8 of life; the duration of embryonic development is 3–4 days;
- regular (every 3–4 days) and numerous appearance of juveniles (the number of juveniles in young females is 10–15; in mature females, it is up to 40 individuals);
- high enough level of organization (existence of the circulatory and nervous systems is especially important), which makes it possible to extrapolate toxicological results to other multicellular representatives of ecosystems and even humans;
- large sizes of individuals, making it possible to conduct visual observations of many responses without the use of special measuring instruments;
- sensitivity of daphnia to a wide range of pollutants;
- elative simplicity of the experiment.

The standard duration of determination of chronic toxic effects by inhibition of fertility of *D. magna* is 21 days according to international protocols (Jonczyk and Gilron 2005) and 24 days according to the Russian bioassay protocol (FR, 2007). However, this duration of experiments allows assessing the effect only on mortality of the first experimental generation (F1) and fertility of F1 individuals (the number of F2). In this work, we aim to show the informativeness and predicted value of multigenerational testing on *D. magna* in model experiments with water pollution with non-radioactive strontium (Sr^{2+}).

Methods

Modeling water pollution with strontium

The model tested environment was groundwater from an artesian well, into which we added strontium chloride $SrCl_2{\cdot}6H_2O$ equal to 1 mg/dm^3 and 2 mg/dm^3 (based on the Sr^{2+} ion). According to Russian standards, these additives correspond to 2.5 and 5 maximum permissible concentrations established for fishery reservoirs. Non-radioactive strontium is a low-toxic element, but its long-term effects are not well understood. Non-radioactive strontium is a companion of calcium; therefore, its pollution of natural waters is observed near gypsum-bearing deposits, dolomites and limestones. It can be leached from raw materials and wastes from chemical industries, contributing to complex pollution of industrial areas (Pathak and Gupta, 2020). The negative effect of strontium on the human body has been proven (Huang et al., 2020). There is information about its toxic effect on plants, in particular, blocking the K^+-channels of root cells, changing the processes of Ca and K transport to leaves, and a decrease in chlorophyll fluorescence (Ivashkina and Sokolov, 2006; Burger et al., 2019).

The control was water without additives. Preliminarily we determined that the used water does not contain harmful impurities (metals Fe, Cu, Cd, Pb, Zn, Ni, pesticides hexachlorane, dichlorodiphenyltrichloroethane, hexachlorobenzene, hexachlorine) and corresponds to drinking-quality waters in terms of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, NO_3^-, NO_2^-, SO_4^- ion content (GD, 2007; FR, 2008a; FR, 2008b; PND F, 2009; PND F, 2010).

Test culture Daphnia magna Straus

For bioassay we used a standardized culture of *Daphnia magna* Straus, which is kept in the laboratory for a long time, has a stable fertility, and $LD_{50}(K_2Cr_2O_7)$ does not go beyond 0.9–2.0 mg/dm^3 (Olkova, 2021). We observed the reactions of 3 generations of Daphnia to water pollution with strontium. Generation F1 was formed from juveniles of the synchronized culture. Generations F2 and F3 were formed from the first mass offspring of the previous maternal generation. Model groups of each generation of crustaceans were formed from juveniles aged 6–24 hours.

Carrying out an experiment

The juveniles were placed in 10 pcs in 100 mL of test or control medium, as is common in the methodology (FR, 2007). The experimental variants were repeated 4 times. Daphnia were fed daily with a suspension of the algae *Chlorella vulgaris*; 2 times a week, individuals were additionally fed with the yeast *Saccharomyces cerevisiae.* We changed the control and test solutions every 5 days, while the solutions with the addition of the toxicant were freshly prepared.

During the experiment, the experimental daphnia were monitored for mortality, fertility, the number of abortive eggs and dead born juveniles. We conducted long-term experiments: the duration of the chronic toxicity test was increased from 24 days (FR, 2007) to 75 days.

Statistical analysis

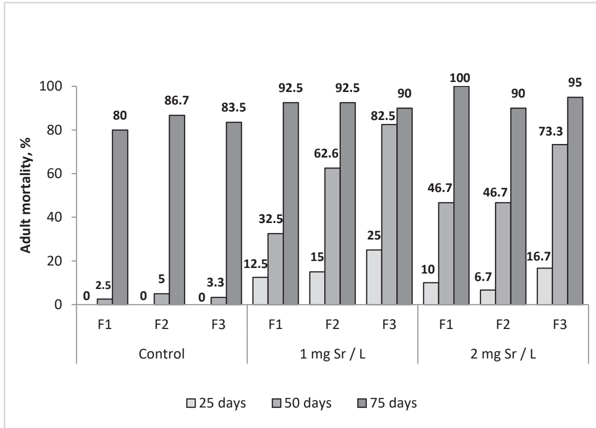
The obtained results were subjected to mathematical processing with further data presentation in the form of M ± S, where M is the arithmetic mean, and S is the standard deviation. The significance of the differences was assessed by the Student test, taking into account the significance levels (p) calculated for the two compared values.

Results and Discussion

Test-function «mortality (death)» in 3 generations

The model solutions containing 1 and 2 mg Sr/L did not cause acute toxicity in *D. magna* in 4 days of exposure. However, in long-term testing, we see that strontium causes lethal effects that increase in generations (*Fig. 1*).

Fig. 1. *Effect of strontium on mortality of adults of D. magna in 3 generations (standard deviations of values are not more than 20% of the mean)*



The lifespan of *D. magna* is about 3.5 months (Tsallolikhin, 1995), so it is quite natural that the deaths of experimental F1 and F2 individuals in the control variants did not exceed 5%, and only in 75 days the control mortality rates become high (80.0%–86.7 %).

Mortality of individuals in 25 days in all experimental variants, except one, did not go beyond the critical 20% recommended by the bioassay method (FR, 2007) as a criterion for chronic toxicity, but an upward trend ($P > 0.05$).

In 50 days of the experiment, the amount of living daphnia in water containing strontium chloride (1 and 2 mg/L) decreased by 50% or more, except for one variant. At the same time, mortality of daphnia in a number of generations significantly increased compared with the control ($P < 0.05$), reaching 82.5% and 73.3% in experiments with F3 when exposed to 1 and 2 mg Sr /L, respectively.

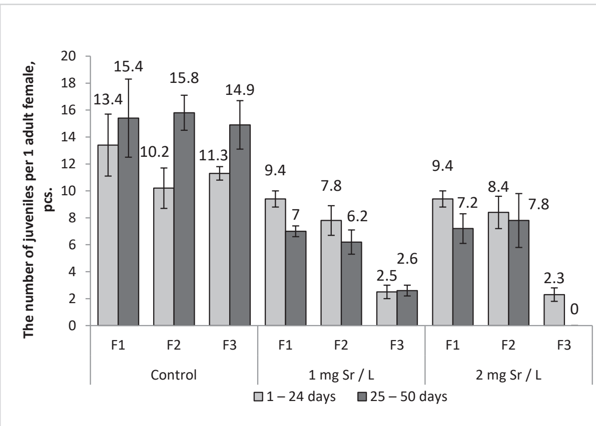
At the end of the experiment – on day 75 – the lethal effects of strontium were close to 100%. In the control variants, mortality in 75 days was also high due to natural causes (maximum – 86.7%). The difference between the control and experimental death during this period was 10%–14% ($P > 0.05$). The effect of strontium at concentrations of 1 and 2 mg/L in terms of mortality in most cases did not differ from each other ($P > 0.05$).

It can be assumed that, in natural ecosystems, individuals that survived after such an impact will not be able to leave full-fledged offspring, even if the entry of the toxicant into the water is stopped. This effect of Zinc oxide nanomaterials was shown in *D. magna* mothers in the multigenerational recovery test (Goncalves et al., 2018). The problem of increasing mortality of freshwater organisms in several generations after exposure is discussed in the literature (Arndt et al., 2014). Therefore, it is necessary to critically evaluate the established safe exposure levels of toxicants, carried out without multigenerational tests.

Test function «fertility» in 3 generations

We give fertility up to the day 50 inclusive (*Fig. 2*), as in the next observation period (50–75 days) the death of individuals was more than 50% (*Fig. 1*). In this case, the calculation of fertility per female gives overestimated values.

Fig. 2. *Influence of strontium on the fertility of adults of D. magna in 3 generations*



Fertility of the control model populations varied from 10.2 ± 1.5 to 13.4 ± 2.3 till day 24 of the experiment inclusive. In the next assessed period (25–50 days), a gradual increase in the ability to reproduce was observed up to 15.8 ± 1.3 individuals per 1 adult female, which is naturally associated with the onset of the peak of the life cycle of crustaceans. There were no significant differences in fertility rates of the control variants of the 3 generations. This indicates the uniform development of each next generation and the possibility of long-term existence of model populations of *D. magna* in the created conditions.

Fertility of daphnia in all experimental variants was lower compared with the corresponding control ($P < 0.05$). Under the influence of strontium, fertility has significantly decreased in generations. For example, in the third generation of the "2 mg Sr/L" variant, the number of juveniles on day 24 of the experiment was only 9% of the control indicator (the differences are trustworthy, $P = 0.03$). In the next 25 days of the experiment, there were no new juveniles here, since mortality of adults on day 50 of the experiment reached 73.3%, and the remaining daphnia did not reproduce. This indicates the incompatibility of the studied level of strontium pollution with the viability of the *D. magna* population. Such a conclusion could not have been made by observing fertility of only the first generation of crustaceans.

Differences in the number of offspring in the first and second generation of crustaceans exposed to strontium are in most cases not reliable. Chronic exposure to strontium was most pronounced in the third generation of *D. magna*. As mentioned above, this is often associated with high mortality in mature F3 females. Comparing fertility of the first and third generations for the two analyzed periods, we can say that the downward trend in fertility reaches a mathematically significant level.

Taken together these facts indicate the delayed action of non-radioactive strontium, which was manifested to a greater extent in the third generation of experimental *D. magna*. Similar delayed effects were shown for different generations of *D. magna* under the combined action of silver and glyphosate nanoparticles (da Silva et al., 2021). The work (Li et al., 2021) shows the mechanisms of formation of delayed effects in *D. magna* tolerance to a single short-term exposure to the insecticide imidacloprid. Most of the toxicant (84%) was distributed in soft tissues and then quickly excreted from the body, and the delayed effect was due to repeated exposure and accumulation of the substance in the exoskeleton (Li et al., 2021). For strontium, this mechanism can also be realized, since it competes with calcium, replacing it in hard tissues (Pathak and Gupta, 2020). The negative effect of strontium can also be observed with a single entry into water, because strontium is much worse removed from water systems due to physicochemical interactions with organic matter and other substrates than many heavy metals (Burton et al., 2019). This property of the chemical element contributes to the continuous effect on aquatic organisms and explains its delayed chronic effects.

Pathological phenomena in tests in 3 generations of *D. magna*

In long-term bioassays for chronic toxicity according to *D. magna* reactions it is quite easy to keep track of abortive eggs and dead juveniles (table). The table also shows the relative values of fertility of crustaceans, calculated for the period of 50 days.

Table 1. *Pathological phenomena of D. magna*

Variant	Generation	Number of abortive eggs *	Number of dead juveniles *	Fertility, % of the control value in 50 days
Control	F1	0	0	-
	F2	2	0	-
	F3	1	0	-
1 mg Sr/L	F1	39	8	56.9
	F2	54	4	53.8
	F3	97	9	19.5
2 mg Sr/L	F1	14	8	57.6
	F2	28	6	62.3
		37	5	8.8

*Note: * – sum for four parallel definitions*

During the experiment in all variants with exposure to strontium, the appearance of dead juveniles was noted. Even low values of this indicator are a sign of the chronic toxic effect of the tested sample (Olkova, 2021).

It is known that pathological changes in development of parthenogenetic eggs can be diagnostic signs of the negative effect on daphnia (Sobral et al., 2001). In our experiment, abortive eggs in the control variants are single, while in the experimental ones their number varies from 14 to 97 (in total for 4 parallel determinations). We observed more rejected (abortive) eggs with when adding 1 mg Sr/L than with 2 mg Sr/L. This is explained by the fact that in the sample with a lower strontium concentration by the middle of the experiment more live crustaceans remained. It was they who provided the increased values of this indicator, as well as of fertility. Dead born juveniles also appeared regularly on exposure to strontium in contrast to control.

The relative values of fertility demonstrate that the maximum effect of non-lethal doses of strontium occurs in a delayed manner, i.e., in the third generation of exposed crustaceans.

Conclusions

In the presented experiment we showed a situation where the toxicant does not have an acute toxic effect on *D. magna* in a short-term experiment (death within 96 hours). No chronic toxic effect was observed for the generally accepted exposure period (21–24 days): the rates of death and decrease in fertility of adults were lower than in the control, but the differences in most variants were not significant. However, when the experiment was 2 times (50 days) and 3 times (75 days) longer, an increase in the death of crustaceans and a decrease in fertility were shown ($P < 0.05$). These effects intensified in subsequent generations of experimental individuals. In the third experimental generation, the effect becomes maximum: from 50 to 75 days of life the crustaceans could not leave offspring (2 mg Sr/L).

Thus, using the example of the effect of strontium on *D. magna,* it was shown that toxicants previously considered low toxic can have delayed toxic effects. High mortality of adults in a number of generations and low fertility of surviving individuals threaten the existence of the population. Therefore, chronic toxicity bioassay using *D. magna* or other reliable test-cultures is a method for detecting and predicting such toxic threats.

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