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Effect of Heavy Metals (Cu, Co, Cd) on the Early Development of *Mytilus edulis* (Mollusca; Bivalvia)

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Abstract—The effect of dissolved heavy metals (Cu, Cd and Co) on the early development of blue mussel *Mytilus edulis* from the White Sea was studied in an acute 72-hour experiment, together with the effect of combinations of abiotic environmental factors: temperature (8, 12, and 16°C) and water salinity (14, 19, and 24‰). Calculated generalized linear models (GLM) show a significant effect of the factors on the number of D-veligers in all series of the experiment. The results provide evidence that the pairwise combination between factors "concentration" and "salinity" is synergistic, while those between factor "temperature" and the above two factors is antagonistic. Dose–effect relationships for copper and cobalt are described by log-logistic curves with an upper limit, and that for cadmium series, by a Weibull curve with an upper limit.

Keywords: Mytilus, Bivalvia, bioassay, toxicology, heavy metals, embryo development, D-veliger
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In the life cycles of hydrobionts, phases of embryonic and larval development are considered most vulnerable to toxic agents. Sensitivity to heavy metals (HMs) in larvae, compared to adults, is higher by two to three orders of magnitude [1]. This is why embryos and larvae of aquatic animals are common objects for toxicology assays [2–7], and larvae of *Mitilus* sp. among them [4, 8–12].

Since *Mytilus* species live in a wide range of temperatures and salinity, a special issue are the possible interactions between the toxicant and abiotic environmental factors. However, only few studies evaluate the combined effect of temperature, salinity, and toxic agents [13, 14].

The purpose of this study was to assess the effect of dissolved HM, temperature, and salinity on the early development of *Mytilus edulis* (Linnaeus, 1758) in an acute experiment.

MATERIAL AND METHODS

The early development of *M. edulis* mussels (the first 72 hours after fertilization) was proposed as a test system, with the number of larvae reaching the D-veliger stage (Figs. 1a, 1b) as the impact criterion.

Adult mussels were collected near the White Sea Biological Station, Moscow State University (66°34′ N, 33°08′ E). Spawning was stimulated by injecting 1 mL of 0.5 M KCl solution into the mantle cavity of each mussel. Eggs and sperm were mixed in fresh sea water (FSW) filtered through a 40-μm membrane at 10–12°C. The embryos were transferred to test plates only after fertilization was successful.

The effect of different concentrations of dissolved HM (Cu^{2+} – 3, 10, 20 μg/L; Co^{2+} – 10, 30, 100 μg/L; Cd^{2+} – 100, 200, 400 μg/L) was evaluated at different temperatures (8, 12 and 16°C) and three variants of salinity: 24‰–the normal White Sea water (100%), 19‰ after its slight desalinization (80%), and 14‰ after strong desalinization (60%). Salinity was measured with a TMC V2 refractometer. To prepare HM test solutions, the following salts were used: $CuCl_2 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$, and $CdCl_2$. HM solutions addition and changes in salinity had no significant effect on water pH, which remained within the range of 7.8–8.1 units throughout the experiment.

The experiment was performed in 12-well culture plates. One well was taken as an accounting unit a replicate. Each well contained 4 mL of a test solution in which the offspring from one pair of mussels was placed. The density of embryos did not exceed 200 ind./mL. Samples for analysis (500 μL) were taken after 48 and 72 hours and fixed with formalin. All tests were conducted in three replicates.

The samples were examined and processed using an Axioplan 2 Imaging microscope with an AxioCam HRm digital camera (Zeiss). Statistical analysis was performed in R [15] with additional program packages MASS ver. 7.3-47 [16], drc ver. 3.0-1 [17], margins ver. 0.3.0 [18], and ggplot2 ver. 2.2.1 [19].

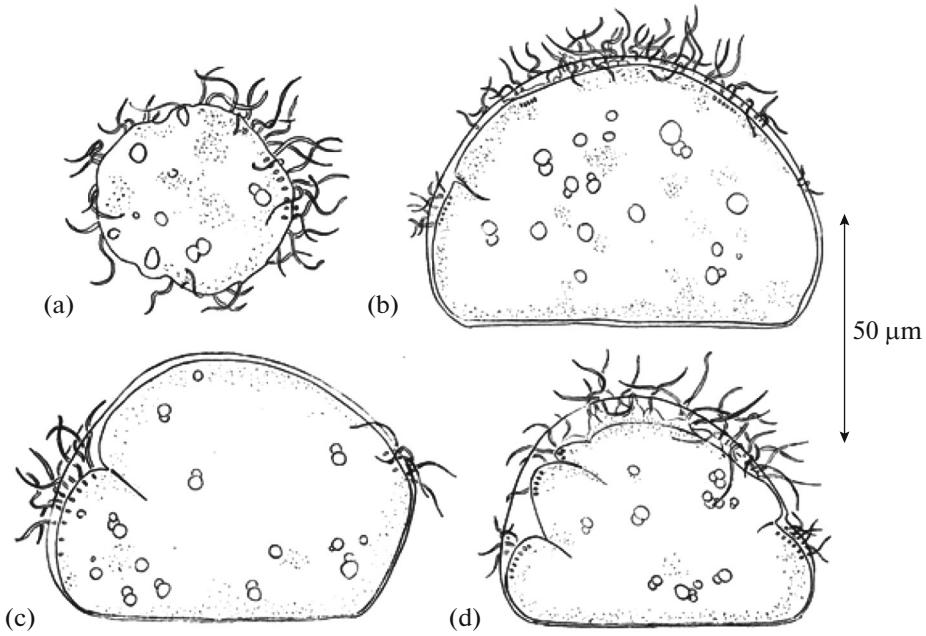

Fig. 1. Different types of larvae observed in experiments with the dissolved HM impact on the early development of *Mytilus edulis*: (a) trochophore larva, (b) D-veliger, (c, d) abnormal shell larvae at (c) 12 and (d) 16°C.

To analyze the results, generalized linear models (GLM) were calculated for experimental groups with different exposure times. The number of D-veligers was taken as response variable; salinity, HM concentration, and temperature, as predictors; and the total number of larvae in a sample, as statistical weights.

Regression analysis and calculation of EC_{50} values were performed using the drc package. The choice of the model was made based on the lowest values of Bayesian and Akiake information criteria (BIC and AIC).

RESULTS AND DISCUSSION

The development of embryos to the D-veliger stage was observed only at a temperature above 12°C and salinity above 14‰. The rate of development at 8°C decreased dramatically, and in some larvae the shell did not appear even after 72 hours. Likewise, only a small proportion of larvae developing at 14‰ salinity had a shell. Therefore, only two temperatures (12 and 16°C) and salinity values (19 and 24‰) were used for GLM modeling. The results are shown in Table 1.

Analysis of the models showed that factors of exposure time, temperature, and salinity had a statistically significant positive influence on the number of D-veligers, while the factor of HM concentration had a significant negative influence (Fig. 2). The marginal effect of salinity had the lowest value among all factors with a positive influence on *M. edulis* early development. Larvae that developed at the low decreased salinity (19‰) could be divided into two types: typical D-veligers and veligers with an abnormal shell structure. The latter had smaller size and distinctive shape with an asymmetric shell (Figs. 1c, 1d). Such larvae were found both in control and in samples treated with HMs after 48 hours but proved to be absent after 72 hours.

Therefore, it appears that abnormal shell larvae appeared primarily in response to a relatively small decrease in salinity, which only slightly slowed down the initial stages of early development. As a result, underdeveloped veligers appeared on the second day, but subsequently they developed into typical D-veligers.

The observed negative influence of decreased salinity is in agreement with data obtained by other authors. The decrease to 65% of the initial values (from 35 to 25‰) affects the development of *M. trossulus* and *M. galloprovincialis* mussels, with the proportion of D-veligers decreasing from 81.80% in the control to 36.15% [12, 14]. The development of larvae with an abnormal shell is also described in some papers [1, 14, 20], but their forms observed in this study had a different structure, which also proved to vary depending on temperature during the development (Figs. 1c, 1d).

Further salinity decrease to 14‰ resulted in a significant suppression of *M. edulis* early development. This is in line with the fact that species of the genus *Mytilus* are almost absent in water bodies with salinities below 15‰, such as Azov and Caspian seas. A comprehensive molecular genetic analysis of *Mytilus* species populations in the Baltic and North seas has shown that the most desalinizated areas of the Baltic Sea are inhabited with *M. trossulus* rather than by *M. edulis*, as was considered previously [21, 22].

Table 1. Results of modeling the effects of test factors on the early development of *Mytilus edulis*

Factors		Coeff.	SD	Z-test	Pr(>|z|)
48 hours					
Exposure time	cmn	**1.6665**	**0.0539**	**30.9290**	**2.00E-16**
Salinity	cmn	**0.4001**	**0.0708**	**5.6550**	**1.56E-08**
Temperature	cmn	**0.4747**	**0.0704**	**6.7440**	**1.54E-11**
HM concertation	Cd	**–0.0044**	**0.0002**	**–17.4980**	**2.00E-16**
	Co	**–0.0175**	**0.0010**	**–16.7510**	**2.00E-16**
	Cu	**–0.1342**	**0.0053**	**–25.4880**	**2.00E-16**
Salinity + temperature	cmn	**–0.2453**	**0.0731**	**–3.3580**	**7.86E-04**
HM concentration + salinity	Cd	–0.0003	0.0003	–1.0990	2.72E-01
	Co	–0.0016	0.0012	–1.3900	1.65E-01
	Cu	0.0017	0.0064	0.2680	7.88E-01
HM concentration + temperature	Cd	**0.0013**	**0.0003**	**4.4990**	**6.83E-06**
	Co	**0.0025**	**0.0012**	**2.1450**	**3.20E-02**
	Cu	**0.0218**	**0.0064**	**3.4170**	**6.32E-04**
72 hours					
Exposure time	cmn	**2.2322**	**0.0609**	**36.6690**	**2.00E-16**
Salinity	cmn	**0.4057**	**0.0800**	**5.0730**	**3.92E-07**
Temperature	cmn	**0.3643**	**0.0772**	**4.7160**	**2.40E-06**
HM concentration	Cd	**–0.0018**	**0.0003**	**–6.1510**	**7.69E-10**
	Co	**–0.0114**	**0.0012**	**–9.7840**	**2.00E-16**
	Cu	**–0.1183**	**0.0050**	**–23.6670**	**2.00E-16**
Salinity + temperature	cmn	**–0.3181**	**0.0791**	**–4.0230**	**5.76E-05**
HM concentration + salinity	Cd	–0.0001	0.0003	–0.3840	7.01E-01
	Co	**–0.0052**	**0.0014**	**–3.8320**	**1.27E-04**
	Cu	0.0049	0.0059	0.8290	4.07E-01
HM concentration + temperature	Cd	**–0.0019**	**0.0003**	**–5.6450**	**1.66E-08**
	Co	–0.0009	0.0014	–0.6600	5.09E-01
	Cu	–0.0086	0.0059	–1.4510	1.47E-01

Designations: Coeff., coefficient of factor; cmn, common to all metals; SD, standard deviation; boldface indicates statistically significant effect on the number of D-veligers, plus marked pairwise combinations.

The value of the marginal effect of temperature is higher than that of salinity (Fig. 2), indicating that increase in temperature has stronger influence on the number of D-veligers. However, the number of veligers with an abnormal shell observed at 12°C significally decreased to a minimum at 16°C and 19‰ salinity (Fig. 3). Probably, raising the temperature to 16°C accelerated the development of larvae, thereby compensating for the slowdown of development low salinity.

According to the results of modeling, only "HM concentration" factor had a significant negative effect on the total number of D-veligers. The adverse effect of HMs decreased in the series Cu > Co > Cd (Fig. 2), confirming the initial data on the relative toxicity of thee dissolved metals [4, 7].

The change in the duration of the experiment (factor "exposure time") had a positive influence on the number of D-veligers: the free term of the model increased as the experiment was prolonged from 48 to 72 hours. The marginal effect of this factor has the highest value, which is the evidence of the strong impact of this factor on the current assay (Fig. 2).

Pairwise combinations. We will firstly describe the dynamics of temperature, HM concentration, and salinity impact.

Although the coefficient of HM concentration decreased on day 3 (Table 1), no significant change in the marginal effects of HMs was revealed. It is possible that this change was so slight that its significance could not be confirmed at a given dataset. The value of the marginal effect of salinity remained unchanged during the experiment the experiment, whereas the same

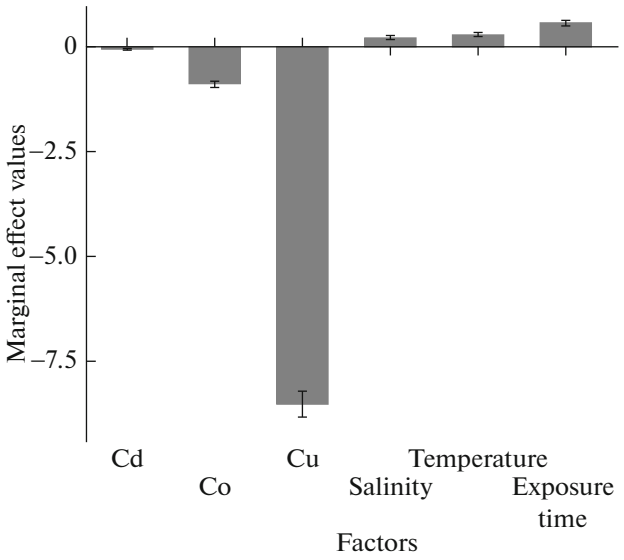

Fig. 2. Marginal effects of model factors on the total number of D-veligers. Error bars show 1.96 standard deviations (here and in Figs. 4–6).

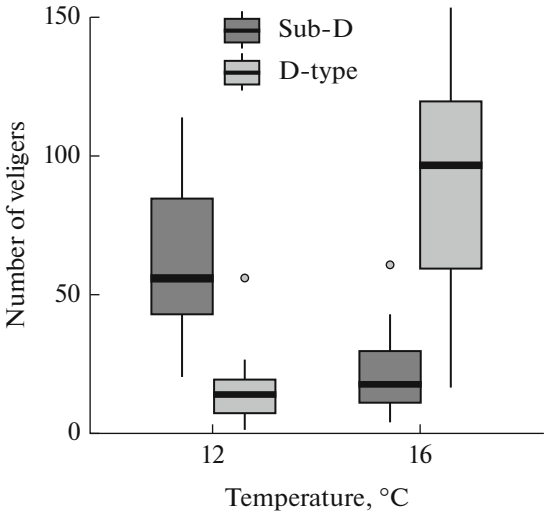

Fig. 3. Boxplot diagram of the numbers of D-veligers (D-type) and larvae with abnormal shell (Sub-D) in 48-hour experiments at 12 and 16°C and 19‰ salinity. Data shown are median values (thick horizontal line), interquartile ranges (boxes), total ranges (whiskers), and outliers (dots).

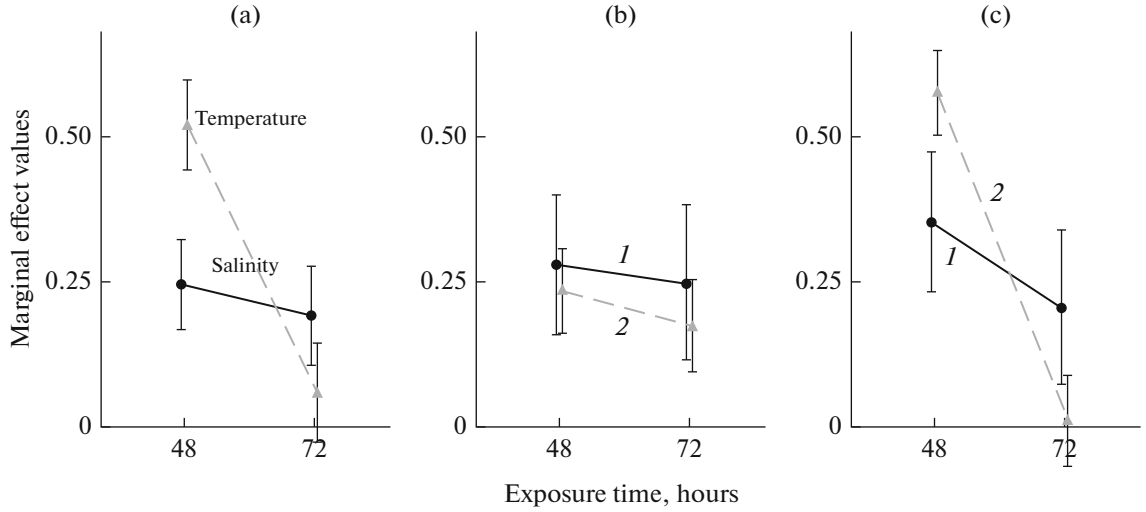

Fig. 4. Marginal effects of temperature and salinity: (a) dynamics of values for the model as a whole; dynamics of marginal effect values of (b) salinity and (c) temperature without (*1*) and with (*2*) dissolved HMs influence.

value for temperature decreased almost to zero (Fig. 4a). Notably, the marginal effects of the temperature and salinity in the control group (no HMs) and the experimental group treated with HMs showed different dynamics (Figs. 4b, 4c).

The values of the effects of salinity in the control and experimental groups on day 2 similar and retained their effect on day 3, with a common dynamics (Figs. 4a, 4b), while the marginal values of temperature differed significantly. On day 3, however, the effect of temperature remained significant in the experimental group but lost statistical significance in the control group (Fig. 4c). It appears that the additional stress factor (increased HM concentration) enhanced the relative effect of temperature by day 2 but abolished it by day 3.

Combination of "temperature + salinity" remained significant throughout the experiment. Each of the factors had a positive effect, especially on day 2, but their combined positive effect dropped down when the values for both factors increased. As it appears from Fig. 5, the positive effect of salinity decreased at higher temperature, and vice versa.

Combination of "HM concentration + salinity" had a statistically significant level only for cobalt after treatment for 72 hours, but its effect was very weak. A shift in the coefficient of HM concentration toward

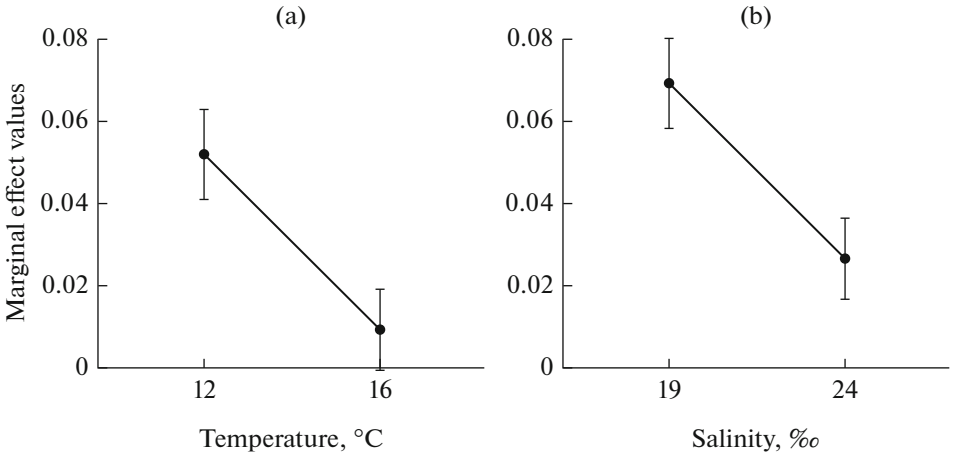

Fig. 5. Pairwise combination of the effects of (a) salinity and (b) temperature.

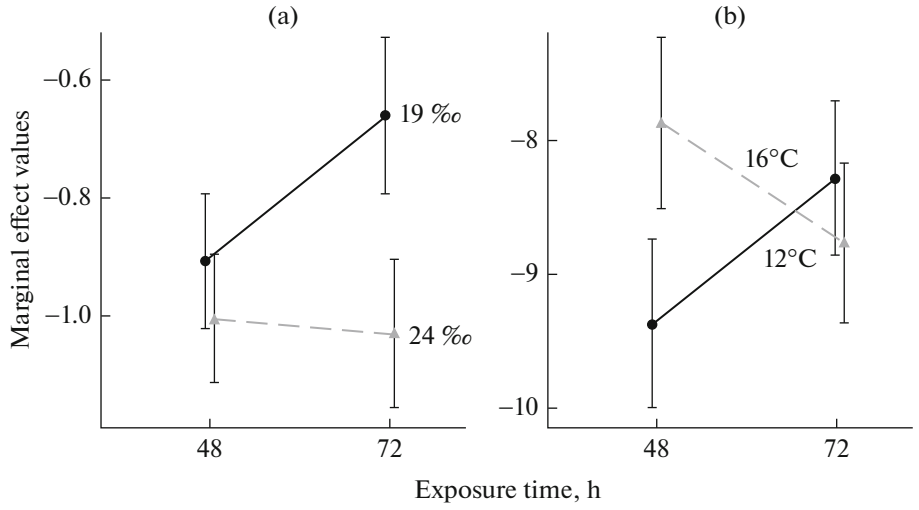

Fig. 6. Marginal effects of (a) cobalt concentration at different salinities and (b) copper concentration at different temperatures.

lower values is statistically significant and indicates that the overall negative effect of HM concentration increases upon change in salinity. It is noteworthy that the maximum effect of HM concentration was observed at normal salinity (Fig. 6a).

Combination of "HM concentration + temperature" had a statistically significant level for all metals on day 2 and demonstrated a positive effect: the coefficient of HM concentration decreased upon rise in temperature. The combined effect of these factors on day 3 was significant only in the cadmium dilution series, and the relation between them became inverse: a rise in temperature enhanced the effect of HM concentration. When assessing the values of marginal effects, significant differences were observed only for copper: its effects at 12 and 16°C were statistically different on day 2, but the difference disappeared at day 3 (Fig. 6b).

Taking into account the positive influence of factors "temperature" and "exposure time" and also the antagonism between factors "HM concentration" and "temperature," we can say that the trend of toxicity increase along the exposure time increase observed previously [4, 9] was not confirmed in the current assay.

Regression analysis. We obtained EC_{50} for HMs under conditions close to natural: 12°C and 24‰ salinity. According to the results of analysis, the dose–effect relationships for the copper and cobalt series are most adequately described by three-parameter log-logistic curves with an upper limit (LL.3u), and that for the cadmium series, by a three-parameter Weibull curve with an upper limit (W.3u).

The EC_{50} values of copper calculated for the White Sea mussel (17.35 ± 0.74 μg/L) proved to be higher

than those obtained for *M. edulis* by other authors (4–10.8 μg/L) [4, 9]. A decrease in EC_{50} at lower salinity (19‰) was observed only on day 2, in agreement with observations of many authors that early developmental stages in bivalves become more sensitive to toxic agents when salinity decreases [1, 14]. Higher EC_{50} values in *M. edulis* from the White Sea, compared to those from water areas with oceanic salinity, may indicate that the main factor is the short-term decrease in salinity rather than the initial difference between conditions in the habitats of these populations.

The effect of cobalt on embryonic development has been studied in a relatively small number of aquatic species [2, 6, 23, 24]. To our knowledge, we were the first to perform experiments on its impact on the embryonic and early postembryonic development of bivalve mollusks. The EC_{50} values calculated in this study (146.22 ± 51.30 μg/L) are almost identical to those for the sand dollar *Dendraster excentricus* (73–147 μg/L) [2] and to EC_{50}/LC_{50} for asexual reproduction and survival of early life stages in the sea anemone *Aiptasia pulchella* (85–154 μg/L) [24].

The EC_{50} values calculated for cadmium ions (461.66 ± 160.00 μg/L) are similar to, or even lower than, the values for other bivalve mollusks including *Mytilus* species [4, 8, 9]. Lower EC_{50}/LC_{50} values among invertebrates have been obtained for asexual reproduction and survival of early life stages in the sea anemone *A. pulchella* (27–185 μg/L) [24] and crab *Cancer magister* (247 μg/L) [4].

CONCLUSIONS

All factors included in this study were found to have a statistically significant effect on the early development of *M. edulis*, if the measure of the effect is the change in the number of D-veligers on the second and third days of experiment.

The combinations between these factors may be synergistic ("HM concentration" and "salinity") or antagonistic ("HM concentration" and "temperature," "temperature" and "salinity"). An interesting fact is the difference of temperature effect dynamics between the control and experimental groups (Fig. 6).

Based on the results of regression analysis, effective concentrations were calculated for HMs included in the study: $EC_{50}(Cu^{2+})$ = 17.35 ± 0.74 μg/L; $EC_{50}(Co^{2+})$ = 146.22 ± 51.30 μg/L; $EC_{50}(Cd^{2+})$ = 461.66 ± 160.00 μg/L.

Taking into account the positive influence of increase in temperature, salinity, and duration of experiment; ambiguous dynamics of the influence of temperature; and antagonistic interaction of HM concentration and temperature, it may be concluded that an acute experiment performed at normal salinity (24‰) and temperature of 12°C for 48 hours is optimal for biotesting in terms of method sensitivity and assessment of effects.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. This article does not contain any studies involving animals performed by any of the authors.

REFERENCES

1. His, E., Beiras, R., and Seaman, M., The assessment of marine pollution-bioassays with bivalve embryos and larvae, *Adv. Mar. Biol.,* 1999, vol. 37, pp. 1–178.
2. Rulon, O., Effects of cobaltous chloride on development in the sand dollar, *Physiol. Zool.,* 1956, vol. 29, pp. 51–63.
3. Nickless, G., Stenner, R., and Terrille, N., Distribution of cadmium, lead and zinc in the Bristol Channel, *Mar. Pollut. Bull.,* 1972, vol. 3, no. 12, pp 188–190.
4. Martin, M., Osborn, K.E., Billig, P., et al., Toxicities of ten metals to *Crassostrea gigas* and *Mytilus edulis* embryos and *Cancer magister* larvae, *Mar. Pollut. Bull.,* 1981, vol. 12, no. 9, pp. 305–308.
5. Kobayashi, N., Marine ecotoxicological testing with echinoderms, in *Ecotoxicological Testing for the Marine Environment: Proc. Int. Symp.,* Persoone, G., Jaspers, E., and Claus, C., Eds., Ghent, Belgium, 1984, vol. 1, pp. 341–405.
6. Dave, G. and Xiu, R., Toxicity of mercury, copper, nickel, lead and cobalt to embryos and larvae of zebrafish, *Brachydanio rerio, Arch. Environ. Contam. Toxicol.,* 1991, vol. 21, pp. 126–134.
7. Spangenberg, J.V. and Cherr, G.N., Developmental effects of barium exposure in a marine bivalve (*Mytilus californianus*), *Environ. Toxicol. Chem.,* 1996, vol. 15, no. 10, pp. 1769–1774.
8. Calabrese, A. and Nelson, D.A., Inhibition of embryonic development of the hard clam, *Mercenaria mercenaria,* by heavy metals, *Bull. Environ. Contam. Toxicol.,* 1974, vol. 11, pp. 92–97.
9. Malakhov, V.V. and Medvedeva, L.A., *Embrional'noe razvitie dvustvorchatykh mollyuskov v norme i pri vozdeistvii tyazhelykh metallov* (Embryonic Development of Bivalve Mollusks under Normal Conditions and under Heavy Metal Exposure), Moscow: Nauka, 1991.
10. Hoare, K., Davenport, J., and Beaumont, A.R., Effects of exposure and previous exposure to copper on growth of veliger larvae and survivorship of *Mytilus edulis* juveniles, *Mar. Ecol. Progr. Series,* 1995, vol. 120, no. 1, pp. 163–168.

11. Yaroslavtseva, L.M. and Sergeeva, E.P., Effect of copper ions on early developmental stages of the mussel *Mytilus trossulus* (Bivalvia), *Russ. J. Mar. Biol.,* 2005, vol. 31, no. 4, pp. 225–231.
12. Nadella, S.R., Fitzpatrick, J.L., Franklin, N., et al., Toxicity of dissolved Cu, Zn, Ni and Cd to developing embryos of the blue mussel (*Mytilus trossulus*) and the protective effect of dissolved organic carbon, *Comp. Biochem. Physiol. C,* 2009, vol. 149, no. 3, pp. 340–348.
13. Hrs-Brenko, M. and Calabrese, A., The combined effects of salinity and temperature on larvae of the mussel *Mytilus edulis, Mar. Biol.,* 1969, vol. 4, no. 3, pp. 224–226.
14. Hrs-Brenko, M., Claus, C., and Bubic, S., Synergistic effects of lead, salinity and temperature on embryonic development of the mussel *Mytilus galloprovincialis, Mar. Biol.,* 1977, vol. 44, no. 2, pp. 109–115.
15. R Core Team, *R: A Language and Environment for Statistical Computing,* Vienna, Austria: R Foundation for Statistical Computing, 2017.
16. Venables, W.N. and Ripley, B.D., *Modern Applied Statistics with S,* 4th ed., New York: Springer-Verlag, 2002.
17. Ritz, C., Baty, F., Streibig, J.C., and Gerhard, D., Dose-response analysis using R, *PLoS One,* 2015, vol. 10, no. 12, e0146021.
18. Leeper, T.J., *margins: Marginal Effects for Model Objects,* R package version 0.3.0, 2017.
19. Wickham, H., *Elegant Graphics for Data Analysis (ggplot2),* New York: Springer-Verlag, 2009.
20. Courtright, R.C., Breese, W.P., and Krueger, H., Formulation of a synthetic seawater for bioassays with *Mytilus edulis* embryos, *Water Res.,* 1971, vol. 5, no. 10, pp. 877–888.
21. Väinölä, R. and Strelkov, P., *Mytilus trossulus* in Northern Europe, *Mar. Biol.,* 2011, vol. 158.
22. Väinölä, R. and Hvilsom, M., Genetic divergence and a hybrid zone between Baltic and North Sea *Mytilus populations* (Mytilidae: Mollusca), *Biol. J. Linn. Soc.,* 1991, no. 43, pp. 127–148.
23. Shevtsova, S.N., Effects of exposure to cobalt sulfate on embryonic development of great pond snail (*Lymnea stagnalis*), *Ekol. Vestn.* 2010, no. 4 (14), pp. 125–134.
24. Howe, P.L., Reichelt-Brushett, A.J., and Clark, M.W., Effects of Cd, Co, Cu, Ni and Zn on asexual reproduction and early development of the tropical sea anemone *Aiptasia pulchella, Ecotoxicology,* 2014, vol. 23, pp. 1593–1606.

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