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**Short-term enrichment of microalgae with inorganic selenium and zinc and  
their effects on the mineral composition of marine rotifer *Brachionus sp.***

**Running Head:** Short-term mineral enrichment of microalgae and rotifers

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**Short-term enrichment of microalgae with inorganic selenium and zinc and their effects on the mineral composition of marine Rotifer**

**Abstract**

Rotifers are widely used in aquaculture for feeding the early stages of finfish and crustacean larvae although their low content in minerals such as selenium (Se, 30 fold lower than in copepods) or zinc (Zn, 5 fold lower) is considered one of the disadvantages of this live prey compared to copepods. Thus, enrichment of rotifers with increasing amounts of these nutrients is a need. The results of the present study provide information about the effects of selenium and zinc enrichment on microalgae (i.e., *Isochrysis* aff. *galbana* and *Nannochloropsis oculata*) and on rotifer growth using a single (experiments 1 and 2) and mixed (experiment 3) minerals. For the mineral enrichment of microalgae, the first step was to centrifuge and re-suspend the microalgae at a concentration of  $18 \times 10^9$  cells/mL of saltwater. Then, different concentrations of both Se and Zn were added to concentrated microalgae in experiments 1, 2 (i.e., 40, 80, and 120 mg/L of Zn or Se) and 3 (i.e., 20, 40, and 80 mg/L of both Zn and Se). The amount of minerals in the microalgae was quantified after 1 and 3 h enrichment, followed by the study of their effect on the growth and mineral composition of the rotifer. Based on the results, the best enrichment time was 1 h and the amount of Se in the enriched microalgae was in line with the concentration of Se in the enrichment media whereas in the case of Zn, the content in enriched algae increased as a function of Zn concentration in the medium until 80 mg/L. In the trial 3, the highest amount of Se and Zn in enriched microalgae was found using 80 mg/L (Zn + Se). In each experiment, the rotifers were fed with the enriched microalgae and the maximum Zn content was detected in the group fed with 80 mg/L Zn-enriched algae. In the second experiment, the maximum Se was observed in rotifers fed with 80 and 120 mg/L Se-enriched algae. Whereas the highest amount of Zn and Se was found in the rotifer fed with 80 mg/L (Zn

+ Se)-enriched algae used in the third experiment. Mineral enrichment of microalgae 44  
contributed to increase their content in rotifers and no antagonistic effect was observed between 45  
the minerals. Thus, both microalgae and rotifers, can be enriched simultaneously with minerals 46  
and used in larval culture. While Zn-enriched microalgae negatively affected the growth of 47  
rotifers, there was no significant effect on rotifer growth when Se was used alone or both 48  
minerals were used together. Overall, rotifer enrichment with Se- and Zn-enriched algae led to 49  
an increase in Se and Zn. Thus, Se- and Zn-enriched rotifers can be used as a mineral delivery 50  
method to cover the nutritional requirements of marine fish larvae. 51

**Keywords:** Enrichment, Growth, Microalgae, Minerals, Rotifer 52

## 1. Introduction 54

According to FAO last report, the aquaculture sector is growing in a sustained way with a 55  
prevision to reach more than 54% of the world production in 2030 with more than 109 million 56  
tonnes. The production of a sufficient number of fish larvae with an adequate quality and 57  
quantity is regarded as one of the bottlenecks of aquaculture. Several factors affect the 58  
improvements in larviculture and the production of suitable live food for the larvae (e.g., 59  
rotifers, *Artemia*, and copepods) is considered one of the main problems (Hagiwara & Marcial, 60  
2019). Although copepods are considered the best live prey for larval culture they are not used 61  
in most of the hatcheries due to problems such as their scaling up and the risk of parasitic 62  
infections if the copepods are captured from the wild (Ajiboye, Yakubu, Adams, Olaji, & 63  
Nwogu, 2011). Rotifers are used in most of the hatcheries for feeding the earliest stages of fish 64  
larvae and crustaceans (Dhont et al., 2013; Hamre, 2016; Loo, Chong, Vikineswary, & Ibrahim, 65  
2016; Mæhre, Hamre, & Elvevoll, 2013; Nordgreen, Penglase, & Hamre, 2013; Sayegh, Radi, 66  
& Montagnes, 2007) due to their small size, slow movements, fast reproduction, and easy 67  
cultivation and handling (Dhont et al., 2013), and their population shows a clear exponential 68

growth if culture conditions are adequate (Mejias, Riquelme, Sayes, Plaza, & Silva-Aciaras, 69  
2018). On the other hand, rotifers have low content of some nutrients, such as polyunsaturated 70  
fatty acids and vitamins, compared to copepods (Hamre et al., 2016; Hamre, Srivastava, 71  
Rønnestad, Mangor-Jensen, & Stoss, 2008; Nordgreen et al., 2013; Samuel Penglase et al., 72  
2013; Srivastava, Stoss, & Hamre, 2011), and, in order to increase the amounts of these 73  
nutrients, enrichment, using direct or indirect methods, is a need. Minerals are a group of micro- 74  
elements, essential to fish larvae that are absent in rotifers. For example, selenium and zinc 75  
(respectively, 0.08 mg/kg and 49 mg/kg in dry weight) are present in the rotifers in lower 76  
quantities (Se, 30 fold and Zn, 5 fold) than the minimum amounts found in copepods (3-5 77  
mg/kg and 340-570 mg/kg in dry weight, respectively) (Nematzadeh, Ahmadifard, Samadi, 78  
Agh, & Ghaderpour, 2018; Nordgreen et al., 2013; Samuel Penglase et al., 2013; J. Wang, Shu, 79  
& Wang, 2019). Furthermore, the National Research Council (NRC, 2011) reported that Se 80  
and Zn in rotifers were below the requirements in fish. 81

Se is an essential trace mineral and a component of the glutathione peroxidase enzyme that 82  
protects cellular tissues and membranes against oxidative damage by destroying hydrogen 83  
peroxide and lipid hydroperoxides (Flohé, 2010; Molina-Poveda, 2016). It is also involved in 84  
the immune system and increases the metabolism of thyroid hormones (Arthur, 1991; Arthur 85  
& Beckett, 1994; Rayman, 2000). An increase in Se in the fish diet increases their stress 86  
resistance (Küçükbay et al., 2009; Rider et al., 2009) and balances the toxicity of heavy metals 87  
such as mercury and cadmium (Raymond & Ralston, 2009; Watanabe, Kiron, & Satoh, 1997). 88  
The lack of Se in the diet reduced growth, survival, and reproduction in animals (Loscalzo, 89  
2014). Selenomethionine (SeMet), as a naturally occurring amino acid present in several 90  
vegetables and cereals, has higher bioavailability, digestibility, and antioxidant properties and 91  
sustainability while lower toxicity compared to its inorganic form (Doucha, Lívanský, 92  
Kotrbaček, & Zachleder, 2009; Hamre et al., 2013; Molina-Poveda, 2016; Silva et al., 2019). 93

Microalgae can take up selenate and selenite from the water and convert them to protein-bound 94  
selenocysteine and SeMet (Gojkovic, Garbayo, Ariza, Márová, & Vílchez, 2015; Neumann, 95  
De Souza, Pickering, & Terry, 2003). Thus, SeMet derived from algae can be used in 96  
aquaculture to fulfil the larval requirements of Se (Gojkovic et al., 2014; Pacitti et al., 2015). 97  
According to previous studies, fish and crustaceans cannot produce SeMet and thus rely on the 98  
organisms of lower trophic levels such as algae and fungi (Pacitti et al., 2015; Williams, Ogle, 99  
Knight, & Burau, 1994). Pacitti et al (2015) reported that Se-fortified *Chlorella vulgaris* 100  
improves the reproduction of Se-enriched rotifer *Brachionus* sp. 101

Zn is also known as an essential trace element for several organisms including live food 102  
and fish (Apines-Amar et al., 2004; Gatlin III & Wilson, 1986; Nordgreen et al., 2013; Satoh, 103  
Takeuchi, Narabe, & Watanabe, 1983). Zn is needed for many physiological functions such as 104  
growth, development, reproduction, immune function, bone formation, and cell proliferation 105  
(Matsumoto, Satoh, Kotani, & Fushimi, 2009; Shet, Patil, Hombalimath, Yaraguppi, & 106  
Udapudi, 2011; Stanevièienė et al., 2008; Yamaguchi & Fukagawa, 2005). Zn also acts as a 107  
catalytic cofactor for more than 300 enzymes (e.g., in carbonic anhydrase, superoxide 108  
dismutase, alkaline phosphatase, along with DNA and RNA polymerases) to maintain plasma 109  
membrane stability (Báscik-Remisiewicz, Tomaszewska, Labuda, & Tukaj, 2009; Molina- 110  
Poveda, 2016; Omar, 2002; Vallee & Falchuk, 1993). Additionally, different chemical forms 111  
and amounts of Zn can affect its absorption and utilization as a nutrient and an antioxidant or 112  
produce toxic effects (Lemire, Mailloux, & Appanna, 2008; Lin, Lin, Yang, Li, & Luo, 2013). 113  
The organic form of Zn has a higher bioavailability and is less toxic and more environmentally 114  
friendly compared to inorganic salts improving larval growth performance (Gharekhani, 115  
Takami, Tukmechi, Afsharnasab, & Agh, 2015; C. Wang & Lovell, 1997; Yang et al., 2012). 116  
Algae can absorb this inorganic form of Zn and incorporate it into proteins. 117

Using a direct enrichment method the rotifers are exposed to high concentrations of a nutrient that might be lethal for the rotifer and/or might reduce the dissolved oxygen levels. If rotifers are enriched several times with different nutrients, they might be susceptible to diseases and endanger the lives of their predators (fish larvae). Therefore, it is essential to establish first which is the best method for rotifer enrichment, being one of the most effective the previous enrichment of microalgae with the nutrients to be tested and then feed the rotifers with them (Dhert, King, & O'brien, 2014).

Zooplankton organisms can meet their nutritional requirement feeding on microalgae, however they are sometimes deficient in some nutrients. Thus, algal cells are able to bioaccumulate metals by controlling the uptake as well as the transfer and deposition of metals into the cell (Yang et al., 2012) and several publications have shown how microalgae can be enriched with minerals such as Zn and Se separately (López-Suárez et al., 2000; Araie & Shiraiwa, 2009; Neumann et al., 2003; Matsumoto et al., 2009; Gómez-Jacinto, García-Barrera, Garbayo, Vílchez, & Gómez-Ariza, 2012; Li, Guo, & Li, 2003; X. Sun, Zhong, Huang, & Yang, 2014; Esmaeili, 2015; Gojkovic et al., 2015; Zhang, Zeng, Ren, Mao, & Qiao, 2017).

In this study, *Isochrysis aff. galbana* (T-ISO) and *Nannochloropsis oculata* were selected, due to its high level of docosahexaenoic acids (DHA) and eicosapentaenoic acids (EPA), for their enrichment with Se and Zn. Thus, after enrichment these microalgae can simultaneously supply high levels of organic minerals and  $\omega$ 3 fatty acids. Zn and Se have antioxidant properties and their use for algae and rotifer enrichment might also help to maintain fatty acid content and avoid oxidation. According to Matsumoto et al. (2009) mineral uptake is faster in the case of microalgae than in rotifers, being even more efficient if the minerals are offered bound to digestible particles than when it is used in a dissolved form (Nordgreen et al., 2013; S. Penglase, Hamre, Sweetman, & Nordgreen, 2011).

Increasing the nutritional quality of microalgae is more important than producing high quantities of biomass because they tailor the biochemical composition of rotifers (Ferreira, Cortina-Burgueño, Freire, & Otero, 2018). So far, no previous studies have focused on feeding rotifers with T-ISO and *Nannochloropsis oculata* co-fortified with Se and Zn in aquaculture. The main objectives of long-term rotifer enrichment with these mineral-rich algae were not only the intestinal filling but also the gradual enrichment of the rotifers without excessive oxygen consumption or endangering the health and reproduction of the rotifers. Therefore, the main purpose of this study was to assess the effect of rotifer enrichment with Zn and Se on population growth, egg ratio, and mineral composition.

## **2. Materials and methods**

### ***2.1. Origin of materials***

In this study, two microalgae, *Isochrysis aff. galbana* (T-ISO) and *Nannochloropsis oculata* and rotifer *Brachionus plicatilis* (adult lorica length = 185  $\mu\text{m}$ ) were obtained from the shrimp research institute of Bushehr, Iran. The seawater with a salinity of 20  $\text{g L}^{-1}$  was autoclaved (120 °C for 20 minutes) for microalgae and rotifer culture. Finally, zinc sulfate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) and sodium selenite ( $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ ) provided by Sigma-Aldrich was used for microalgae enrichment.

### ***2.2. Microalgae culture***

*Isochrysis aff. galbana* (T-ISO) and *Nannochloropsis oculata* were cultured in 10 L flasks at  $26 \pm 1$  °C and 24 h of light photoperiod using f/2 Guillard medium (Guillard, 1975). Once the microalgae got the exponential phase they were harvested and concentrated using a high volume centrifuge (Sigma model) at 4000 rpm for 5 minutes.



### 2.3. Mineral absorption by microalgae

The concentrated microalgae (i.e., *I. galbana* and *N. oculata*) were then re-suspended in 20 ppt saline water at  $18 \times 10^9$  cell mL<sup>-1</sup> and then enriched in three separate experiments based on the protocol of Matsumoto et al. (2009) with few modifications. These experiments were designed to analyse the effects of single and combined addition of (Se) and zinc (Zn) on the enrichment of mixed microalgae.

In experiments, 1 and 2, three different levels (4000, 8000, and 12000 mg L<sup>-1</sup>) of zinc (ZnSO<sub>4</sub>.7H<sub>2</sub>O) and selenium (Na<sub>2</sub>SeO<sub>3</sub>.5H<sub>2</sub>O) were used. One mL of each stock solution was poured into 100 mL of enrichment vessels containing  $18 \times 10^9$  cell mL<sup>-1</sup> of *I. galbana* and *N. oculata*. The vessel was then kept at 25 °C with continuous aeration, and the enrichment carried out in triplicates. The resultant solutions were designated as 40, 80 and 120 mg L<sup>-1</sup> Zn (in experiment 1) and 40, 80 and 120 mg L<sup>-1</sup> Se (in experiment 2). Algae with no mineral solution added were considered as control (0 mg Zn or Se).

In experiment 3, the combined effects of zinc and selenium were studied and three different levels (2000, 4000, and 8000 mg L<sup>-1</sup>) of both minerals were prepared separately, followed by pouring 1 mL of each stock solution in 100 mL vessels containing  $18 \times 10^9$  cell mL<sup>-1</sup> of the mixed microalgae. The enrichment was performed as in the first and second experiments at 25 °C in triplicates and the resultant solutions designated as 20, 40 and 80 mg L<sup>-1</sup> of both Zn and Se.

In each experiment, two groups of enrichment containers were prepared and after 1 and 3 h, the whole material was centrifuged at 4000 rpm for 5 minutes to remove the remaining soluble mineral in the medium. Then, the concentrated, enriched microalgae were washed twice with 20 ppt sterile saltwater and centrifuged at 4000 rpm for 5 minutes, followed by storing the

materials at -20 °C for future analysis. The effects of the enriched microalgae on rotifers was investigated once the best time for microalgae enrichment was established. For this second step (rotifer enrichment), the same processes for microalgae enrichment were conducted daily and the concentrated mineral-enriched microalgae was re-suspended in sterile saline water for feeding the rotifers.

### 2.3. Rotifer culture

Rotifer culture was carried out in Urmia University wet lab using 7 L cylindrical glass containers with 24 h light and 1000 lux. Continuous aeration was used to avoid the accumulation of rotifers in the bottom and to produce a slow water movement in the upper part of the containers. The temperature was kept at  $28 \pm 0.5$  °C, dissolved oxygen at  $> 5$  mg L<sup>-1</sup>, pH at ~ 8.3, and salinity at 20 g L<sup>-1</sup> with an initial density of 40-50 rotifers mL<sup>-1</sup>. The rotifers were cultured in a batch system with no water exchange during 4-day culture cycles. The 3 separate experiments were carried out as mentioned earlier in the microalgae enrichment section, and the rotifers were fed in triplicates, twice a day, using a mixture of *N. oculata* and *I. galbana* (1:1 with  $3 \times 10^6$  cell mL<sup>-1</sup>) enriched microalgae.

### 2.4. Mineral composition analysis (Analytical methods)

The frozen samples of microalgae and rotifers were dried in the oven at 40 °C for 24 h. Thereafter, the dried sample was placed in a 10 mL Pyrex for digestion. Then, 9 mL of 65% HNO<sub>3</sub> (three times using a 10-minute interval) was added to 125 µL of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to digest the biomass completely. The digestion was carried out in a water bath at 80 °C for 30 minutes. The concentration of Zn, Se, copper (Cu), and manganese (Mn) was measured

in the digested samples using a novAA® 400 PAtomic Absorption (Analytic Jena, Germany) 213  
by the method of Lowry and Lopez (1946). 214

215

### **2.5. Growth parameters of rotifer** 216

Three samples of 3 mL from each conical container were used to count the number of rotifers 217  
and total eggs using the Bogorov Counting Chamber. Then, the specific growth rate and the 218  
doubling time were calculated using the following formulae: 219

Specific growth rate (Abbasi, Ahmadifard, & Tukmechi, 2019; Krebs, 1995): 220

$$SGR = \frac{\ln N_t - \ln N_0}{t} \quad 221$$

where  $N_0$  and  $N_t$  are the initial and final population of rotifers and (t) stands for the experiment 222  
period (days). The SGR value was calculated in the exponential phase of the population. 223

Doubling time (Vallejo, Newmark, & Mercedes Criales, 1993): 224

$$(DT) = \frac{\ln 2}{SGR} \quad 225$$

At the end of the feeding period, all the rotifers were filtered through a 50 µm mesh, rinsed 226  
with tap water, and transferred to microtubes and the samples stored at -20 °C until analysis. 227

### **2.6. Statistical analysis** 228

The data were statistically analyzed by SPSS, version 21 using Levene's and Shapiro-Wilk 229  
tests to check the homogeneity of variances and normality, respectively ( $p < 0.05$ ). The means 230  
of treatments were compared by the analysis of variance (ANOVA), followed by performing 231  
Tukey-Kramer HSD for post-hoc multiple comparisons. Differences among the means were 232  
considered significant at  $p < 0.05$  and the data were displayed as the means of three replicates 233  
± standard deviations (SD). 234

### **2.7. Ethics Statement:** 235

No ethical approval was required for this study, as no specific permission is needed for rotifer studies in Iran.	236 237 238
<b>3. Results</b>	239
<b>3.1. Mineral composition of algae</b>	240
<i>3.1.1. Zn enrichment</i>	241
Zinc (Zn) content in the microalgae increased as a function of Zn concentration in the medium for both 1 and 3 h with the exception of 120 mg/L Zn that showed a decrease in microalgae content. A significantly (ANOVA, $P < 0.05$ ) higher Zn content ( $690.97 \pm 11.16 \mu\text{g g}^{-1}$ DW) was found in the microalgae enriched with 80 mg/L Zn for 1 h. This treatment (80 mg/L Zn) was found to be more cost-effective and needed less enrichment time, being selected as the best treatment for experiment 3 (Fig. 1). Other minerals were also analysed in 1h enriched microalgae and copper (Cu) was found to be in significantly decreasing amounts in 120 mg/L Zn-enriched algae, and in all the 3h enriched microalgae Cu content was lower than the control group (Table 1). Additionally, manganese (Mn) content was lower in all the treatments with the highest Mn amount found in 80 mg/L Zn-enriched algae and the control for both 1 and 3 h enrichment time (Table 1).	242 243 244 245 246 247 248 249 250 251 252
<i>3.1.2. Se enrichment</i>	254
The highest selenium (Se) content was found in 1-h enriched microalgae and, as in the case of Zn, its content increased in the microalgae in parallel to the concentration in the medium. Maximum ( $544.84 \pm 6.62 \mu\text{g g}^{-1}$ DW) and minimum ( $153.23 \pm 9.03 \mu\text{g g}^{-1}$ DW) Se content were observed in microalgae after 1 h enrichment with 120 mg/L and in the control group, respectively ( $p < 0.05$ ). In the 3h enriched microalgae the highest Se content was found for	255 256 257 258 259

120 mg/L Se-enriched group, being the content 3 times lower than that obtained enriching the microalgae with 120 mg/L Se for only 1 h. The mixed microalgae was a bit flocculated when it was enriched with 120 mg/L Se. Thus, 80 mg/L Se treatment was selected for designing the next experiment, especially considering that Se content ( $403.99 \pm 4.36 \mu\text{g g}^{-1}$  DW) in this treatment was optimum (Fig. 2). No differences were found in Cu content among the Se-enriched groups for 1 h being the highest amounts of Cu those found in 3h enriched control group (Table 1). Finally, Mn content of Se-enriched algae decreased by increasing the concentration of Se in enrichment vessels with the control group showing the highest Mn content for both 1 and 3 h enrichment time (Table 1).

### 3.1.3. Mixed enrichment (Zn + Se)

In the trial using both Zn and Se together for microalgae enrichment Zn content was found to increase in algae as a function of the Zn concentration in the medium for 1 h. The maximum content of Zn ( $518.11 \pm 3.26 \mu\text{g g}^{-1}$  DW) when the enrichment was carried out for 3 h was obtained in 40 mg/L (Zn + Se)-enriched algae being significantly higher than the amounts found in 80 mg/L (Zn + Se)-enriched microalgae ( $p < 0.05$ , Fig 1). In the case of Se, the content was always increased by increasing Se concentration in the medium, for both enrichment times 1 and 3h ( $p < 0.05$ , Fig 2). Fig. 1 and 2 clearly show that the maximum amounts of Zn ( $638.91 \pm 4.96 \mu\text{g g}^{-1}$  DW) and Se ( $1275 \pm 25 \mu\text{g g}^{-1}$  DW) were found in the 1h, 80 mg/L (Zn + Se)-enriched microalgae ( $p < 0.05$ ). Regarding Cu content, no significant differences were found among the groups enriched for 1 h being the highest content found in the control group enriched for 3-h (Table 1). Mn highest content was observed in the algae enriched with 80 mg/L (Zn + Se) for 1 h although in general Mn content in enriched algae decreased significantly by increasing the concentration of minerals in the media (Table 1).

## 3.2. Mineral composition of rotifer 285

According to the results presented in the previous sections, the maximum amounts of Zn and 286  
Se were obtained in all the 1h enriched algae, being these treatments selected for the enrichment 287  
of rotifers carried out during 4 days. 288

### 3.2.1. Zn enrichment 289

Although the mixed microalgae were a bit flocculated when it was enriched with 120 mg/L 291  
Zn, the rotifers fed with them showed the maximum Zn ( $110.45 \pm 1.92 \mu\text{g g}^{-1}$  DW) and Cu 292  
( $20.12 \pm 0.23 \mu\text{g g}^{-1}$  DW) content. The minimum amount of Mn ( $5.53 \pm 0.38 \mu\text{g g}^{-1}$  DW) and 293  
Cu ( $14.92 \pm 0.10 \mu\text{g g}^{-1}$  DW) were observed in the rotifer fed 80 mg/L Zn-enriched algae (Table 294  
3). 295

Mn content ( $14.13 \pm 0.31 \mu\text{g g}^{-1}$  DW) was significantly higher in the rotifer fed 40 mg/L 296  
Zn-enriched microalgae ( $p < 0.05$ ). No significant differences were observed (Table 3) 297  
between the groups fed 120- and 40 mg/L -Zn enriched algae in terms of Cu content ( $20.12 \pm$  298  
 $0.23$  and  $19.04 \pm 0.10 \mu\text{g g}^{-1}$  DW, respectively) having the rotifers fed Zn-enriched algae a 299  
significantly higher Cu content than the untreated group ( $p < 0.05$ ) 300

### 3.2.2. Se enrichment 301

Se content in the rotifer fed with Se-enriched microalgae increased significantly by increasing 303  
the Se concentration ( $p < 0.05$ ). No significant difference was found between the groups fed 304  
80 mg/L and 120 mg/L Se-enriched algae. Mn content ( $12.58 \pm 0.19 \mu\text{g g}^{-1}$  DW) was found to 305  
be significantly higher in the control and 40 mg/L Se groups ( $p < 0.05$ ) whereas Cu content 306  
was found to increase in the rotifers in parallel to the increase in Se concentration ( $p < 0.05$ ). 307

No significant differences were found in Cu content between 80 mg/L and 120 mg/L Se-enriched groups (Table 3).

### 3.2.3. Mixed enrichment (Zn + Se)

In the rotifers fed with (Zn + Se)-enriched microalgae, the content of Zn in the rotifer increased as Zn increased in the diet. The highest amount of Zn ( $96.89 \pm 1.36 \mu\text{g g}^{-1}$  DW) was observed in the rotifer fed mg/L (Zn + Se)-enriched algae ( $p < 0.05$ ). Se significantly decreased in the rotifer fed 40 mg/L (Zn + Se)-enriched algae compared to those fed 20 mg/L (Zn + Se)-enriched algae ( $p < 0.05$ ), whereas the highest Se content ( $169.10 \pm 4.83 \mu\text{g g}^{-1}$  DW) was detected in the groups fed 80 mg/L (Zn + Se)-enriched algae ( $p < 0.05$ ). On the other hand, the highest level of Mn and Cu was found in 20 mg/L (Zn + Se) group ( $p < 0.05$ ) while the minimum amount of Cu was obtained in the control group ( $p < 0.05$ ) (Table 3).

## 3.3. Rotifer growth

### 3.3.1. Zn enrichment

Rotifer population increased at a higher rate during the whole trial (4 days) in the control group (Fig. 4). The groups fed 40 mg/L Zn-enriched microalgae showed a similar growth, without any significant difference with the control group. No significant difference was also detected between 80 mg/L Zn fed group and the control groups on days 2 and 4, although there was a significant difference between them on day 3 ( $p < 0.05$ ). No significant difference was found in  $N_{\text{max}}$ , specific growth rate (SGR), and doubling time (DT) among treatments (Table 2) with the highest SGR ( $0.47 \pm 0.034 \text{ day}^{-1}$ ) registered in the control group. Finally, the lowest population growth was observed during the whole duration of the trial in the rotifer fed 120 mg/L Zn-enriched microalgae ( $p < 0.05$ ).

332

3.3.2. *Se enrichment* 333

No significant difference among the treatments was detected in population growth at day 4 334  
(Fig. 4,  $p < 0.05$ ) although lower population growth than the control group was detected in 335  
rotifers fed Se-enriched microalgae in the days 1-3 ( $p < 0.05$ ). The highest population growth 336  
( $349 \pm 45.55$  ind mL<sup>-1</sup>) and N<sub>max</sub> were recorded in rotifers fed 80 mg/L Se-enriched algae on 337  
the fourth day (Fig. 4, Table 2) also showing the highest, although no statistically significant, 338  
SGR and the lowest DT among the treatments (Table 2). The rotifers fed 40 mg/L and 120 mg/L 339  
Se-enriched microalgae showed a lower population growth compared to control and 80 mg/L 340  
Se groups at day 4 ( $p < 0.05$ ). 341

342

3.3.3. *Mixed enrichment (Zn + Se)* 343

No significant differences among the groups fed (Zn + Se)-enriched algae were detected in all 344  
days compared to the control group (Fig. 4). The highest N<sub>max</sub> ( $339 \pm 11.06$  ind mL<sup>-1</sup>) and SGR 345  
( $0.48 \pm 0.008$  day<sup>-1</sup>) and the lowest DT were observed in 40 mg/L (Zn + Se) treatment (Table 346  
2). 347

348

**4. Discussion** 349

**4.1. Mineral composition of algae** 350

Microalgae are an example of microorganisms with a relatively high metal-binding capacities 351  
that arise from the intrinsic composition of their cell walls, which contain negatively charged 352  
functional groups. Biosorption of metals by microorganisms is a process that typically proceeds 353  
through a two-stage pathway: (i) an initial rapid, reversible, and passive adsorption onto the 354  
cell surface (where metal ions adsorb via electrostatic interactions to cell wall functional 355



groups), followed by (ii) a much slower, irreversible, active process, involving transport of metal cations across the cell membrane into the cytoplasm, with posterior binding to intracellular compounds (Yang et al., 2012). Understanding this metal bioaccumulation ability of mixed microalgae and using them as feed for rotifers is of high interest, and the present study was designed, not only to study the rotifer enrichment with minerals but also to establish the effects of increasing zinc and selenium (individually and together) levels in the microalgae before used them for rotifer enrichment. Most studies published until now have been focused on studying the mineral content in rotifers fed with enriched yeast, rotifer diets, or indirectly by the addition of minerals (Hamre, 2016; Kim, Nakamura, & Hagiwara, 2014; Matsumoto et al., 2009; Nordgreen et al., 2013; S. Penglase et al., 2011; Samuel Penglase et al., 2013; Srivastava, Hamre, Stoss, & Nordgreen, 2012; Srivastava et al., 2011) without considering that green microalgae can accumulate metals obtained from culture media even under high concentrations (Sandau, Sandau, & Pulz, 1996; Wilde & Benemann, 1993).

Aquatic organisms cannot absorb Zinc (Zn) and selenium (Se) since these minerals are water-soluble and thus unavailable. Matsumoto et al. (2009) indicated that the rotifer is unable to absorb and retain an optimum level of Zn in the body. Thus, in the present study, two algal species were enriched with minerals as a first step before the rotifers were fed with this enriched microalgae. In the case of *Chlorella* two methods have been designed for microalgae enrichment with minerals (López-Suárez et al., 2000; Matsumoto et al., 2009) and used in the present study with some modifications. According Matsumoto et al. (2009) and Moreno-Garrido, Codd, Gadd, and Lubián (2002) the accumulation of metals by microalgae is carried out in 2 steps. In the first and fast step the metals are adsorbed on the cell surface whereas in the second and slower step an intracellular transport is carried out. In the present study, the mixed microalgae (*N. oculata* and *I. galbana* 1:1) were enriched by binding the metal ions on their cell surface prior to be used as feed for rotifers. Based on the unknown interactions

between individual metals, the composition of several metals may have variable effects on the 381  
organism, which differs from those caused by exposure to single metals (Norwood, Borgmann, 382  
Dixon, & Wallace, 2003; Ríos-Arana, Walsh, & Ortiz, 2007). Therefore, it is necessary to re- 383  
evaluate the effects of a single metal on microalgae and rotifers when other metals are present. 384

In experiment 1, the highest content of Zn in enriched mixed microalgae was observed using 385  
80 mg/L Zn for 1 h (Fig. 1), that was 7-fold lower than the highest Zn content (almost 5000  $\mu\text{g}$  386  
 $\text{g}^{-1}$  DW) published by Matsumoto et al. (2009). The differences in Zn bioaccumulation might 387  
be due to the species used in the studies, microalgae size, and/or the enrichment method used. 388  
In our study microalgae cells tended to agglomerate when they were exposed to 120 mg/L Zn, 389  
indicating a stronger stressful effect of Zn on microalgae cells during 1 h of exposure 390  
preventing or even impeding the adsorption of metal ions. This is in line with the findings by 391  
Esmaeli (2015) studying the effects of Zn on *C. vulgaris* when treated with 30 mM of  $\text{ZnCl}_2$  392  
for 72 h. According to our data, mixed microalgae can efficiently accumulate Zn by adding 80 393  
mg/L Zn to the medium for only 1h. Zinc content of enriched microalgae at 3 hours was lower 394  
than at 1 hour probably due to the high amounts of Zn accumulated on the cell surface together 395  
with a slower Zn absorption and accumulation into the microalgae cells. Furthermore, 396  
according to our results, increasing the enrichment time did not contribute to a higher metal 397  
accumulation in microalgae cells, they seem to reject some of the adsorbed metal ions, or their 398  
binding is so weak that they are easily separated. Similar results were obtained by Matsumoto 399  
et al. (2009) comparing *Chlorella* enriched with Zn for 1 and 3 hours. Thus, algal short-time 400  
(1 h) enrichment with 80 mg/L Zn might be considered good enough to increase Zn content in 401  
microalgae, which was 6.9 times more than the untreated algae. 402

Most of the studies in Se accumulation in microalgae have been focused in the long-term 403  
enrichment process (Araie & Shiraiwa, 2009; Gojkovic et al., 2015; Gómez-Jacinto, García- 404  
Barrera, Garbayo, Vílchez, & Gómez-Ariza, 2012; Li, Guo, & Li, 2003; X. Sun, Zhong, Huang, 405

& Yang, 2014; Zhang, Zeng, Ren, Mao, & Qiao, 2017), and no study is available based on the short-term. According to these studies, the effect of Se relies on the concentration of Se, algal species, and the source of the metal. The results of the present study show that Se content in mixed microalgae increased sharply within 1-h enrichment, independently of the concentration used, probably due to the high surface uptake of in the first minutes when the metal is irreversibly fixed onto the cell surface (De Alcantara, Lopes, & Wagener, 1998). Se can be attached to algal cell very fast, but its absorption and accumulation inside the cells is very slowly or inefficient, having in mind the results showing that Se content in the microalgae was lower after 3 h enrichment than after only 1h. According to Neumann et al (2003) Se can be transported into the green alga cells as anionic macronutrients via a transport system with a slight affinity. The results of the present study show that although the uptake of Se by the mixed microalgae increased significantly using the highest Se concentration, the intracellular content was lower than the Zn amount in Zn-enriched microalgae (Experiment 1). This indicates that microalgae adsorption is higher and faster for Zn than for Se and the behavior of microalgae towards different ions should be explored in future studies. In addition, our findings revealed that Zn had stronger stress effects on microalgal cells due to the formation of more agglomerations compared to Se (see the results obtained using 120 mg/L Zn).

It should be noted that no study has evaluated microalgae enrichment using simultaneously Zn and Se. The results obtained in experiment 3 clearly demonstrate that Zn content of mixed microalgae was almost equal to that obtained when microalgae was enriched with the mineral alone (experiment 1) using the same enrichment time. The repeatability of these findings demonstrates that this enrichment method is effective, and the presence of Se in the culture medium has no negative effect on Zn adsorption. Fig. 1 shows that the simultaneous use of Zn and Se had a significant effect on Se uptake. The bioaccumulation of selenium after 1 and 3 h enrichment with 40 mg/L (Zn + Se) was 2.4 and 4.2 times, and in the case of 80 mg/L (Zn +

Se) was 3.5 and 4.7 times, higher than the amounts obtained with 40 mg/L and 80 mg/L Se in 431  
experiment 2. However, the maximum content of both metals was observed in 80 mg/L (Zn + 432  
Se)-enriched algae ( $p < 0.05$ ). Hence, the presence of Zn in the algae enrichment medium had 433  
a synergistic effect on Se uptake while the content of Zn in the algae did not decrease 434  
significantly. The efficiency of Se and Zn bio-adsorption in the combined enrichment of mixed 435  
microalgae was Se > Zn. Some studies demonstrated that the bioavailability of metals is 436  
affected by free ion activity compared to its concentration (Campbell, Errécalde, Fortin, 437  
Hiriart-Baer, & Vigneault, 2002; Tessier & Turner, 1995) and it might be the case that Zn can 438  
increase the activity of free Se ions. Furthermore, our findings are in accordance with those 439  
concerning the cell wall acting as a controlled barrier for the absorption of metal ions into algal 440  
cells (Esmaeili, 2015). However, more studies are needed to understand the pathways and 441  
mechanisms of metal ions transport across the cell membrane (simple diffusion or protein- 442  
mediated) and the internalization in microalgal cells. 443

444

#### **4.2. Mineral composition and growth factors of rotifer**

445

The highest amount of Zn was obtained in the rotifers fed 120 mg/L Zn-enriched microalgae 446  
that unfortunately induced a significant reduction in rotifer growth compared to the other 447  
treatments. Thus, this treatment cannot be recommended for larval feeding. Our findings are in 448  
line with those by Nordgreen et al. (2013) showing that Zn content in the rotifers fed with 449  
fortified microalgae had a non-linear response and might be the reason why Zn content 450  
decreased in the rotifers fed 80 mg/L Zn-enriched microalgae. In our study, the highest amount 451  
of Zn in rotifers was 6 to 7-fold lower than the amounts published by Matsumoto et al. (2009) 452  
and Nematzadeh et al. (2018) that might be due to the different microalgae species, or the live 453  
food (yeast) used as well as the rotifer enrichment time. 454

According to the reports of Matsumoto et al. (2009), the content of manganese (Mn) in the rotifer decreased as Zn levels increased in the microalgae, which contradict our findings. Likewise, Nguyen et al. (2008) reported a decrease in Mn in *Artemia* nauplii enriched with Zn and Mn. On the other hand, an increase in Zn and copper (Cu) content found in the rotifers may reflect a synergistic interaction, which is in contrast with the findings of the above-mentioned studies. Thus, further investigations are required to explain these findings.

The highest amount of total eggs was obtained when rotifers were fed 40 mg/L Zn-enriched algae on day 3 whereas the rotifer fed 120 mg/L Zn-enriched algae showed the lowest total eggs. Therefore, enrichment with Zn can affect egg production in rotifers, being 40 mg/L Zn the optimum level needed in the enriched microalgae. No significant differences in rotifer growth from day 3 to 4, were obtained between this group and the control. Although Zn content in the enriched rotifers was lower than in the copepods, the bioavailability of Zn from the enriched microalgae is much higher than the direct enrichment (Nordgreen et al., 2013). Hamre et al. (2008) mentioned that the level of micronutrients and biological availability are important for assessing the nutritional value of different feeds of marine larvae. Therefore, 40 mg/L of Zn might be the best treatment in terms of Zn, Cu, Mn, K, and sodium (Na), as well as for rotifer growth. Based on the findings of Penglase et al. (2013), 40 mg/L of Zn fulfil the early larval cod requirements for Mn and Cu (8 mg Mn and 5 mg Cu kg<sup>-1</sup> DM).

According to previous studies (Hamre, Mollan, Sæle, & Erstad, 2008; NRC, 2011), Se content in the rotifers fed Se-enriched microalgae increased in parallel to the concentrations used in the microalga enriching medium being considerably higher than both the fish requirements and the copepod levels (0.25-0.3 and 3-5 µg. g<sup>-1</sup>, respectively). The synergistic interactions among vitamins and antioxidants present in Se-enriched microalgae might be the reason for the higher Se bio-accessibility for the rotifer (Oraby, Allababidy, & Ramadan, 2015). Mn level in the rotifers was significantly affected by the Se added to microalgae, leading to a

decrease in Mn compared to the control group. The bioavailability of minerals from the diet is 480  
affected by multiple factors such as the amount and form of the nutrient, particle size and 481  
digestibility of the food, as well as nutrient interaction, which might be either synergistic or 482  
antagonistic (Watanabe et al., 1997). Thus, the increase in Se and the decrease in Mn content 483  
in the rotifers might be due to antagonistic interactions to maintain mineral homeostasis. On 484  
the contrary, increasing Se in the rotifers, like the increase in Zn, resulted in an increased Cu 485  
uptake in the rotifer, which was an ascending function. Therefore, rotifer enrichment with Na 486  
selenite had a synergistic effect on the content of Cu. The K content was similar in all the rotifer 487  
treatments and not significantly different from the control. Additionally, there were no 488  
significant differences among the groups regarding rotifer growth, which is in line with the 489  
findings obtained on enriched rotifer by Se-yeast and -fortified *Chlorella vulgaris* (Kim et al., 490  
2014; S. Penglase et al., 2010). Rotifers fed 80 mg/L Se-enriched microalgae showed the 491  
highest, although no statistically significant, SGR, total egg production and  $N_{max}$  and the lowest 492  
doubling time. No significant difference was observed in rotifer growth between this group and 493  
the control group for the whole culture period (4 days). Thus, the enrichment of rotifer with Se 494  
had no negative effect on egg production and a small increase in rotifer growth parameters 495  
were found when the rotifer was enriched with 80 mg/L selenium. 496

The results of the combined Zn sulfate and Na selenite enrichment show no significant 497  
differences in rotifer growth parameters and total egg production. In this experiment, rotifer 498  
Se content increased more than that found in copepods (15-169 and 3-5  $\mu\text{g. g}^{-1}$  in rotifers and 499  
copepods, respectively) and it was even higher than that found in rotifers fed yeast, Culture 500  
Selco 3000, and AlgaMac 2000 (Hamre, Srivastava, et al., 2008). However, Zn content (48-96 501  
 $\mu\text{g. g}^{-1}$ ) of rotifers fed (Zn + Se)-enriched algae was below the copepod levels (340-570  $\mu\text{g. g}^{-1}$  502  
<sup>1</sup>) and above the fish requirement values of 20-30  $\mu\text{g/g}$  (NRC, 2011). According to our results 503  
rotifers cannot be enriched with higher amounts of Zn due to the stress effects observed both 504

in microalgae and rotifers. Thus, a study is needed to assess the effects of the Zn+Se enriched rotifers enriched on fish larvae to find if they meet their needs because of its high bioavailability.

Although Cu and Mn sources were not manipulated in this study, some changes were observed in their values. According to Ezoe et al (2002) Cu is mainly found in rotifer sexual organs playing an important role in reproduction. No significant difference in Cu content was detected in enriched microalgae but significant differences were observed among rotifer treatments. Thus, the interaction among Se, Zn, and Cu led to an increase in the Cu levels in the rotifers covering the Cu requirement of larvae (3-5  $\mu\text{g. g}^{-1}$ , NRC, 2011). Several studies have evaluated the rotifer deficiency on Cu using different enrichments (Hamre, Srivastava, et al., 2008; Samuel Penglase et al., 2013; S. Sun, Chen, Ge, & Qin, 2013). For instance, Hamre et al. (2008) found no increase in Cu content in rotifers enriched with Culture Selco 3000 with a high level of Cu ( $8.1 \pm 2.2$ ). Taking all these results together there is no need to enrich the rotifers with Cu if it is already enriched with Zn and Se.

Mn rotifer content decreased significantly in all the treatments except for 20 mg/L (Zn + Se) group, in agreement with the results obtained by Hamre et al (2008) and Matsumoto et al (2009). Nordgreen et al. (2013) concluded that rotifers fed with microalgae had enough Mn, within the range found in copepods (8-25  $\mu\text{g. g}^{-1}$ ), and the enrichment with other minerals, especially Zn and Se, may lead to its excretion or consumption. The same authors (Nordgreen et al., 2013) also found that Mn levels had a 3-fold increase in the rotifer that was enriched with Cu, Mn, and iodine (Nordgreen et al., 2013). Given that the Mn content failed to increase despite the enrichment with Se and Zn, it was preferred not to enrich the rotifer with Mn, as previously observed for copper enrichment (Nordgreen et al., 2013).

The effects of multi-mineral mixtures on rotifer growth and reproduction are very little known. Xu et al (2015) in a study carried out *B. calyciflorus* found that the reproductive

parameters such as the population growth and the reproductive rate were influenced by a multi- 530  
metal mixture (i.e., Cu, Zn, Cd, Cr, and Mn). In the present study no significant differences in 531  
population growth could be found among the treatments although some differences in egg 532  
production were found by mixing Zn and Se. In another study, Norwood et al. (2003) examined 533  
the toxicity of these metal mixtures and concluded that there were no strict rules and the results 534  
appear to be inconsistent. According to their report, the egg production of rotifers was lower 535  
using 20 mg/L (Zn + Se) than the control whereas the egg production of 40 mg/L (Zn + Se) 536  
rotifers was higher than the control. The mechanisms of uptake, storage, and excretion of 537  
minerals vary in rotifers, but the reason is unclear (Nordgreen et al., 2013). 538

539

## 5. Conclusion 540

The use of selenium (Se) and zinc (Zn) mixture in the medium increases their uptake by the 541  
microalgae, and contribute to their uptake by the next trophic level, the rotifers. It should be 542  
noted that three elements: Zn, Se, and copper (Cu), had a similar increasing accumulation 543  
pattern whereas manganese (Mn) showed an opposite pattern. Thus, the enrichment with these 544  
3 elements contributes to a reduction in Mn levels. Rotifer content in Cu, Zn, and Se can be 545  
manipulated very easily, but the Mn accumulated in the microalgae was very little retained by 546  
the rotifers when these minerals are present in high amounts (Srivastava et al, 2012). Thus, 547  
enrichment techniques should be improved in the future if hatcheries managers want to obtain 548  
rotifers with adequate amounts of all the nutrients, including minerals. 549

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	554
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Ghaderpour S., Ahmadifard N., and Vahabzadeh, Z. designed the study. Ghaderpour S. and	556
Ahmadifard N., participate in curation and analysis of data, and drafting the manuscript. Agh	557
N. supported the determination of mineral analysis. Vahabzadeh, Z. helped in Methods of	558
instrumental analysis. All authors read and approved the final manuscript.	559
	560
<b>Data availability statement:</b>	561
The authors confirm that the data supporting the results in the paper are included in the tables	562
and figures in the paper and research data are not shared.	563
	564
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**Table 1.** Changes of Cu and Mn in enriched microalgae with different concentrations of Zinc Sulfate (experiment 1), Selenium Selenite (experiment 2), and a combination of the effect of Zinc Sulfate and Selenium Selenite (experiment 3) in two times 1 and 3 h (mean  $\pm$  SD; n = 3).

Experimental Groups		Cu ( $\mu\text{g g}^{-1}$ )		Mn ( $\mu\text{g g}^{-1}$ )	
		1 h	3 h	1 h	3 h
Experiment 1	0 mg/L Zn	31.02 $\pm$ 0.47 <sup>a</sup>	32.05 $\pm$ 0.39 <sup>a</sup>	24.04 $\pm$ 0.73 <sup>a</sup>	26.78 $\pm$ 1.83 <sup>a</sup>
	40 mg/L Zn	31.78 $\pm$ 0.36 <sup>a</sup>	24.85 $\pm$ 0.17 <sup>b</sup>	0.94 $\pm$ 0.34 <sup>c</sup>	8.87 $\pm$ 3.17 <sup>d</sup>
	80 mg/L Zn	31.00 $\pm$ 0.40 <sup>a</sup>	25.95 $\pm$ 0.06 <sup>b</sup>	3.61 $\pm$ 0.23 <sup>b</sup>	14.37 $\pm$ 0.14 <sup>b</sup>
	120 mg/L Zn	25.27 $\pm$ 0.70 <sup>b</sup>	25.64 $\pm$ 0.08 <sup>b</sup>	0.14 $\pm$ 0.01 <sup>c</sup>	11.90 $\pm$ 0.69 <sup>c</sup>
Experiment 2	0.0 mg/L Se	31.02 $\pm$ 0.47	32.05 $\pm$ 0.39 <sup>a</sup>	24.04 $\pm$ 0.73 <sup>a</sup>	26.78 $\pm$ 1.83 <sup>a</sup>
	40 mg/L Se	31.31 $\pm$ 0.56	26.25 $\pm$ 0.10 <sup>b</sup>	18.52 $\pm$ 0.63 <sup>b</sup>	17.32 $\pm$ 2.77 <sup>b</sup>
	80 mg/L Se	32.63 $\pm$ 0.46	25.61 $\pm$ 0.13 <sup>b</sup>	12.50 $\pm$ 0.41 <sup>c</sup>	10.69 $\pm$ 2.85 <sup>c</sup>
	120 mg/L Se	32.82 $\pm$ 0.67	25.98 $\pm$ 0.10 <sup>b</sup>	1.95 $\pm$ 1.56 <sup>d</sup>	18.67 $\pm$ 0.42 <sup>b</sup>
Experiment 3	0 mg/L Zn + 0 mg/L Se	31.02 $\pm$ 0.47 <sup>a</sup>	32.05 $\pm$ 0.39 <sup>a</sup>	24.04 $\pm$ 0.73 <sup>b</sup>	26.78 $\pm$ 1.83 <sup>a</sup>
	20 mg/L Zn + 20 mg/L Se	32.32 $\pm$ 0.42 <sup>a</sup>	25.95 $\pm$ 0.10 <sup>b</sup>	23.74 $\pm$ 1.27 <sup>b</sup>	16.24 $\pm$ 0.10 <sup>b</sup>
	40 mg/L Zn + 40 mg/L Se	31.35 $\pm$ 0.08 <sup>a</sup>	27.06 $\pm$ 0.23 <sup>b</sup>	21.39 $\pm$ 0.34 <sup>b</sup>	13.80 $\pm$ 0.23 <sup>c</sup>
	80 mg/L Zn + 80 mg/L Se	29.39 $\pm$ 0.45 <sup>a</sup>	26.96 $\pm$ 0.15 <sup>b</sup>	27.58 $\pm$ 0.57 <sup>a</sup>	13.95 $\pm$ 0.15 <sup>c</sup>

*Note.* Cu: Copper; Mn: Manganese; Zn: Zinc; Se: Selenium. Different letters on the each column indicate significant differences by Tukey's test ( $P < 0.05$ )

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**Table 2.** Specific Growth rate (SGR), maximum rotifer density ( $N_{max}$ ), and doubling time (DT) in rotifers fed short-term enriched microalgae for 4 days (mean  $\pm$  SD; n = 3).

Experimental Groups		$N_{max}$ (ind mL <sup>-1</sup> )	SGR (day <sup>-1</sup> )	DT (days)
Experiment 1	0 mg/L Zn	335 $\pm$ 46.98 <sup>a</sup>	0.47 $\pm$ 0.034	1.47 $\pm$ 0.101
	40 mg/L Zn	303 $\pm$ 8.08 <sup>ab</sup>	0.45 $\pm$ 0.007	1.54 $\pm$ 0.023
	80 mg/L Zn	296 $\pm$ 29.91 <sup>ab</sup>	0.44 $\pm$ 0.026	1.56 $\pm$ 0.092
	120 mg/L Zn	256 $\pm$ 26.23 <sup>b</sup>	0.41 $\pm$ 0.025	1.71 $\pm$ 0.105
Experiment 2	0.0 mg/L Se	335 $\pm$ 46.98 <sup>a</sup>	0.47 $\pm$ 0.034	1.47 $\pm$ 0.101
	40 mg/L Se	302 $\pm$ 19.30 <sup>a</sup>	0.45 $\pm$ 0.016	1.54 $\pm$ 0.053
	80 mg/L Se	349 $\pm$ 45.55 <sup>a</sup>	0.48 $\pm$ 0.034	1.44 $\pm$ 0.105
	120 mg/L Se	331 $\pm$ 25.52 <sup>a</sup>	0.47 $\pm$ 0.019	1.47 $\pm$ 0.061
Experiment 3	0 mg/L Zn + 0 mg/L Se	335 $\pm$ 46.98 <sup>a</sup>	0.47 $\pm$ 0.034	1.47 $\pm$ 0.101
	20 mg/L Zn + 20 mg/L Se	320 $\pm$ 18.82 <sup>a</sup>	0.46 $\pm$ 0.014	1.49 $\pm$ 0.046
	40 mg/L Zn + 40 mg/L Se	339 $\pm$ 11.06 <sup>a</sup>	0.48 $\pm$ 0.008	1.45 $\pm$ 0.025
	80 mg/L Zn + 80 mg/L Se	322 $\pm$ 60.86 <sup>a</sup>	0.46 $\pm$ 0.047	1.51 $\pm$ 0.153

*Note.* SD: Standard deviation.

**Table 3.** Changes in Cu and Mn content in rotifers fed enriched microalgae using different concentrations of Zinc Sulfate (experiment 1), Selenium Selenite (experiment 2), and a combination of the effect of Zinc Sulfate and Selenium Selenite (experiment 3) for 4 days (mean  $\pm$  SD; n = 3).

Experimental Groups		Cu ( $\mu\text{g g}^{-1}$ )	Mn ( $\mu\text{g g}^{-1}$ )
Experiment 1	0 mg/L Zn	16.30 $\pm$ 0.96 <sup>b</sup>	12.58 $\pm$ 0.19 <sup>b</sup>
	40 mg/L Zn	19.04 $\pm$ 0.10 <sup>a</sup>	14.13 $\pm$ 0.31 <sup>a</sup>
	80 mg/L Zn	14.92 $\pm$ 0.10 <sup>c</sup>	5.53 $\pm$ 0.38 <sup>d</sup>
	120 mg/L Zn	20.12 $\pm$ 0.23 <sup>a</sup>	8.91 $\pm$ 0.41 <sup>c</sup>
Experiment 2	0.0 mg/L Se	16.30 $\pm$ 0.96 <sup>c</sup>	12.58 $\pm$ 0.19 <sup>a</sup>
	40 mg/L Se	17.87 $\pm$ 0.06 <sup>b</sup>	13.51 $\pm$ 0.03 <sup>a</sup>
	80 mg/L Se	18.95 $\pm$ 0.20 <sup>a</sup>	4.48 $\pm$ 0.29 <sup>b</sup>
	120 mg/L Se	19.08 $\pm$ 0.08 <sup>a</sup>	4.76 $\pm$ 0.21 <sup>b</sup>
Experiment 3	0 mg/L Zn + 0 mg/L Se	16.30 $\pm$ 0.96 <sup>c</sup>	12.58 $\pm$ 0.19 <sup>b</sup>
	20 mg/L Zn + 20 mg/L Se	20.31 $\pm$ 0.30 <sup>a</sup>	23.47 $\pm$ 0.64 <sup>a</sup>
	40 mg/L Zn + 40 mg/L Se	17.45 $\pm$ 0.10 <sup>b</sup>	4.43 $\pm$ 0.13 <sup>c</sup>
	80 mg/L Zn + 80 mg/L Se	18.11 $\pm$ 0.08 <sup>b</sup>	5.03 $\pm$ 0.15 <sup>c</sup>

*Note.* Cu: Copper; Mn: Manganese; Zn: Zinc; Se: Selenium. Different letters on the each column indicate significant differences by Tukey's test ( $P < 0.05$ )

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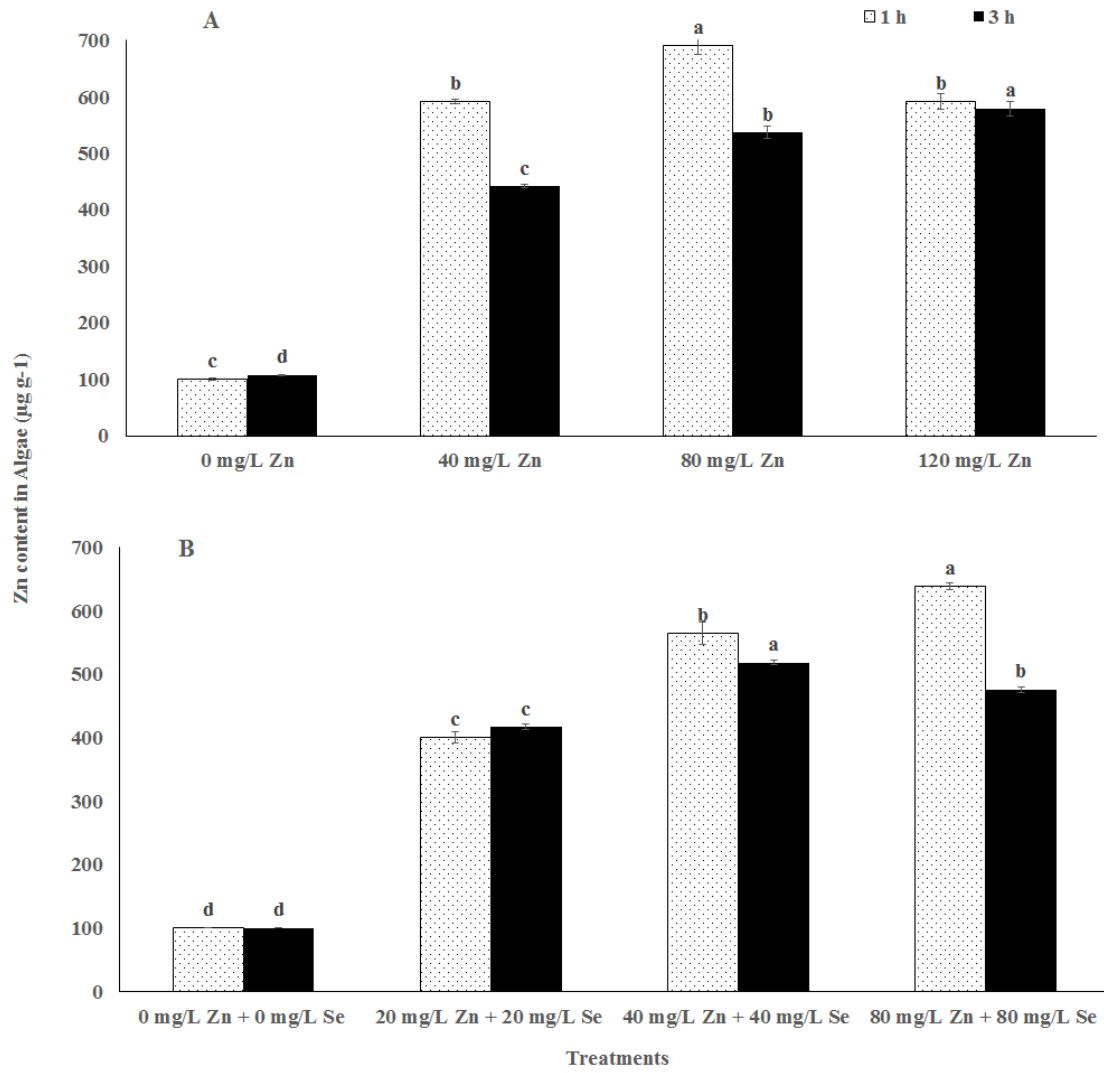
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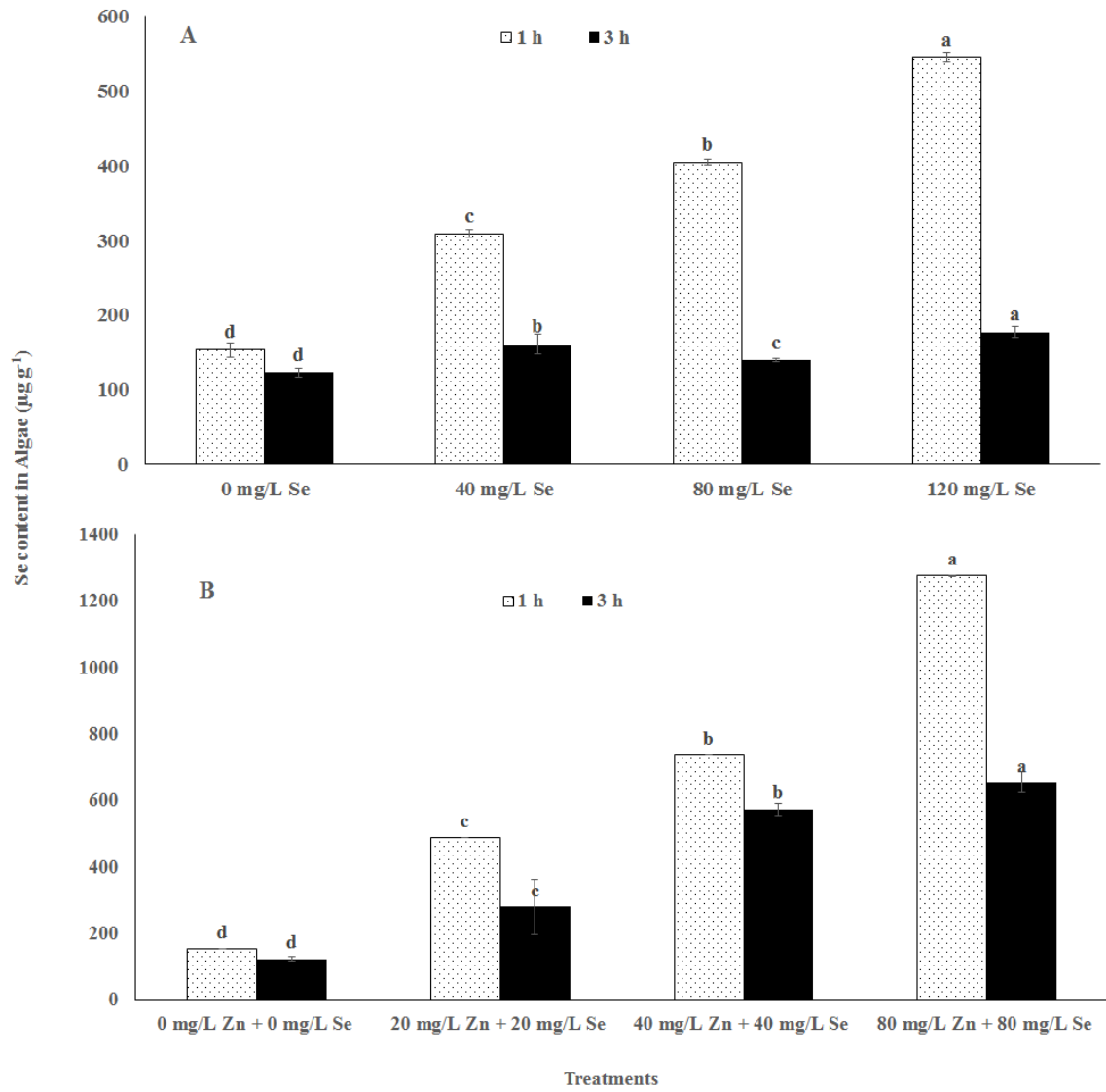




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**Fig. 1.** Changes in Zinc (Zn) content in microalgae with single (A) (experiment 1) and 840  
 combination (B) (experiment 3) enrichment, after 1 and 3 h 841

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**Fig. 2.** Changes in Selenium (Se) content in microalgae with single (A) (experiment 1) and combination (B) (experiment 3) enrichment, after 1 and 3 h

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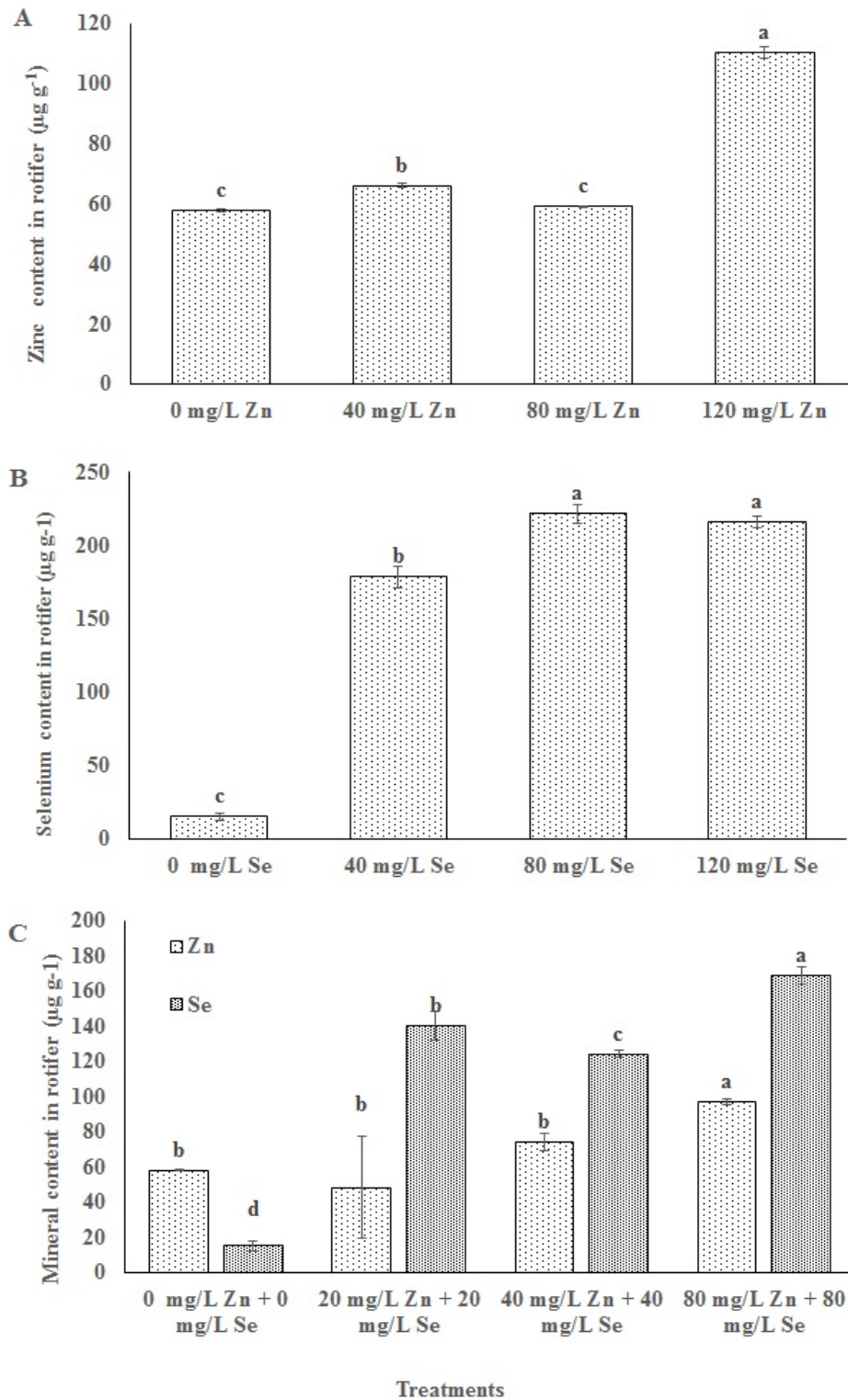
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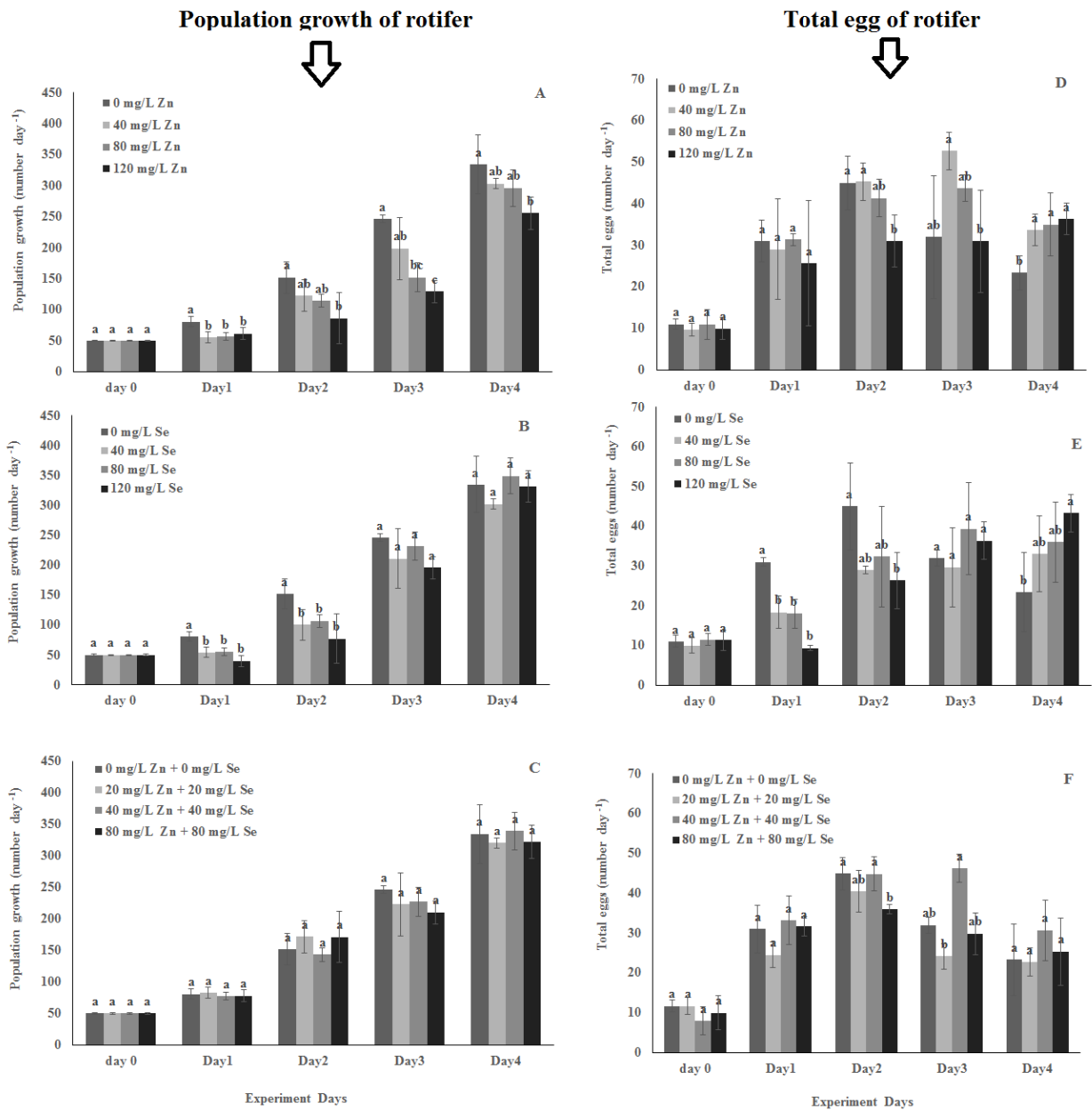
**Fig. 3.** Changes in mineral content of rotifers fed enriched microalgae with A: Zinc (Zn), B: Selenium (Se), and C: a combination of Zn and Se for 4 days.

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**Fig. 4.** Population growth (A, B and C) and total eggs (D, E and F) production of rotifers fed enriched microalgae with Zinc (Zn) (experiment 1), Selenium (Se) (experiment 2), and a combination of Zn and Se (experiment 3) for 4 days (mean± standard error, n=3). Different letters indicate significant differences by Tukey's test ( $P < 0.05$ )