Long-term incorporation of Selenium and Zinc in microalgae *Isochrysis galbana* and *Nannochloropsis oculata* and its effects on rotifer

Running Head: Long-term minerals enrichment of algae and rotifer

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Abstract

Rotifers are widely used in hatcheries to feed small-sized aquatic larvae although one of their disadvantages is the lack of zinc and selenium 5 and 30 fold lower than in copepods, respectively. To improve the rotifers quality, different concentrations of zinc and selenium (2, 4, 5, and 10 mg L^{-1} of each mineral) were added to the medium of the microalgae *Isochrysis* aff. *galbana* and *Nannochloropsis oculata* for 4 days, then the microalgae were harvested and concentrated to feed the rotifers. *N. oculata* accumulated a greater amount of Zn and Se into cells than *I. galbana*. The cell size of algae given 0, 2, and 4 mg L^{-1} of minerals did not change in both microalgae, but enrichment of the microalgae with the 5 and 10 mg L^{-1} decreased the sizes and paled the color of cells and increased cell division. The 2 mg L^{-1} was the best group for rotifers in terms of growth (population density, number of eggs, egg ratio, Specific growth rate, the maximum number and doubling time), and contained the second-highest level of Zn (69.26 ± 0.60) and Se (103.5 ± 5.0) content within a safe limit. Thus, rotifers enriched with Se and Zn can be used as a mineral delivery method to cover the nutritional requirements of marine larvae.

Keywords: long-term enrichment, Live food, Microalgae, Cell size, Minerals, Rotifer.

1. Introduction

Rotifers are widely used for feeding aquatic larvae with small-sized mouths because of their small size, slow swimming, rapid reproduction, easy culture and nutrient enrichment capability (Yanes-Roca et al. 2018; Wang et al. 2019) although their content in polyunsaturated fatty acids, vitamins, and some minerals such as selenium and zinc (Se, 30 and Zn, 5 fold) is lower than in copepods (Nordgreen et al. 2013; Penglase et al. 2013; Wang et al. 2019) and, in the case of selenium and zinc content, (Zn, 49 and Se, 0.08 μg g-1 DW) it is also lower than fish requirements (Zn, 20-30 and Se, 0.25-0.3 μg g^{-1}) (NRC, 2011). The amount of nutrients in rotifers can be increased by direct or indirect enrichment using other microrganisms like microalgae and yeast (Hamre et al. 2008a, 2008b, 2016; Nordgreen et al. 2013; Penglase et al. 2013). Live food enrichment is important because of its reproducibility and predictability, so high quality live food can be produced on a large scale (Samat et al. 2020).

Microalgae are considered acceptable sources of protein, carbohydrates, fatty acids, carotenoids, antioxidants, vitamins, and minerals for herbivorous zooplankton such as *Daphnia* (Rasdi et al. 2020), rotifer (Koiso et al. 2009; Kandathil Radhakrishnan et al. 2020), *Artemia* (Ma and Qin, 2014; Dhaneesh and Kumar, 2017), and copepods (Rasdi et al. 2021) as well as for feeding finfish and shellfish larvae (Chen et al. 2021; Dineshbabu et al. 2019; Nagappan et al.

2021). The chemical composition of microalgae depends on a wide range of species and culture conditions and is not an inherently fixed factor. Some microalgae have the capacity to adapt to changes in environmental conditions by changing their chemical composition in response to environmental variability (Bonachela et al. 2011; Ghaderpour et al. 2021). By changing environmental factors such as temperature, brightness, pH, CO_2 supply, salt and nutrients, the desired products can be largely accumulated in microalgae. Also, they have properties such as high surface to volume ratio and a high affinity for metal-binding groups (Nagappan et al. 2021). They can adsorb soluble materials such as minerals from the culture medium (fast process) or, in a slow process, concentrate soluble ions from the water in organic forms in specific organs (Yang et al. 2012).

In aquaculture, despite the use of microalgae as food for zooplankton, the larvae to which the zooplankton are fed, lack some nutrients such as minerals. Selenium and zinc are essential trace minerals with a beneficial effect on human as well as on aquatic animal health (Monteiro et al. 2011, Nordgreen et al. 2013, Wang et al. 2019). According to Samat et al. (2020) the use of selenium-enriched zooplankton increased the growth, survival and thyroid hormone status of larval fish whereas using Zn and Mn-enriched Artemia the growth and normal skeletal development was improved in sea bream (*Pagrus major*) larvae (Satoh et al. 2008). These minerals have organic and inorganic forms. The water-soluble inorganic form is toxic and cannot be directly used for zooplankton and fish (Molina-Poveda, 2016; Silva et al. 2019), on the other hand the organic form has higher bioavailability and lower toxicity. According to published results, Se-enriched *Isochrysis galbana* (Santos, 2015) and *Chlorella vulgaris* (Kim et al. 2014) have higher efficiency for Se as a Se-methionine form. Higher trophic level organisms such as finfish and shellfish cannot produce organic forms of minerals, but microalgae can take up soluble inorganic forms and bio-accumulate these minerals in an organic form. In this way, microalgae can be used as biotransferring organisms. In general, enrichment of zooplankton with mineral-enhanced microalgae is an effective method compared to direct enrichment to meet the requirement of fish larvae (Samat et al. 2020).

Marine microalgae *Nannochloropsis oculata* and *Isochrysis galbana* were selected due to their high amounts of fatty acids along with mineral enrichment to meet the needs of rotifers. These microalgae are well known for their high levels of polyunsaturated fatty acids (PUFA) with *Isochrysis galbana* containing high amounts of docosahexaenoic acid (DHA), and *N. oculata* a higher percentage of eicosapentaenoic acid (EPA) (Nuño et al. 2013). The importance of EPA and DHA in fish diets has been reviewed by Patil et al. (2005, 2007).

This experiment was designed to understand the capacity of marine microalgae *I. galbana* and *N. oculata* to long-term enrichment with inorganic zinc and selenium. The negative effects of these inorganic minerals on the growth and cell size of microalgae were also studied. This is the first time that long-term enriched microalgae with the combination of Se and Zn was used for rotifer enrichment and the first study showing their effects on rotifer populations, their egg production, and mineral content.

2. Materials and methods

2.1. *Origin of materials*

Microalgae *Isochrysis* aff. *galbana* (T-ISO) and *Nannochloropsis oculata* and rotifer *Brachionus plicatilis* (adult lorica length = 185 µm) were obtained from the shrimp research institute of Bushehr (Iran). The culture medium at 20 g L^{-1} of salinity for microalgae and rotifer culture was previously autoclaved at 120 °C for 20 min. Zinc sulfate ($ZnSO_4.7H_2O$) (Merck) and sodium selenite (Na_2SeO_3) used for enrichment of microalgae were provided by Sigma-Aldrich (USA).

2.2. Long-term enrichment of microalgae

Microalgae *I. galbana* and *N. oculata* were cultured in F/2 Guillard medium in an indoor wet-lab for 4 days as in Abbasi et al. (2019) using 10 L glass containers with continuous aeration at 26 ± 1°C and 24h light photoperiod. Selenium as sodium selenite ($Na_2SeO_3.3H_2O$) and zinc as zinc sulfate ($ZnSO_4.7H_2O$) were added at 0 (control), 2 Zn + 2 Se, 4 Zn + 4 Se, 5 Zn + 5 Se, and 10 Zn + 10 Se mg L^{-1} to the nutrient medium for microalgae at the beginning of the culture. Growth of microalgae was daily determined by counting the cell density under a microscope using a Neubauer chamber (hemocytometer). At the end of the experiment, the biomass of cultured microalgae in different concentration of combined minerals was determined using the method of Babaei et al. (2017). In brief, 50 mL of cultured samples were filtered in a pre-weighed fiberglass filter, dried in an oven at 105 °C for 3 h, and finally weighed to the nearest 0.0001 g using a digital balance (Table 1). The microalgae species were harvested at the exponential growth phase using a high volume centrifuge (Sigma model). The slurries were dried at 40 °C until constant weight for further analysis.

2.3. Rotifer culture

Rotifer batch culture was carried out at Urmia University wet lab using 7 L cylindrical glass conical containers with continuous aeration and light (24h L, 1000 lux) and live *Nannochloropsis oculata* as food. Continuous aeration was used to avoid rotifer accumulation at the bottom and to produce a slow water movement at the top of the conical containers. The temperature was maintained at 27 ± 0.5 °C, pH ~ 8.3, and salinity 20 g L^{-1} with an initial density of 50 rotifers mL^{-1}.

2.4. Rotifer enrichment with Long-term Zn- and Se-enriched algae

Rotifers were enriched using mineral-enriched microalgae using the following treatments: (i) non-enriched mixed microalgae (50:50 of *I. galbana* and *N. oculata* without adding Zn or Se), (ii) 2 mg L^{-1} Zn + 2 mg L^{-1} Se -enriched mixed microalgae, (iii) 4 mg L^{-1} Zn + 4 mg L^{-1} Se -enriched mixed microalgae, (iv) 5 mg L^{-1} Zn + 5 mg L^{-1} Se -enriched mixed microalgae and (v) 10 mg L^{-1} Zn + 10 mg L^{-1} Se -enriched mixed microalgae. Triplicate rotifer cultures were fed twice per day using a mixture of the microalgae with density of 1.5×10^3 cell mL^{-1} *N. oculata* and 1.5×10^3 cell mL^{-1} *I. galbana* without any water exchange during 4-day cycles. Due to the difference in size of algal cells, the microalgae were given to the rotifers based on their dry weight.

2.5. Growth rate and Egg ratio

Three samples of 1 mL were taken from each conical container for counting the number of rotifers and eggs using the Bogorov Counting Chamber. Specific growth rate (SGR) was calculated (Krebs, 1995) using the following formula:

$$SGR = (Ln\ N_t - Ln\ N_0)/t$$

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

Doubling time (Vallejo et al. 1993):

$$DT = \frac{Ln\ 2}{SGR}$$

The egg ratio (females carrying amictic eggs, ER) of rotifers was calculated as a basis to quantify rotifer health, using the following equation (Nematzadeh et al. 2018):

$$ER = \text{rotifers with eggs} / \text{total rotifers}$$

At the end of the feeding period, all the rotifers were filtered through a 50 μm mesh, rinsed with tap water, and transferred to microtubes. The samples were stored at –80 °C until analysis.

2.6. Determination of mineral content

The concentration of minerals (Se and Zn) in samples were determined using a novAA® 400 PAtomic Absorption (Analytic Jena, Germany) (Lowry and Lopez, 1946). In short, the frozen microalgae and rotifers were dried in an oven at 40 ˚C for 24 h. Then, 100 mg of dried samples were weighed and digested with 65% nitric acid and 125 µL of hydrogen peroxide at 80 ˚C for 30 minutes in the water bath. Samples were allowed to cool and the volume of them was adjusted to 20 mL with distilled water for analyzing.

2.7. Statistical analysis

Statistical analysis was performed with the SPSS software, version 21 using Levene's and Shapiro-Wilk tests to check the homogeneity of variances and normality, respectively ($P<0.05$). Arcsine transformations were conducted in case of all data and were expressed in terms of percentages. The comparisons among means of treatments were done by the analysis of variance (ANOVA) followed by Tukey–Kramer HSD for post-hoc multiple comparisons. Differences among the means were considered significant at $p < 0.05$. The data are displayed as means of three replicates ± standard deviation (SD).

2.8. Ethics Statement

No ethical approval was required for this study, as no specific permission is needed for rotifer studies in Iran.

3. Results

3.1. Cell density and the size of microalgae

The cellular density of *Isochrysis galbana* enriched with a combination of different concentrations of zinc (zinc sulfate) and selenium (sodium selenite) is presented in Fig. 1A. Statistically significant differences in cellular density of *I. galbana* were observed in the treatments with high concentrations of minerals (5 and 10 mg L^{-1}) compared to those with low concentrations (2 and 4 mg L^{-1}) on the first and third days. Treatments 0, 2, and 5 mg L^{-1} show a clear increase in cellular density during cultivation with the highest values observed on the fourth day of culture ($p < 0.05$). Although the treatment of 4 mg L^{-1} also showed an increase in the number of cells, it did not reach its peak during this period. On the other hand, the 10 mg L^{-1} treatment showed a decrease in cell density at the end of the culture period.

The cellular density of *N. oculata* enriched with different concentrations of zinc (zinc sulfate) and selenium (sodium selenite) is presented in Fig. 1B. The treatment of 5 mg L^{-1} showed an increase in cell density during the culture period with the highest algal cell density obtained on day 4. The highest cell density of *N. oculata* enriched with 2 and 4 mg L^{-1} was observed on the first day of culture, with 2 mg L^{-1} treatment showing a constant trend during the 4 days of culture and 4 mg L^{-1} a decrease in cell density. The algal cell density of 10 mg L^{-1} treatment increased until the second day of culture when it reached the stationary phase. The cellular density of *N. oculata* in the control group showed an inconsistent trend during the whole culture period.

The dry weight of non-enriched and enriched *I. galbana* and *N. oculata* microalgae with different concentrations of mixed minerals (zinc sulfate and sodium selenite) is presented in Table 1. The results showed that the dry weight of both microalgae increased by increasing the minerals in the culture medium until 5 mg L^{-1}, except for the dry weight of 10 mg L^{-1} treatment that decreased significantly ($p < 0.05$).

The cell size of enriched *I. galbana* and *N. oculata* with different concentrations of zinc sulfate (Zn) and sodium selenite (Se) is presented in Fig. 2. High concentrations of minerals (5 and 10 mg

L^{-1}) significantly reduced the size of *N. oculata* cells, while only the highest amount of these minerals (10 mg L^{-1}) reduced the size of *I. galbana* cells ($p < 0.05$). The lower concentrations of minerals had no significant effect on the cell size of both microalgae ($p > 0.05$).

3.2. Mineral content in microalgae

Changes in zinc (Zn) and selenium (Se) content in enriched *N. oculata* and *I. galbana* with different concentrations of the combination of zinc sulfate and sodium selenite are shown in Fig. 3 (A and B, respectively). The highest Zn content was observed in enriched *I. galbana* with 2 and 4 mg L^{-1} of the minerals in the medium, while the highest Zn content in enriched *N. oculata* was obtained using 4 and 10 mg L^{-1} of minerals ($P < 0.05$) (Fig. 3A). Interestingly, increasing the mineral concentration in the culture medium of *I. galbana* produced a decrease in the amount of zinc retained.

The amounts of selenium in both microalgae increased in parallel to the concentration of this mineral in the culture medium, with the accumulation of Se in *I. galbana* being significantly different among the treatments except for those using 2 and 4 mg L^{-1} of minerals (Fig. 3B).

3.3. Effects of the use of Long-term enriched microalgae on rotifer growth

The population density of rotifers fed long-term enriched microalgae with zinc sulfate and sodium selenite is shown in Fig. 4. After 48 h feeding, the population density of rotifers significantly changed in all the treatments. The highest population density was obtained in the rotifers fed 2 mg L^{-1} enriched microalgae, being significantly higher at days 3 and 4 compared to the control group ($p < 0.05$). The lowest number of rotifers in the last three days of culture was obtained in the rotifers of the 10 mg L^{-1} treatment ($p < 0.05$) that even at day 2 showed the lowest values.

Total egg production of rotifers fed long-term enriched microalgae with zinc sulfate and sodium selenite is presented in Fig. 5. The highest number of eggs (97 ± 13 eggs day^{-1}) was observed in the rotifer fed with microalgae enriched with 2 mg L^{-1} of both minerals and this was achieved on the second day of rotifer culture, reaching its reproductive peak this day and then declining. The number of eggs in the control group was not significantly different from that obtained in the 2 mg L^{-1} fed group except at day 3, that was lower than the mentioned treatment ($p < 0.05$). The results in egg production of rotifers fed 4 and 5 mg L^{-1} were not significantly different during the whole duration of the trial ($p < 0.05$) whereas rotifers fed 10 mg L^{-1} of minerals showed the lowest number of eggs without any egg production detected on days 1 and 3.

The results of egg ratio are presented in Fig. 6. The highest egg ratio in the first and second days was observed in the 2 mg L^{-1} treatment. In the case of the 4 and 5 mg L^{-1} treatments the highest ratio was observed on the second day although this was not significantly different from the 2 mg L^{-1} group. The lowest egg ratio was observed in the rotifers fed 10 mg L^{-1} minerals during the whole trial.

SGR, N_{max} and DT results of rotifers fed long-term enriched microalgae with zinc sulfate and sodium selenite are shown in Table 2. The highest SGR value (0.614 ± 0.026day^{-1}) and N_{max} (584 ± 62 ind mL^{-1}) were observed in the 2 mg L^{-1} treatment on the 4th day of culture, which also showed the shortest time for doubling the rotifer population ($p < 0.05$). SGR and DT in this treatment did not show any significant difference with respect to the control group ($p < 0.05$). SGR was higher and DT was lower than the control group, whereas N_{max} was significantly higher for this 2 mg L^{-1} group of rotifers compared to all the treatments ($p < 0.05$).

3.4. Effect of Long-term enriched microalgae on the mineral content of rotifer

The highest content of zinc was obtained in rotifers fed 4 and 10 mg L^{-1} of minerals (zinc sulfate and sodium selenite) (78.93 ± 0.19 and 82.28 ± 0.37 µg g^{-1} DW, respectively) which was significantly different from the other experimental groups ($p < 0.05$) (Fig. 7, A). The lowest content of zinc (64.17 ± 0.34 µg g^{-1} DW) was observed in the control group which was significantly different from all the other treatments ($p < 0.05$).

The highest amount of selenium (171.31 ± 5.83 µg g^{-1} DW) was obtained in rotifers fed enriched microalgae with 5 mg L^{-1} of minerals (zinc sulfate and sodium selenite) and the lowest amount (15.03 ±2.77 µg g^{-1} DW) in the rotifers from the control group, which were significantly different from the other treatments ($p < 0.05$) (Fig. 7, B).

4. Discussion

Despite the importance of Se and Zn as a key part of metalloenzymes in aquatic physiology (Eryalçın et al. 2020), oxidative stress (Betancor et al. 2012; Pacitti et al. 2013; Saleh et al. 2014; Izquierdo et al. 2017), ossification (Yamaguchi, 1998), and improvements in larval growth and survival (Satoh et al. 2008), information regarding mineral nutrition in marine fish larvae is very limited. Moreover, the availability of the minerals depends on their molecular form such as Izquierdo et al. (2017) observed using different forms of minerals (inorganic, organic and nanoparticles) in fish larvae feeding. According to their results, fish larvae fed with organic minerals showed the best growth and early mineralization while preventing deformities in branchial arches. Taking into account (1) the importance of selenium and zinc, (2) the lack of these minerals in live food such as Artemia and Rotifer compared to copepods (Hamre et al. 2013), and

(3) the availability of different minerals form, we have used a combination of these minerals to investigate the effect of simultaneous enrichment on microalgae and then on rotifers. The ability of rotifers to absorb minerals from digestible materials is much higher using microalgae than using a direct feeding with those minerals as Matsumoto et al. (2009) and Thiry et al. (2012) indicated.

There are two methods to enrich microalgae: short-term and long-term enrichment. In the present study we have selected long-term enrichment taking into account that one of the important benefits of long-term enrichment is the incorporation of nutrients inside the microalgal cells facilitating their transfer to a higher trophic level. According to Dhert et al (2014), the dietary composition of rotifers can be improved not only through enrichment but also by ameliorating the feeding and culture conditions in a way that continuous enrichment forms an integral part of the culture. The purpose of the long-term and simultaneous enrichment of rotifers with two minerals in this experiment was to reduce rotifer mortality due to the high manipulation (harvest from culture, cleaning, enrichment, harvest, cleaning) and the high risk of bacterial and fungal infections of rotifers in the case that an enrichment using 2 different minerals was carried out. Therefore an indirect enrichment of rotifers with zinc sulfate and sodium selenite was performed in the present study using *I. galbana* and *N. oculata* that had accumulated these minerals during 4 days.

The reaction of microalgae to minerals depends on their amounts in the surrounding environment. Growth has been cited as the best and sensitive indicator for detecting excess minerals in the enrichment (Lin and Shiau, 2005; Jaramillo et al. 2009). If the amount of minerals in the culture medium is low, it has a stimulating effect on microalgal growth but when it is high, it has an inhibitory effect (Chan and Chiu 1985, El-Sheekh et al. 2000). Enrichment of *I. galbana* using different zinc and selenium concentrations caused different reactions. Thus, the use of 5 and

10 mg L^{-1} minerals increased the cell density of this microalgae compared to the control group, whereas 2 and 4 mg L^{-1} had no significant effects on growth as it is shown in Fig 1.

The enrichment of minerals for *N. oculata* with 5 and 10 mg L^{-1} increased the density and cell division, although not significantly different from the control group. Treatments with 2 and 4 mg L^{-1} of minerals in the culture medium of *N. oculata* caused a peak of reproduction earlier than in the control group and, as a consequence, their density was lower than the control group in the final days of culture.

In most of the microalgae studied a steady increase in cell density has been observed using concentrations of 0.01-0.05 mg Se L^{-1}. Higher concentrations can induce ultrastructural injuries like those observed in *Dunaliella salina* using 0.1 mg L^{-1} Se (Reunova et al. 2007). In the present study, the two species of microalgae used tolerated higher concentrations of Se and Zn and showed an increase in cell density at 5 and 10 mg L^{-1} in parallel to a reduction in cell size. The increasing number of cells might be the result of the algal cell response to stressful conditions enhancing its survival despite cell shrinkage.

Pale microalgae cells were observed in the microalgae cultured with 5 and 10 mg L^{-1} of Se and Zn as signs of stress conditions. This reduction in color might be due to the production of reactive oxygen species (ROS) by Zn or Se, with the consequent release of free radicals that can attack thylakoid lipids damaging the structural pigments of algal cells (Shi and Dalal 1990, Esmaeili 2015, Petsas and Vagi 2017). Furthermore, this might be due to a decrease in the content of magnesium in the chloroplasts, as a consequence of increasing selenium in the medium, although Se didn’t affect the uptake of other minerals (Guimarães et al. 2021).

The algal cell wall is porous and composed of polysaccharides, lipids and proteins, that allow algal cells to absorb metals (Hope and Walker 1975; Davis et al. 2003). The amount of zinc in cultured *Chlorella* can reach up to 8.8 µg g^{-1} DW (Matsumoto et al. 2009), much higher than the amount of zinc in the control group of the microalgae used in the present experiment due to the differences in the microalga species and/or their reaction to the same nutrient (zinc sulfate). Algal species show different reactions in terms of stress sensitivity, which can be due to differences in pigment type and photosynthetic capacity, cellular lipid and protein content, and cell size (DeLorenzox et al. 2004).

The amounts of zinc in the long-term enrichment of *N. oculata* and *I. galbana* with Zn sulfate and Na selenite obtained in the present study are lower than those found using a short-term enrichment (Ghaderpour et al. 2021). However, it should also be noted that, despite the very high amounts of zinc bio-concentrated in the microalgae in previous experiments, the Zn content in rotifers fed with them was not high enough. Therefore, it can be concluded that rotifers cannot reach the same levels of zinc concentration observed in copepods (340-570 µg g^{-1} DW) by using microalgae enriched with zinc as food (NRC, 2011).

In the case of the low levels of zinc observed in *I. galbana* enriched with 5 and 10 mg L^{-1} minerals, it has been probably a consequence of the smaller size of the cells and the decrease in their surface area for absorption. This was not observed in *N. oculata* enriched with 10 mg L^{-1} minerals despite the smaller cell size and reduced absorption area, but it was observed using 5 mg L^{-1}. *N. oculata* absorbed relatively higher amounts of zinc compared to *I. galbana,* especially in the treatments using high mineral inclusion, thus a different behavior exists when different microalgae species are exposed to zinc, with their reaction being quite complex and needing further investigation.

The toxic effects of selenium on microalgae might be a consequence of: (1) the use of an inorganic form of Se, (2) the Se concentration in the media, (3) and its similarity with sulphur and in this case Se can disrupt proteins via substituting sulphur in sulphur bonds, resulting in incorrect protein shape and dysfunctional enzymes (Ponce et al. 2018) and (4) the specific response of microalgal species (Guimarães et al. 2021). Also, the high levels of Se produces reactive oxygen species (ROS) that stimulate oxidative stress (Lemly 2002). Given that the free form of this mineral was added to the microalgal culture medium, all these events might have occurred.

The uptake of selenium by *N. oculata* is much higher than in the case of *I. galbana* using the same treatments, probably be due to intracellular processes. For example, Se accumulation in the control group of *N. oculata* was 1.95 times higher than the same treatment in *I. galbana*. Also, the amount of this element in *N. oculata* from treatments 2 and 4 mg L^{-1} was approximately 1.8-1.9 times higher than in *I. galbana*, which was almost the same as the control group. Se accumulation in 5 and 10 mg L^{-1} groups increased up to 3.37 and 2.5 times, respectively. Eryalçın et al. (2020) mentioned that increasing copper in the diet can increase the uptake of other +2 ions by upregulating the solute transporter protein, so it is possible that increasing zinc or selenium has increased the uptake of another ions in the organisms.

The decrease observed in the 10 mg L^{-1} treatment might indicate some regulation to maintain homeostasis inside the cells. Interestingly, Se content in *N. oculata* and *I. galbana* enriched with 10 mg L^{-1} was 1.17 and 1.57 times higher than observed using 5 mg L^{-1}, respectively. Overall, the Se content in *N. oculata* and *I. galbana* (separately and in combination) increased in parallel to the concentration in their culture medium.

The levels of Se in *I. galbana* were higher than those of the control group in short-term Se-enriched mixed microalgae and the combination of Zn and Se, while in *N. oculata* (10 and 5 mg L^{-1} treatments) the level was much higher than treatments of 40, 80 and 120 mg L^{-1} in the short-term Se-enriched mixed microalgae (*N. oculata* and *I. galbana*), but lower than Zn and Se-enrichment treatments of 80 and 40 mg L^{-1}. Therefore, there might be an inhibitory factor in *I. galbana* cells when exposed to Se. Thus *N. oculata* is a better option for mineral enrichment than *I. galbana*. Oraby et al. (2015) mentioned that the interaction between antioxidants and vitamins increases the bioavailability of Se, thus the presence of these in two microalgae can also increase the Se-bioavailability.

Other application of these minerals-enriched microalgae can be in the nutrition of aquatic animals. Concentrated and dried microalgae can be included in aquafeeds to provide minerals (or other nutrients) to meet their demands and avoid i.e. skeletal abnormalities or other nutritional deficiencies.

In the case of rotifers (see Fig. 7), the highest Se content was observed in those fed microalgae enriched with 5 mg L^{-1}. This treatment used in both *N. oculata* and *I. galbana* resulted in the second-highest level of selenium among all other algal treatments. Although the highest amount of selenium in the microalgae was obtained using 10 mg L^{-1}, the results observed in rotifers were lower than those obtained with microalgae enriched with 5 mg L^{-1}, probably as a result of a lower feeding rate of rotifers due to the toxicity of high selenium levels, that can also be confirmed taking into account the reduction in rotifer density when 10 mg L^{-1} treatment was used.

The content of Se (171.31 ± 5.83 µg g^{-1} DW) was higher in the rotifers fed the 5 mg L^{-1} treatment being the results higher than those found in a previous study (Ghaderpour et al. 2021) using short-term Zn and Se-enriched microalgae (140.38 - 169.10 µg g^{-1} DW). On the other hand, Se content in the present trial was lower than its content in the rotifers fed algae enriched using a Se short-term enrichment protocol.

Due to the unknown interactions between minerals, the use of mixtures using several compounds might have different effects on the organisms compared to treatments using a single mineral (Ríos-Arana et al. 2007, Hamre et al. 2008b). The mechanisms of mineral uptake, storage and excretion in rotifers are variable (Nordgreen et al. 2013), in agreement with the results of this experiment. Comparing the Se content in the previous trial (140.38 – 5101.44 µg g^{-1} DW) using short-term enriched microalgae (Ghaderpour et al. 2021) with the results of the present one, we can conclude that long-term enrichment of microalgae with these minerals reduces the amount of Se in the rotifer.

Se content of 1.4-3 µg g^{-1} DW in the rotifers is enough to cover the Se requirements of fish larvae (Ribeiro et al. 2012, Kim et al. 2014) and juvenile and adult fish (0.15-0.25 µg g^{-1}) (NRC 2011). According to previous studies, increasing the Se levels from 1.3 to 6.27 mg selenomethionine kg^{-1} resulted in growth increase and a reduction in muscular dystrophy in fish larvae (Betancor et al. 2012), whereas increasing from 0.73 to 8 mg kg^{-1} did not affect the growth in rainbow trout (Rider et al. 2009). Saleh et al. (2014) observed that increasing Se from 1.7 to 11.65 increased survival and stress resistance, and mineralization of bones in fish larvae.

Previous research show that increasing Zn levels up to 245 mg/kg in rotifer had no negative effects on larval fish survival and even using zinc supplementation up to 306 mg/kg did not affect fish larval growth (Satoh et al. 2008; Yamamoto et al. 2013; Eryalçın et al. 2020). Moreover, low levels of zinc in the diet (85-100 mg kg^{-1}) and enriched rotifers (119-306 mg kg^{-1}) did not affect the growth of fish larvae (Izquierdo et al. 2017). It is noteworthy that organic zinc increases alkaline phosphatase activity in rainbow trout (Kucukbay et al. 2006).

Simultaneous enrichment of larvae with manganese along with zinc and selenium reduced larval survival to 50% (Izquierdo et al. 2017; Eryalçın et al. 2020), which contrasted with increased survival of larvae fed manganese-enriched rotifers (Satoh et al. 2008). This difference might be due to the used inorganic form of manganese.

Therefore, the use of long-term Se enriched microalgae, even using low amounts of the mineral, can be considered the best way for enriching the rotifers, especially considering that Se uptake by rotifers was also very high, covering the requirements of larvae.

The highest Zn content was obtained in the rotifers fed with *N. oculata* and *I. galbana* enriched with 4 and 10 mg L^{-1} of minerals. The treatment of 10 mg L^{-1} showed the highest content in *N. oculata* and the lowest in *I. galbana*. Feeding rotifers with microalgae enriched on 5 and 2 mg L^{-1} showed the second-highest level of zinc, the 2 mg L^{-1} treatment in *I. galbana* had the highest Zn value (134 ± 0.38 µg g^{-1} DW), the same treatment used for *N. oculata* showed a lower zinc content in rotifers compared to the control group. Interestingly, the rotifers fed *N. oculata* enriched using the 5 mg L^{-1} treatment had the lowest content among all the other treatments, whereas using the same treatment in *I. galbana* gave a lower value compared only to the control group. Therefore, the uptake of Zn by live food did not follow a general rule and depends on the microalgae species used. Despite the higher amount of Zn in the microalgae medium, especially in the case of the 5 mg L^{-1} and 2 mg L^{-1} treatments, the amount of zinc in the rotifers was almost the same, which is similar to the results by Nordgreen et al. (2013). They found that despite the higher amount of Zn in Oriculture commercial feed compared to Origreen, an equal amount of Zn was observed in the rotifers fed on them. They concluded that the amount of zinc in rotifers fed diets enriched with copper, Se, manganese and Zn, despite the increase in zinc in the rotifer diet, would not exceed a certain level and the Zn level decreased in rotifers.

The zinc values obtained in the rotifers were higher than the combination of commercial products e.g. Algamac 2000 with yeast, Culture Selco 3000 and the combination of algae *Chlorella* and yeast (range 64-62 µg g^{-1} DW) according to Hamre et al. (2008a). However, the Zn content in the control group was 49 ± 3 µg g^{-1} DW in the study by Hamre et al. (2008a), which was much lower than the amounts (64.18 ± 0.34 μg g^{-1} DW) found in rotifers from the control group of the present study. Furthermore, the Zn content of rotifers in all the treatments of this experiment was considerably higher than the recommendation given by NRC (1993), and the requirements for juvenile or adult cold-water fish (20-30 μg g^{-1} DW).

Long-term Zn and Se enrichment of microalgae did not increase the Zn content in the rotifers if we compare with rotifers fed microalgae enriched with Zn for one hour. The highest amount of zinc in the rotifers was obtained using 10 mg L^{-1} enriched microalgae (82.28 ± 0.37 μg g^{-1} DW), which was lower than the maximum value obtained in the short-term enrichment (Ghaderpour et al. 2021) with 80 mg L^{-1} of zinc sulfate and sodium selenite (96.89 ± 1.36 μg g^{-1} DW) and 120 mg Zn L^{-1} treatment (110.45 ± 1.92 μg g^{-1} DW). It should be noted that rotifers metabolize and excrete the ingested nutrients during the enrichment period leading to changes in the composition of rotifers after enrichment. For example, rotifers have been shown to lose essential fatty acids (Rodriguez et al. 1996, Naz 2008) and zinc (Matsumoto et al. 2009) after enrichment. Wang et al. (2019) also observed changes in the retention of minerals in the rotifers loosing about 35% of the stored zinc in the first hour after feeding.

Se absorption in the rotifer depends on the amount of the mineral in the food, on the contrary, the accumulation of Zn in the rotifer can be much lower than the amount in the diet even when high levels are used (Nordgreen et al. 2013). The presence of other substances in enrichment formulations and the duration of enrichment may affect the final concentration of Se in zooplankton (Samat et al. 2020). Some aquatic cells are able to catalyze the organic form of Se into alternative forms that can produce superoxides (Spallholz et al. 2004, Ponce et al. 2018). It might be possible that the rotifers, after absorbing Se at low levels of toxicity from enriched microalgae, convert it to toxic metabolites resulting in a decrease in rotifer density despite the good culture conditions (Ponce et al. 2018).

Efficient reproduction, and consequently population density, are important factors to be controlled in rotifer culture and both are affected by the concentration and variety of metals to which they are exposed (Hamre et al. 2008a; Xu et al. 2015). Rotifers select food in the range of 4-10 µm, and using *I. galbana* and *N. oculata* enriched with minerals is more appropriate than Se enriched *C. vulgaris* with a size of more than 15 µm (Sun et al. 2020). In this study, Sun et al (2020) showed that rotifer fed on *Chlorella pyrenoidesa* with 4 µm size gave better results than using *C. vulgaris*. Thus, one of the reasons for the low growth of the rotifer fed the 5 and 10 mg L^{-1} treatments, especially the latter, might be that the microalgae size was lower than the optimal to feed rotifers.

Rotifers fed on enriched microalgae with 10 and 5 mg L^{-1} minerals have a larger size than the control group. This might be due to their inability to lay eggs, confirmed by the lower egg number and egg ratio than the other groups on the same days of culture (see Fig 6). Similar results were found by Penglase et al (2013) in enriched rotifers with 67.5 mg Se-yeast that have a larger size and slower motion.

Rotifer population density decreased in the 4, 5, and 10 mg L^{-1} treatments compared to the control group during the 4 days of culture. Considering that 10 mg L^{-1} treatment was the worst in terms of rotifer growth (population density, egg number, egg ratio, SGR and N_{max}), and despite the highest amount of Zn in the rotifers, it cannot be recommended for marine fish aquaculture. Similar negative effects of high Se levels on rotifer population growth were already obtained by other authors (Penglase et al. 2011, Ponce et al., 2018) using rotifer fed on Se-yeast. The 4 mg L^{-1} treatment produced the highest amount of zinc (78.93 ± 0.19 µg g^{-1} DW) and showed the third-highest level of Se (116.38 ± 5.61 μg g^{-1} DW), but the population density, N_{max} and SGR were lower than those of the control group. The number of eggs was also lower than the egg number in the reproductive peak of the control group, as well as the egg ratio (0.11-0.42) and the time needed to double the rotifer population. For these reasons, 4 mg L^{-1} cannot be selected as the best treatment. On the other hand, 2 mg L^{-1} was the best group in terms of growth (population density, egg number, egg ratio, SGR, N_{max} and DT), contained the second highest level of Zn (69.26 ± 0.60 μg g^{-1} DW) and the amount of Se was 103.45 ± 4.99 μg g^{-1} DW. According to published results (Penglase et al. 2011, Sun et al. 2020), the amount of Se that is non-toxic in rotifers is 22-113 μg g^{-1} DW, with the amount obtained using the 2 mg L^{-1} enrichment treatment being in this range. The highest specific growth rate (0.61 ± 0.03 day^{-1}) was observed in the group fed with 2 mg L^{-1} of minerals, which is equal to the population growth rate of the rotifer fed with 3.3 μg g^{-1} DW Se-enriched *C. vulgaris* (Kim et al. 2014).

The highest egg ratio (0.98) was observed in the 2 mg L^{-1} treatment at 24 h after feeding, which was higher than the control group on day 3. Similar egg ratios were found in rotifers enriched with Se-yeast and Oriculture (0.19-0.28 and 0.25-0.4, respectively) by Penglase et al. (2011) and Dhert et al. (2014).

Minerals were added to the culture medium of microalgae at day 0, with the microalgae being harvested after 4 days before being used for rotifer feeding for a further 4-day period. Taking this into account, these minerals might be bound to proteins inside the algal cells and provided to rotifers in an organic form. On the other hand, the last feeding dose was given to rotifers 19 hours before harvest and Se-methionine might have been the Se form in the rotifer (Ponce et al. 2018).

Enrichment of microalgae with Cu has been shown (Moreno-Garrido et al. 1999) to delay rotifer population density reaching its maximum by 1 or 2 days. In the present study, the use of 2 mg L^{-1}

of zinc sulfate and Na selenite in the microalgae culture medium accelerated the increase in rotifer population density. Therefore, it can be concluded that selecting the correct amounts and choosing the right combination of minerals for inclusion in each microalga can be beneficial both for rotifer enrichment and for larviculture of marine organisms.

5. Conclusion

The use of minerals (Zn and Se) added to the culture medium of microalgae increased the amounts of these minerals in microalgae and in higher trophic levels (i.e. rotifers). Although 5 and 10 mg L^{-1} treatments increased microalgae cell density, the cells become smaller and with a pale color. Two and 4 mg L^{-1} treatments might be a better option for microalgae enrichment taking into account that they reached their maximum cell density earlier without showing any sign of stress. Each microalgae species has different capacities for mineral uptake, being *N. oculata* a better option for mineral enrichment than *I. galbana*. The highest Se content was observed in rotifers fed microalgae enriched with 5 mg L^{-1}. The highest Zn content was found in 4 and 10 mg L^{-1} rotifer treatments. However, rotifer density (and harvested quantities) along with enrichment with valuable nutrients are very important in aquaculture, and 2 mg L^{-1} treatment can be considered the best for rotifers. The best method for rotifer and microalgae enrichment in order to obtain the highest amount of minerals (Zn and Se) is short-term enrichment, although long-term enriched microalgae are safer, more cost-effective, and eco-friendly than short-term enriched ones, especially if they are going to be used for rotifer enrichment. Thus, hatchery managers can use rotifers cultured using long-term enriched microalgae to meet the mineral requirements of aquacultured fish larvae.

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Data availability statement

The authors confirm that the data supporting the results in the paper are included in the tables and figures in the paper and research data are not shared.

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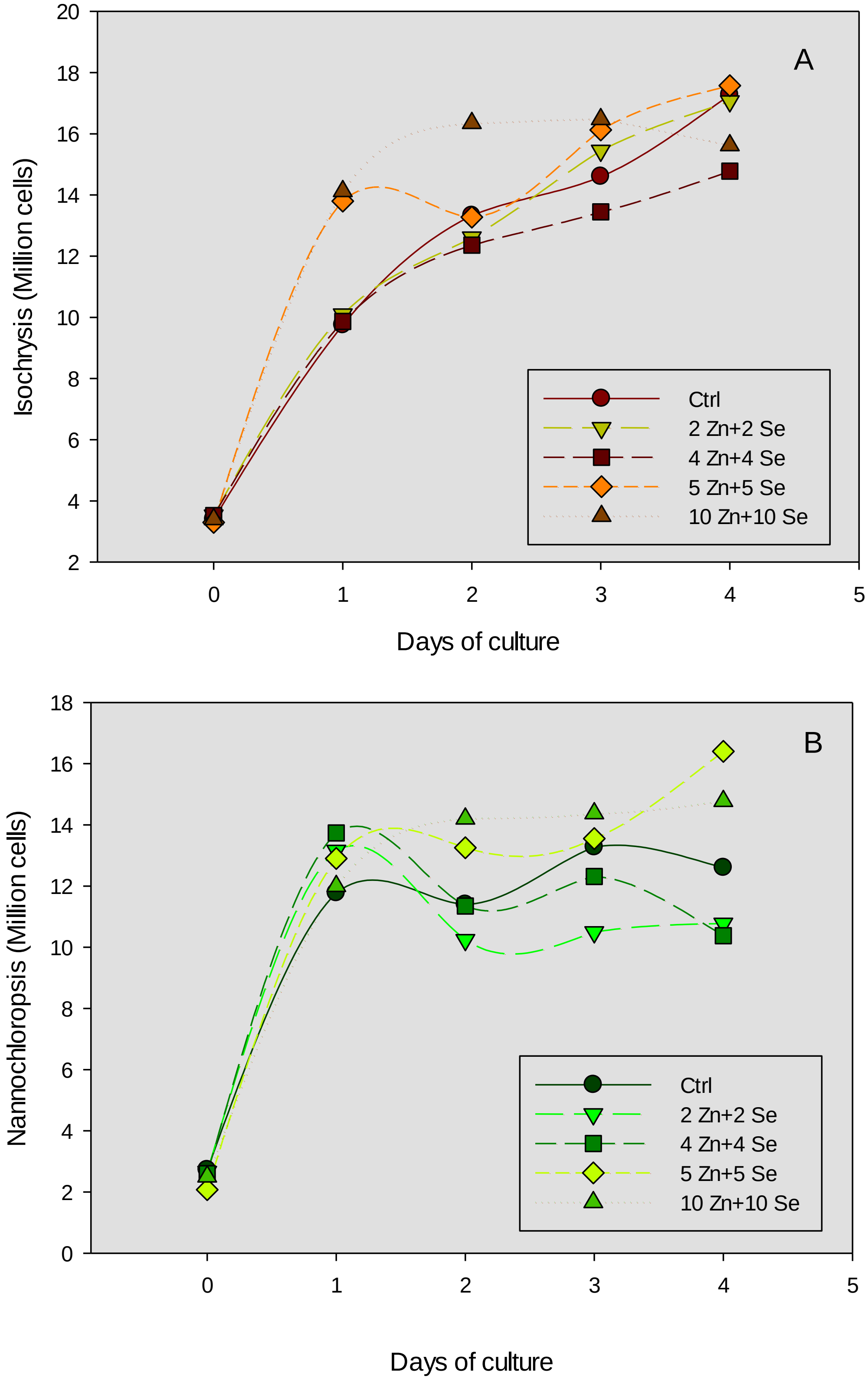

Fig. 1. Cellular density of *Isochrysis galbana* (A) and *Nannochloropsis oculata* (B) (Cells mL^{-1}) cultured with different concentrations of mixed zinc (zinc sulfate) and selenium (sodium selenite) during 4 days. The numbers 2, 4, 5 and 10 indicate the amount of zinc sulfate and sodium selenite as mg L^{-1} in the culture media.

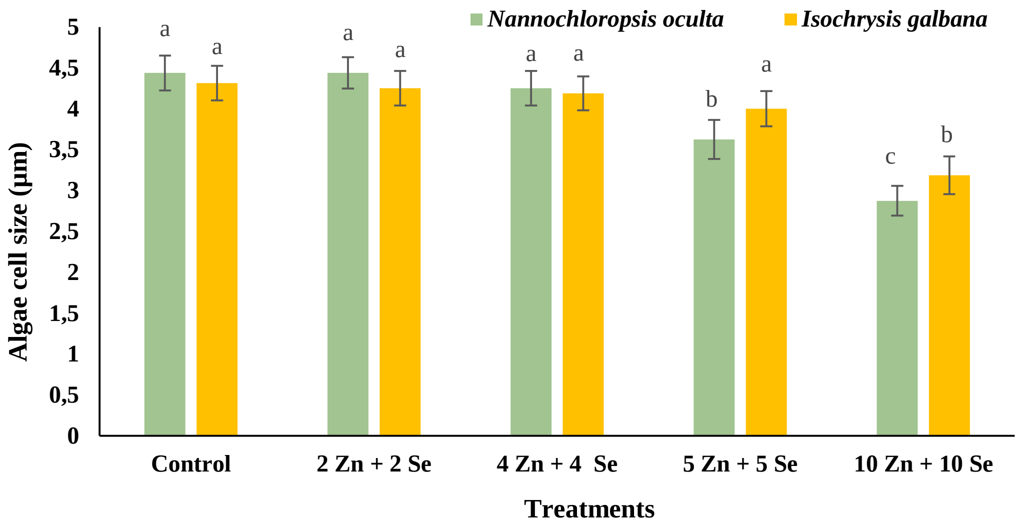

Fig. 2. Cell size of *Isochrysis galbana* and *Nannochloropsis oculata* cultured with different concentrations of mixed zinc sulfate (Zn) and sodium selenite (Se). The numbers 2, 4, 5 and 10 indicate the amount of zinc sulfate and sodium selenite as mg L^{-1} in the culture medium. Different letters indicate significant differences (ANOVA $P<0.05$)

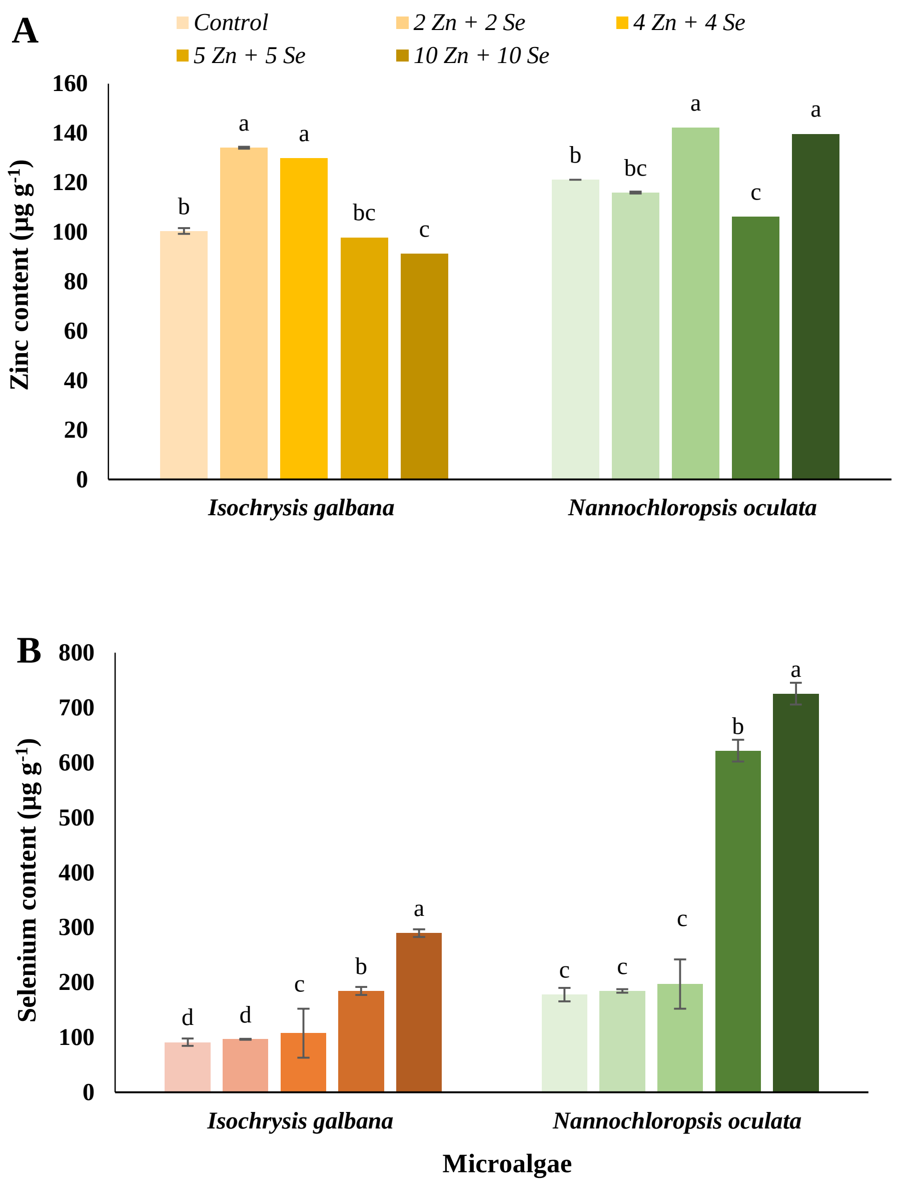

Fig. 3. Changes in Zinc (Zn, A) and Selenium (Se, B) content in *Isochrysis galbana* and *Nannochloropsis oculata* cells cultured with different concentration of mixed zinc sulfate (Zn) and sodium selenite (Se). Different letters indicate significant differences (ANOVA P<0.05)

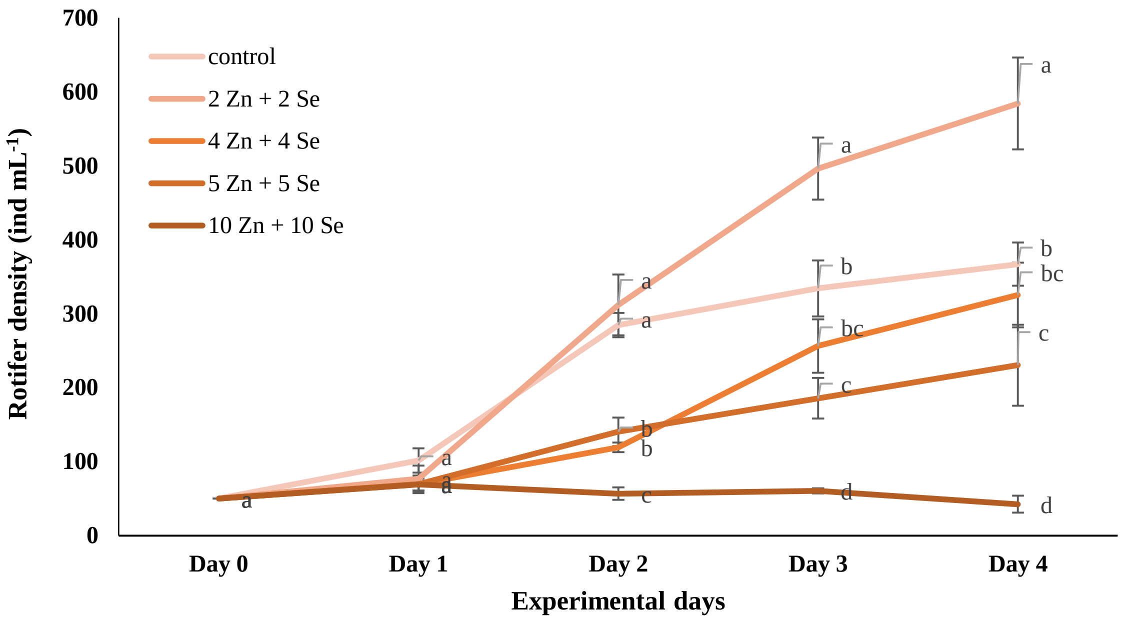

Fig. 4. Population density of rotifers (ind/mL) fed with long-term enriched microalgae (0, 2, 4, 5 and 10 mg L^{-1} of $ZnSO_4.7H_2O$ and Na_2SeO_3) (mean± SD, n=3). Different letters indicate significant differences (ANOVA, P<0.05)

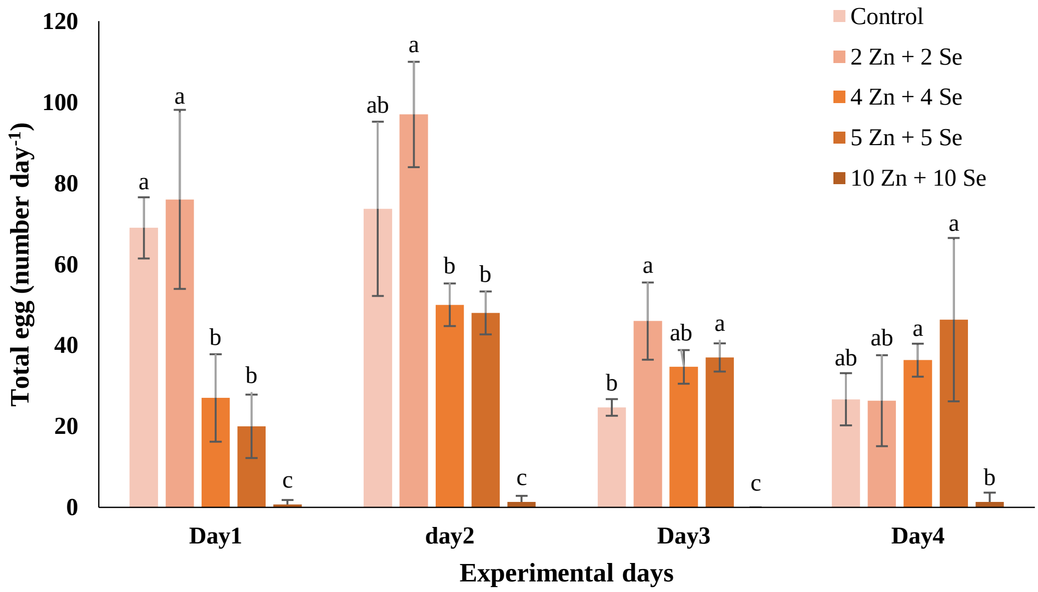

Fig. 5. Eggs produced (number per day) by rotifers fed with long-term enriched microalgae (0, 2, 4, 5 and 10 mg L^{-1} of $ZnSO_4.7H_2O$ and Na_2SeO_3) (mean± SD, n=3). Different letters indicate significant differences (ANOVA, P<0.05)

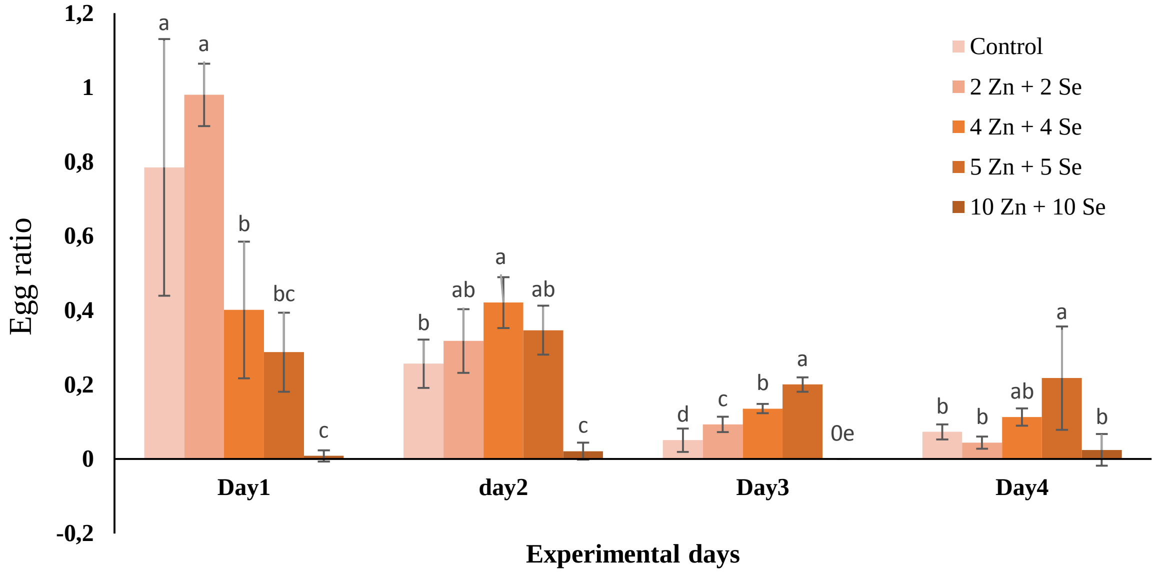

Fig. 6. Egg ratio of rotifers fed with long-term enriched microalgae (0, 2, 4, 5 and 10 mg L^{-1} of $ZnSO_4.7H_2O$ and Na_2SeO_3) (mean± SD, n=3). Different letters indicate significant differences (ANOVA, P<0.05)

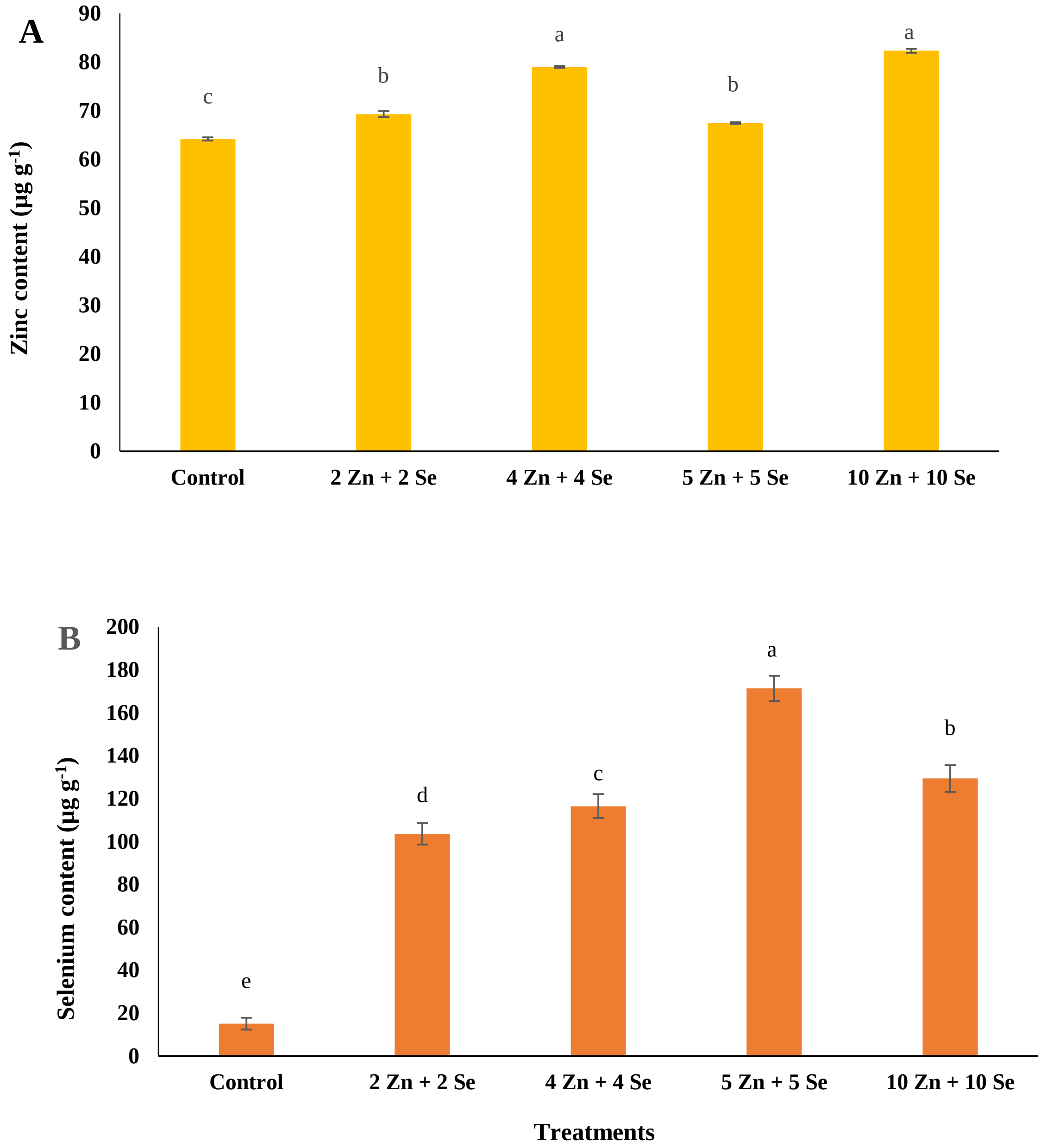

Fig. 7. Changes of A: Zinc (Zn) and B: Selenium (Se) content in rotifers fed with long-term enriched microalgae (0, 2, 4, 5 and 10 mg L^{-1} of $ZnSO_4.7H_2O$ and Na_2SeO_3). Different letters indicate significant differences (ANOVA; $P<0.05$)

Table 1: Dry weight of non-enriched and Zn + Se-enriched microalgae (g L^{-1}) (Mean ± SD).

Microalgae	Treatment	Dry weight (g L^{-1})
Nannochloropsis oculata	0 Zn mg L^{-1} + 0 Se mg L^{-1}	0.44 ± 0.01^{a}
	2 Zn mg L^{-1} + 2 Se mg L^{-1}	0.44 ± 0.02^{a}
	4 Zn mg L^{-1} + 4 Se mg L^{-1}	0.46 ± 0.02^{a}
	5 Zn mg L^{-1} + 5 Se mg L^{-1}	0.48 ± 0.01^{a}
	10 Zn mg L^{-1} + 10 Se mg L^{-1}	0.28 ± 0.01^{b}
Isochrysis galbana	0 Zn mg L^{-1} + 0 Se mg L^{-1}	0.50 ± 0.03^{a}
	2 Zn mg L^{-1} + 2 Se mg L^{-1}	0.52 ± 0.02^{a}
	4 Zn mg L^{-1} + 4 Se mg L^{-1}	0.54 ± 0.02^{a}
	5 Zn mg L^{-1} + 5 Se mg L^{-1}	0.62 ± 0.01^{a}
	10 Zn mg L^{-1} + 10 Se mg L^{-1}	0.24 ± 0.02^{b}

Table 2: Specific growth rate (SGR), N_{max} and doubling time (DT) (Mean ± SD) of rotifers fed on long-term enriched microalgae for 4 days.

Experimental groups	N_{max} (ind mL^{-1})	DT (days)	SGR (day^{-1})
0 Zn mg L^{-1} + 0 Se mg L^{-1}	367 ± 29.3^{b}	1.39 ± 0.059^{bc}	0.50 ± 0.020^{ab}
2 Zn mg L^{-1} + 2 Se mg L^{-1}	584 ± 62.0^{a}	1.13 ± 0.047^{c}	0.614 ± 0.026^{a}
4 Zn mg L^{-1} + 4 Se mg L^{-1}	325 ± 43.8^{c}	1.49 ± 0.105^{b}	0.467 ± 0.033^{b}
5 Zn mg L^{-1} + 5 Se mg L^{-1}	230 ± 54.6^{d}	1.87 ± 0.316^{a}	0.377 ± 0.061^{c}
10 Zn mg L^{-1} + 10 Se mg L^{-1}	42.3 ± 11.5^{e}	-	-0.048 ± 0.069^{d}