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Effect of Different Concentrations of Waterborne Sodium on the Hatching Rate and Ions Content of Rainbow Trout (*Oncorhynchus mykiss*) Eggs

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Abstract

With the aim of finding the optimum concentration of waterborne sodium for use during egg incubation period and higher production of the rainbow trout larvae in a water recirculation system, a completely randomize experiment was conducted. The effect of different levels of waterborne sodium (2, 14, 50 and 100 mg L^{-1}) on eye pigmentation, hatching rates, ions (Na, K, P, Cu, and Mn) and water content of eggs in rainbow trout fish, *Oncorhynchus mykiss*, were determined. The newly fertilized eggs were introduced in water recirculation incubators as experimental units. The water temperature was between 7.5-9 °C during the incubation period. In treatments with Na^+ concentration of 2 and 14 mg L^{-1}, hatching rates were significantly ($P<0.05$) higher than other treatments. Increase of waterborne Na^+ up to 100 mg L^{-1} significantly ($P<0.05$) decreased eye pigmentation. In all the treatments, water content (%) of egg, compared to the newly fertilized egg significantly ($P<0.05$) decreased at the hatching time. Egg ions content showed that the elevation of Na^+ level to 14 mg L^{-1} motivate sodium uptake by eggs from water and highest sodium uptake occurred in treatment with [Na^+] 100 mg L^{-1}. In all the treatments, potassium uptake occurred by eggs from environmental water. Egg potassium content in [Na^+] 100 mg L^{-1} was significantly ($P<0.05$) higher than other treatments. In treatments with [Na^+] 50 and 100 mg L^{-1}, prevention of Cu uptake by egg from ambient water occurred. Mn uptake by egg from water was only recorded in treatment with [Na^+] 2 mg L^{-1}. An increase in phosphorus content of the eggs was also recorded in treatments with [Na^+] 50 and 100 mg L^{-1}. It was concluded that eye pigmentation, hatching rates and ions content of rainbow trout eggs depend on the rate of waterborne sodium and concentration of 14 mg L^{-1} can be introduced as a standard level, for hatching the rainbow trout eggs in a water recirculation system

Keywords: Rainbow trout, egg, hatching rate, waterborne sodium, ion content.

Introduction

The embryogenic development is the main stage of the rearing cycle of fish, especially in captivity (Depeche and Billard, 1994). Ionic composition of ambient water is known as the key factor for the development of fertilized eggs (Vandervelden *et al.,* 1991). Mineral requirements of fish embryo fulfill either from the existing storage during vitellogenesis or from the ambient water during incubation periods (Lee and Hu, 1983). Calcium and sodium are the main abundant cations in ambient water (Maekareth *et al.,* 1978) and can be uptake from water by the embryos of rainbow trout (Barrett *et al.,* 2001). However, Peterson *et al.,* (1982) showed that ions were not uptake by rainbow trout eggs prior to hatching. It is known that, mitochondrion-rich cells (MRCs) are present in yolk sack membrane and other body surfaces are the main ion-regulatory sites in embryonic stage (Kaneko *et al.,* 2002). A numerous number of MRCs in the yolk sac membrane of embryos and larvae of Mozambique tilapia (*Oreochromiss mossambicus*) were identified (Kaneko *et al.,* 2002). It is known that an increase in the Na^+ content of embryonic rainbow trout in the few days prior to hatching occurred, resulting in the presence of mitochondria rich cells in epithelia of both yolk sack and the developing gills (Shen and Leatherland, 1977). HA electrically linking with the epithelial Na channel (ENaC), and the electroneutral NHE as two models were proposed for apical Na^+ uptake/acid secretion functions in FW fish gills (Hwang, 2011). A specific NHE inhibitor, EIPA, was found to decrease the Na^+ uptake activity (by SIET) in medaka (*Oryzias latipes*) embryonic skin ionocytes (Wu *et al.*, 2010). Horng and colleagues (2007) identified the vacuolar H^+-ATPase (V-ATPase, H^+ pump) in the skin of zebrafish (*Danio rerio*) embryo

that distributed mainly in the apical membrane of H^+-pump-rich cells and knockdown of this pump revealed abnormalities, including suppression of acid-secretion from skin, growth retardation, trunk deformation, and loss of internal Ca^{2+} and Na^+.

The physiological roles of calcium; influence on permeability of biological membranes, uptake of certain metals by competitive inhibition which modulates their toxicity to fish (Matsuo *et al.,* 2004) and preventing ion losses from the tissues to water (Bijvelds *et al.,* 1998) are well known. Ketola *et al* (1988) reported that survival of rainbow trout eggs incubated in waterborne calcium concentration of 34-49 mg L^{-1} increases and higher Ca^{2+} concentration reduces post-hatch survival of this species. Sodium is known as the key factor regulating osmotic and ionic gradient of egg plasma membrane and also absorption of some ions by egg is depended on sodium concentration in embryo tissues and ambient water (Alderdic, 1988). Negative correlation of copper accumulation in rainbow trout (Sloman *et al.,* 2003) and manganese uptake by brook charr (*Salvelinus fontinalis*) (Gonzalez *et al.,* 1990) regarded with sodium uptake has been reported. There is a clear need to find whether ions accumulation in embryonic tissues correlates with external sodium concentration or not. Therefore, this research was conducted to examine whether or not external Na^+ concentration affects the rainbow trout eggs hatching efficiency and uptake of other ions (Na, K, P, Cu and Mn) by egg during the incubation period.

Materials and Methods

Experimental Treatments

A completely randomized experimental design was carried out at the Faculty of Natural Resources, University of Tehran, Karaj, Iran. Fertilized rainbow trout eggs were obtained from Salmonids Research Institute, Kelardasht, Iran. The 270 newly fertilized eggs were introduced in each experimental unit in which waterborne recirculation through incubators. The experimental treatments were four waterborne sodium media (2, 14, 50 and 100 mg L^{-1} [Na]) in triplicates. The incubation media (treatment) were prepared by adding analytical salt (Merck) [NaCl, $CaCl_2$, KCl, $(MgCO_3)_4.Mg(OH)_2.5H_2O$, $FeCl_3.6H_2O$, $ZnCl_2$, $MnCl_2.4H_2O$, $CuSO_4.5H_2O$, $Na_2HPO_4.12H_2O$] in to double-deionized distilled water. Waterborne ions concentration was determined by ICP (GBC Integra XL) (Table 1). Dissolved oxygen, temperature and pH of water were measured using PC_{300} instrument, total ammonia of water were measured using photometer instrument (Palintest 8000 model). The pH, temperature, dissolved oxygen and non ionized ammonia varied between 7.1-7.22, 7.5-9ºC, 10.39-11.42 (mg L^{-1}) and 0.20-0.82 (mg L^{-1}) respectively, among treatments during incubation period.

Experimental Procedure

The newly fertilized eggs were introduced into the treatments, incubation trays in a recirculation waterborne sodium media. Each experimental unit was supplied with 45 L aerated water media and sterilized by a UV lamp (246 nm, 20 w). Eye pigmentation and hatching time of the eggs in each treatment was measured during incubation period. The hatching rate (H) was calculated by the method of Geertz Hansenand and Ramassen (1994): H = (number of incubated eggs – number of dead eggs)/ (number of incubated eggs) × 100

Sampling Protocol

Prior to allocating the fertilized eggs to trays and over the time of hatching, 100 eggs were sampled from the eggs batch and their wet mass, water and ions content were measured. For measurement of the Na, K, P, Cu and Mn concentration of the waterborne, one liter of (1 L) waterborne was also sampled.

Measurement of Whole Egg Water and Ion Content

Wet mass (W_w, mg) of eggs was determined after blotting dry by absorbent paper to remove the surface moisture. Dry mass (W_D, mg) was determined after placing the eggs in a 60ºC oven for 48 h ,then cooling them in a desiccator. Dried eggs were weighed and whole body water content (%) was calculated by the method of Barrett *et al.* (2001):

Table 1. Ions concentration in different waterborne sodium treatments

Treatments (mg L^{-1})	Waterborne ions concentration (mg L^{-1})								
	Na	Ca	Mg	K	P	Fe	Cu	Zn	Mn
2	2.03± 0.06	54.55± 1.19	9.11± 0.22	1.94± 0.05	1.44± 0.01	0.055± 0.0008	0.034± 0.0006	0.026± 0.002	0.014± 0.0003
14	14.18±0. 18	54.63± 0.83	9.12± 0.26	1.91± 0.05	1.45± 0.01	0.054± 0.001	0.033± 0.0009	0.026± 0.0004	0.015± 0.0002
50	50.95±0. 16	54.51± 0.60	9.19± 0.15	1.92± 0.03	1.46± 0.01	0.053± 0.001	0.034± 0.0008	0.024± 0.0003	0.015± 0.0003
100	99.41±0. 58	55.62± 0.14	9.23± 0.13	1.93± 0.05	1.45± 0.02	0.054± 0.002	0.032± 0.0006	0.025± 0.0005	0.014± 0.003

(Mean ± SD)

water content = 100 (W_w- W_D) W_w^{-1}.

The eggs were dried in a 105ºC oven for 48 h and 1g egg digested in 6 ml 1 N HNO_3 (Moopan, 1983), then concentrations of Na, K, P, Cu and Mn were measured by ICP (GBC Integra XL).

Statistical Analysis

Data were presented as Means±SD. Percent values were transformed by arcsine method to be normalized, and then analyzed statistically. Differences in waterborne Na, K, P, Cu and Mn concentrations at the start and end of the experiment were recorded, egg mortality rate prior and after eye pigmentation were tested as well, using paired-sample T test. Differences between the means were analyzed using one way analysis of variance (ANOVA). Duncan's new multiple range test was conducted to determine the significant differences between the means using SPSS software (version 12.0). Statistical significance was accepted at the level of $P<0.05$.

Result

Eye Pigmentation and Hatching Rates

Eye pigmentation was apparent 22-26 days post fertilization and hatching occurred 35-41 days post fertilization. Eye pigmentation rate of eggs which were exposed to the highest Na^+ concentration (100 mg L^{-1}) was significantly ($P<0.05$) lower than eggs exposed to other Na^+ concentrations (Table 2). The hatching rate of eggs was significantly ($P<0.05$) different among waterborne sodium treatments. The highest hatching rate was observed in the [Na^+] 2 mg L^{-1} treatment. The hatching rates of eggs were not significantly ($P>0.05$) different between [Na^+] 2 and 14 mg L^{-1} treatments (Table 2).

The variation of eggs mortality rate prior and after eye pigmentation were shown in the present study (Figure 1). In all treatments, egg mortality percent after eye pigmentation significantly increased ($P<0.05$) compared to the prior of eye pigmentation.

Wet Mass and Water Content of Egg

Wet mass of eggs which were incubated in different waterborne sodium concentrations did not show any significant ($P>0.05$) change compared to the newly fertilized eggs. Different waterborne sodium concentrations did not significantly ($P>0.05$) influence the wet mass of eggs (Table 3).

Incubation of eggs in different waterborne sodium concentrations significantly ($P<0.05$)

Table 2. Hatching and eye pigmentation rates of rainbow trout eggs as a function of different Na^+ concentrations utilization in different treatments

Treatments (mg L^{-1})	Eye pigmentation (%)	Hatching rate (%)
2	94.06±0.60^a	78.72±3.00^{ab}
14	95.20±0.36^a	82.41±1.04^a
50	93.64±1.40^a	72.83±4.38^b
100	90.50±1.82^b	72.80±1.65^b

(Mean ± SD)
Means with different letters in a column are significantly different ($P<0.05$). n =270.

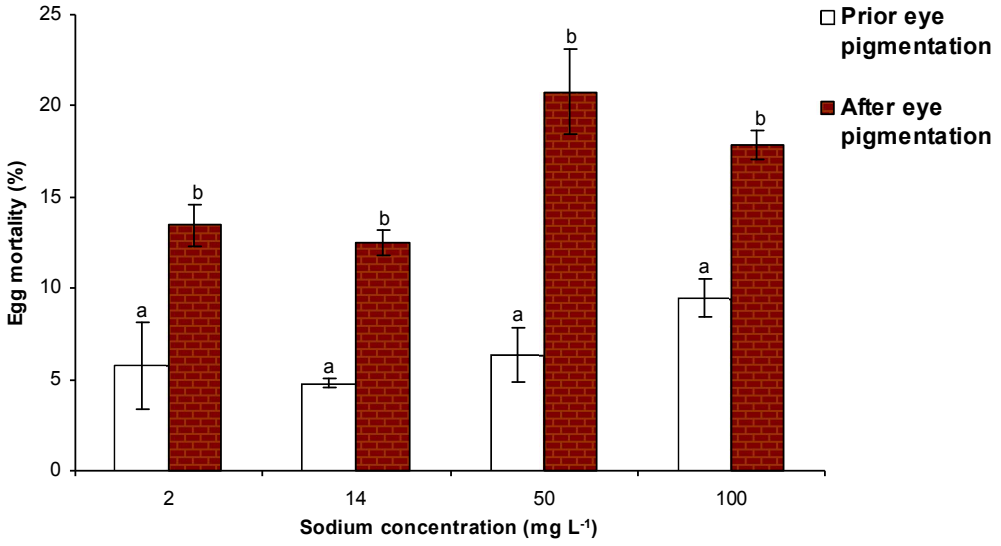

Figure 1. Comparison between rainbow trout eggs mortality rate prior and after eye pigmentation incubated in different sodium concentrations. Bars represent Mean ± SD. $P<0.05$. n=270

decreased water content (%) of eggs at hatching time in relation to the newly fertilized eggs. Egg water content was not significantly different among treatments (Table 3).

Whole Egg ion Content

The whole egg ions content are shown in Table 4. The whole egg Na^+ in treatment with [Na^+] 14, 50 and 100 mg L^{-1} significantly ($P<0.05$) increased 40.89, 43.91 and 64.64% compared to the newly fertilized egg respectively. In all the treatments, K content of whole egg significantly ($P<0.05$) increased compared to the newly fertilized egg. In treatments with [Na^+] 2 and 14 mg L^{-1}, no significant ($P>0.05$) alteration in whole egg P was observed relative to the newly fertilized egg, but incubation of eggs in [Na^+] 50 and 100 mg L^{-1} increased whole egg P content compared to the newly fertilized egg. Cu content of whole egg in [Na^+] 2 and 14 mg L^{-1} were ten and six time higher than the newly fertilized egg respectively. Incubation of egg in treatment with [Na^+] 2 mg L^{-1} increased Mn content of whole egg by 48.8% compared to the newly fertilized egg. Increasing the waterborne sodium to 14, 50 and 100 mg L^{-1} did not significantly ($P>0.05$) influenced Mn content of whole egg relative to the newly fertilized egg.

Waterborne Ions Content

The variations of waterborne ions content in different sodium treatments at the beginning of the experiment and hatching stage are shown in Table 5. In all treatments, waterborne Na and K significantly ($P<0.05$) decreased during eggs incubation period. Alteration of waterborne P was not significantly ($P>0.05$) different among treatments during the incubation period. Waterborne Cu significantly

Table 3. Whole egg wet mass and water content (%) in the newly fertilized egg and incubated egg in different sodium treatments at hatching stage

Parameter	Newly fertilized egg	Sodium Treatments (mg L^{-1})			
		2	14	50	100
Wet mass (mg)	84.77±2.42^a	84.97±1.16^a	81.68±1.63^a	81.62±1.98^a	86.37±4.9^a
Water content (%)	69.58±0.14^a	67.18±0.32^b	67.22±0.24^b	67.11±0.96^b	68.12±0.95^b

(Mean ± SD).
Means with different letters in the same row are significantly different ($P<0.05$). n = 20.

Table 4. Whole egg ions content (mg/g dry weight) in newly fertilized egg and incubated egg in different treatments at hatching stage

Mineral	Newly fertilized egg	Sodium Treatments (mg L^{-1}			
		2	14	50	100
Na	0.74±0.021^a	0.84±0.064^a	1.04±0.051^b	1.06±0.07^b	1.22±0.08^c
K	4.63±0.51^a	5.25±0.48^b	5.58±0.26^b	5.28±0.21^b	6.24±0.29^c
P	29.61±0.27^a	30.09±0.20^a	29.82±0.18^a	32.26±0.07^b	31.90±0.29^b
Cu	0.01±0.003^a	0.11±0.001^c	0.065±0.014^b	0.016±0.001^a	0.015±0.001^a
Mn	0.0045±0.0003^a	0.0067±0.0005^b	0.0048±0.001^a	0.005±0.0003^a	0.0043±0.0003^a

(Mean ± SD).
Means with different letters in the same row are significantly different ($P<0.05$).

Table 5. Variations in waterborne ions concentration (mg L^{-1}) in different treatments at the beginning of the experiment and hatching stage

Mineral	Time	Sodium Treatments (mg L^{-1})			
		2	14	50	100
Na	Beginning of Exp.	2.03±0.06^a	14.18±0.18^a	50.59±0.16^a	99.41±0.58^a
	Hatching stage	1.16±0.13^b	9.12±0.26^b	43.05±0.22^b	79.08±0.46^b
K	Beginning of Exp.	1.94±0.052^a	1.91±0.050^a	1.92±0.030^a	1.93±0.054^a
	Hatching stage	0.56±0.052^b	0.57±0.010^b	0.53±0.010^b	0.50±0.011^b
P	Beginning of Exp.	1.44±0.015^a	1.45±0.010^a	1.46±0.013^a	1.45±0.025^a
	Hatching stage	1.32±0.30^a	1.32±0.092^a	1.22±0.30^a	1.38±0.058^a
Cu	Beginning of Exp.	0.034±0.0006^a	0.033±0.0009^a	0.034±0.0008^a	0.032±0.0006^a
	Hatching stage	0.017±0.0028^b	0.012±0.004^b	0.026±0.009^a	0.026±0.006^a
Mn	Beginning of Exp.	0.014±0.0003^a	0.015±0.0002^a	0.015±0.0003^a	0.014±0.0003^a
	Hatching stage	0.009±0.0007^b	0.015±0.0018^a	0.014±0.0024^a	0.014±0.0025^a

(Mean ± SD).
Means with different letters in beginning of trial and hatching stage in each sodium treatment are significantly different ($P<0.05$).

($P<0.05$) decreased in treatments with $[Na^+]$ 2 and 14 mg L^{-1}, but Cu reduction was not significant ($P>0.05$) in treatments with $[Na^+]$ 50 and 100 mg L^{-1}. Waterborne Mn significantly ($P<0.05$) decreased only in treatments with $[Na^+]$ 2 mg L^{-1} during the incubation period.

Discussion

Eye Pigmentation and Hatching Rates

Reduction of eye pigmentation in treatment $[Na^+]$ 100 mg L^{-1} may be due to premature bursting of eggs from excessive water absorption, so that water content (%) of eggs in this treatment was higher than other treatments (Table 3). In all treatments, higher eggs mortality occurred after eye pigmentation (Figure 1), this phenomenon can be attributed to Na^+ uptake by egg after eye pigmentation.

The findings of this study revealed that different waterborne Na^+ l evels affect the eggs hatchability. The eggs exposed to waterborne $[Na^+]$ of 2 and 14 mg L^{-1} had the highest hatching rates whereas reduction on hatch rate in $[Na^+]$ 50 and 100 mg L^{-1} was recorded. Early development of fish embryo involves the dorsal axis formation and the movement of epiboly. It has shown that embryos of *Misgurnus fossilis* (Cobitidae) which were placed in 100 mM NaCl solution can not begin normal epiboly and axis formation at early blastula stage and embryos had irregular structures and different cell type. Influence of NaCl on embryogenesis appear to be specific for Na^+ and is not osmotic (Minin and Ivanova, 1996).

Whole Egg Water Content

Whole egg water content in different waterborne sodium decreased in relation to the newly fertilized egg which indicates the water loss during the incubation period. In all treatments, waterborne calcium concentration was approximately constant, 50 mg L^{-1}, led to the prevention of water absorption. Water hardness has a direct effect on the swelling of the newly fertilized egg (Spade and Bristow, 1999). It has shown that higher Calcium concentration directly affected the swelling of rainbow shark minnow (*Epalzeorhynchus frenatum*) eggs due to an increase in the osmotic concentration of the incubation water (Abernathy, 2004). The result of the present study confirmed that different waterborne sodium concentrations had not any significant ($P>0.05$) effect on water uptake by egg. Egg water content of 67.11-68.11% was recorded among treatments. Barrett et al (2001) reported the rate of 64.4% egg water content for rainbow trout.

Ion Uptake by Embryos

Increasing the whole egg sodium content exposed to $[Na^+]$ treatment 14, 50 and 100 mg L^{-1} compared to the newly fertilized egg and reduction of waterborne sodium concentration during the incubation in these treatments indicate that the trout eggs can uptake sodium from waterborne. The linear relationship between $[Na^+]$ uptake rate by rainbow trout egg and external $[Na^+]$ suggest that Na^+ can enter into the egg (Barrett *et al.* 2001). Sodium uptake by eggs was increased along with an increase in waterborne $[Na^+]$, suggesting that the rate of sodium uptake by egg related to waterborne $[Na^+]$ levels.

The results of whole egg K content and waterborne K concentration showed that potassium uptake by egg from water in all treatments and the highest potassium uptake observed in treatment with $[Na^+]$ 100 mg L^{-1}. This phenomenon may be related to the consequence of greater sodium uptake by egg (Table 4). These findings suggest that the probable mechanism of K uptake relate to the sodium uptake by egg from water. Potassium uptake by river lamprey egg (*Lampetra fluviatilis*) mediated by two basic energy dependent transport mechanism Na-K-pump and Na^+, K^+, $2Cl^{-1}$ co-transport (NKCC) (Sherstobitov *et al.* 2004). The NKCC is a class of integral membrane proteins that mediate the movement of Na^+, K^+ and Cl^- ions across the plasma membrane of animal cells in an electrically neutral manner (Payne and Forbush, 1995). Some studies revealed that Na^+, K^+, $2Cl^{-1}$ co-transport involved in sodium uptake function in aquatic environments rich in sodium ions (Kirschner, 2004).

Uptake of phosphorus by embryo and larvae of fish from water has reported. For example, Win penny (1998) recorded phosphorus absorption by brown trout (*Salmo trutta* L.) from water. The present data conversely indicate lack of phosphorus uptake from environmental water and different external $[Na^+]$ do not affect P uptake by eggs (Table 4 and 5). Lack of P uptake may be due to sufficient storages of P within the newly fertilized egg and active P uptake may not be necessary. An increase in whole egg P content incubated in $[Na^+]$ 50 and100 mg L^{-1} in relation to the newly fertilized egg may be as a consequence of increasing activity of ATP pumps for ions transporting, so that uptake of Na, K and Mg (not shown) from water were higher than eggs incubated in $[Na^+]$ 2 and 14 mg L^{-1}.

Cu uptake from water environment occurred in eggs incubated in $[Na^+]$ 2 and 14 mg L^{-1}, as Cu content of the whole egg increased in relation to the newly fertilized egg and waterborne Cu decreased during the incubation period. Incubation of eggs in $[Na^+]$ 50 and 100 mg L^{-1} were prevented Cu uptake by eggs from water. A theory suggests that copper at least in a part cross the gill through sodium channel on the apical member and some part of transported copper can be excluded by high external sodium levels (Grosell and Wood, 2002). Sloman *et al.* (2003) reported that the differences in copper accumulation in rainbow trout may be due to differences in the sodium uptake rate. Sodium and copper may share a

common pathway for up-taking through epithelial cells from external medium. Copper absorption via Cu-specific pathway somehow was modulated by the presence of external Na^+ (Handy *et al.,* 2002). The data of this study suggest that Na^+ and Cu absorption by egg from the external water probably take place via the same pathway. Lower Na^+ uptake rate by egg in $[Na^+]$ 2 and 14 mg L^{-1} can be attributed to Na^+ and Cu competition to entry through the same pathway and higher Cu uptake rate.

The results of whole egg Mn content and waterborne Mn showed that only eggs in treatment with $[Na^+]$ 2 mg L^{-1} absorbed Mn from the waterborne whereas incubation of eggs in treatments with $[Na^+]$ 14, 50 and 100 mg L^{-1} prevented Mn uptake from water (Table 4 and 5). Manganese is an acutely toxic at relatively high aqueous concentration and its toxicity is affected by water hardness (Stubblefield *et al.* 1996). Gonzalez et al (1990) found that manganese uptake by gills of brook charr (*Salvelinus fontinalis*) inversely correlated with body sodium concentration. Manganese uptake by eggs in $[Na^+]$ 2 mg L^{-1} may be due to the lack of sodium uptake from water and less sodium accumulation in whole body egg.

Conclusion

According to the results of this study, it is recommended that the incubation of rainbow trout eggs in waterborne $[Na^+]$ from 14 to 100 mg L^{-1} and $[Ca^{2+}]$ concentration of 55 mg L^{-1} environmentally are useful to reduce accumulation and toxicity of Cu and Mn ions during the rainbow trout embryogenesis. These results also indicated the important role of waterborne Na^+ levels in the improvement efficiency of rainbow trout egg hatching rate and successful culture of this species in captivity.

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