# Different Ratio C/N with Sugar Beet Raffinate and Algae-Limit in Artemia franciscana (Kellogg, 1906) Diets on Growth and Reproductive Performance, Biomass Production, Proximate Compositions and Digestive Enzymes

Saeid Vahdat ( Saeidvahdat1989@gmail.com )
Urmia University

Research Article

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## Abstract

The supply of food is one of the most crucial factors in the culture of *Artemia*because the larviculture of fish and shellfish is inextricably dependent on live food. This study focused on adjusting the C/N ratio in *Artemia franciscana* diets in-vivo by adding raffinate and limiting of algae. The nauplii of *Artemia* were fed seven feeding treatments, including a control diet and six experimental feeds with varying concentrations of raffinate and algae. The findings demonstrated that *Artemia* fed a diet containing 10% algae and 1.25% raffinate performed marginally better in terms of survival, but with significantly higher growth, the total number of offspring, alkaline protease, amylase, and lipase activities were detected compared to control. When *Artemia* was fed 10% algae and 1.25% raffinate did the higher biomass and lower FCR become statistically significant. The body protein showed a significant increase when compared to C/N 3.5 (high diet protein). In conclusion, diets containing algae (from 60–80 percent less than control) and raffinate improved growth, biomass production, and reproductive productivity in C/N 9.5 to 10.5.

## Introduction

*Artemia* accounts for an important live feed in the aquaculture industry for the feeding stages of aquatic species (Lavens and Sorgeloos 1996; Anh et al. 2009). The application of various techniques to increase extensive, intensive, and super-intensive mass production in large earthen salt ponds, outdoor environments, as well as in indoor, recirculation, and biofloc systems is the result of reductions occurring in the natural resources as well as rising demand for *Artemia* cysts. (Baert et al. 1997; Naegel 1999; Zmora et al. 2002; Zmora and Shpigel 2006; Van Hoa et al. 2011; Ronald et al. 2014; Vahdat et al. 2018, 2022). *Artemia* species are typically fed on agricultural waste products, organic manures, brans (rice, wheat, and corn), and/or industrially processed feed that includes a variety of pelleted soy protein, whey, algal dried powder, yeasts, and bacteria (single-cell proteins) as the only foodstuffs and/or supplemental feeds in addition to other sources like microalgae (Basil et al. 1995; Zmora and Shpigel 2006; Anh et al. 2009; Vahdat and Oroujlou 2021). Different live and dried unicellular algae, including Dunaliella, are frequently used as food for *Artemia*; however, the pricey and time-consuming process of algal production is considered to be one of the major limitations in the mass culture of *Artemia* (Coutteau et al. 1992; Lavens and Sorgeloos 1996; Naegel 1999; Maldonado-Montiel et al. 2003; Vahdat et al. 2022). Low growth and survival rates, however, were reported when agricultural byproducts (e. g., wheat bran, rice bran, and soybean meal) were used as *Artemia*'s sole diets (Sorgeloos 1982; Anh et al. 2009; Vahdat et al. 2021).

The ethanol and sugar industries that distill fermented molasses have the highest residual pollution output from raffinate and vinasse. A salty liquid was extracted from the base of the distillation columns (Decloux and Bories 2002; Diaz et al. 2002, Montoya et al. 2021). Raffinate and vinasse from beet molasses fermentation is expressly rich in betaine (Decloux and Bories 2002; Caqueret et al. 2008). On the contrary, it has the minerals including potassium, sulfate, phosphorus, calcium, and chloride (Da Silva et al. 2007). Raffinate is a byproduct of vinasse, which is also used to produce bioenergy (Montoya et al. 2021).

It has been shown that using bacterial bioflocs as an additional food source in aquaculture systems can significantly reduce the amount of protein feed needed (Avnimelech 2007; Verdegem et al. 2005; Crab et al. 2007). In general, tilapia and shrimp cultures could produce more bacteria if the carbon/nitrogen (C/N) ratio was raised, for instance by adding starch (Burford et al., 2004; Avnimelech 2007; Verdegem et al. 2005; Crab et al. 2007). Improvements in water quality and a general decrease in environmental pollution are brought about by the significant reduction in nitrogen products in effluents caused by the stimulation of bacterial biofloc production, both in the field and in the lab (Verdegem et al. 2005; Crab et al. 2007).

The aims of this study were manipulating ratio C/N with sugar beet raffinate and algae-limit in *Artemia franciscana* diets on water parameters, growth and reproductive performance, biomass production, proximate compositions and digestive enzymes in-vitru.

## Materials And Methods

The raw Raffinate used in this study had been prepared from the West Azerbaijan Province, Noshin Shahr (Sugar factory). The dried cysts of *A. franciscana* were prepared from the INVEH. The cysts were incubated under standardized hatching conditions (28 $^{\circ}$C, 33 g.L$^{-1}$; pH: 8.5) (Sorgeloos et al. 1986).

### Experimental design

After hatching, *Artemia* instar-I nauplii (n=500) were transferred immediately to a cylindroconical glass tube containing 1.0 L water with 80 g.L$^{-1}$ salinity; the experiment was manipulated by three replications (Vahdat et al. 2018).The experimental *Artemia* were fed in seven different treatments based on the following percentages of supplemental dietary combinations of raffinate:

### Table 1 Should Be Here

The density of the *Artemia* was reduced to one individual (meta-nauplius) per 2 mL in the culture media (Abatzopoulos et al. 2003; Vahdat et al. 2018). Aeration was applied from the bottom of the culture containers with a filtered pipet.

Temperature, pH, and dissolved oxygen (DO) were determined daily by ATC-686 (made in China). Salinity was measured daily by an optic salinity meter (ATC 0-300 ppt). A spectrophotometer was used to measure the levels of total nitrogen (TAN), nitrite, and nitrate (APHA 1998). TDS was measured by ATC-686 (made in China) portable device weekly. Total organic carbon was calculated every week using by Heated-Persulfate Oxidation Method (5310-C)(American Society for Testing and Materials 1994).

### Growth and survival of *Artemia*

The growth rate through the total length (mm) and survival rate (%) of *Artemia* was measured on Days 8, 11, 14, 17 and 20 of culturing with a random sampling of 10 *Artemia* using stereomicroscope (Model; Nikon SMZ, 1500, Tokyo, Japan).

Survival (%) = (final number of *Artemia*/initial number of *Artemia*) × 100.

### *Artemia* reproductive characteristics and life span

To study the females' reproductive performance upon the onset of sexual maturity, 30 broodstocks (male and female) were isolated from rearing media and individually placed in cylindroconical falcon tubes (50 mL) (Vahdat et al. 2018). Each falcon tube was taken as a replication and fed by the same concentration in the previous stage. In this stage, the water quality was held like beforehand. To evaluate the reproductive performance of female broodstock under different conditions, the following reproductive and lifespan characteristics were studied: the total number of offspring (cysts + nauplii), the number of nauplii, the number of cysts, the percentage of encysted embryos, the number of broods, number of offspring per brood, pre-reproductive, reproductive periods, post-reproductive period, offspring per female and lifespan.

### Mass Culture of *Artemia*

Six tanks, each with a working volume of 1000 L, were used for *Artemia* mass culture in the current study along with a control tank to produce bioflocs. Equal amounts of feed from each treatment (T2 to T7) were added to each tank along with the TSS source at starting concentrations of roughly 5000 mg/L (Yao et al. 2018). Saline water (80 ppt) has flowed into each tank. The tanks' internal temperature was kept at 25 0C. There was no water discharged from the tanks because they were set up as an air-water lift (AWL) system. The tanks were 1 point 5 m in length, 1 m in width, and 1 m in height. The water in the tanks was agitated vigorously by the pipes (Lavens et al. 1980). The air blowers were supplied with a 3 hp air pump (Calmo® Side Channel, China) operating at a rate of approximately 215 $m^3$/h. Dissolved oxygen (DO) was maintained at 7.5 ± 0.5 mg/L. The organic carbon to nitrogen ratio (C/N) in the biofloc tanks were maintained at > 9.5-11 by adding raffinate as the external carbohydrates. The bioflocs (the size between 8-56 micron) reached a steady state on day 26 (Hargreaves 2013). With a density of 5000 nauplii per liter starting on day 27, the hatching *Artemia* cysts (INVEH) were added directly to the tanks. There were two tanks in each experimental diet. Fresh water was added each day to make up for the water lost to evaporation. During the culture period, no water was discharged. The feed conversion ratio (FCR) (New 1987) was evaluated for each tank whenever the biomass production was assessed.

Some physicochemical parameters of the culture medium during the rearing period of *Artemia* in all situation were as follows: water temperature 25 °C, dissolved oxygen 7.0–7.5 mg $L^{-1}$, pH 7.8–8.3, salinity 80 g $L^{-1}$, total alkalinity 159 mg $L^{-1}$ $CaCO_3$, total hardness 213 mg $L^{-1}$ $CaCO_3$,$Ca^{2+}$ 29 mg $L^{-1}$,and $SO_4^{2-}$ 107 mg $L^{-1}$.

### Body biochemical composition and total carotenoids

### Crude protein content

To determine the protein content in a whole body of adult *Artemia*, two grams of *A. franciscana* dry matter from each replicate were minced for analysis according to A.O.A.C (2002). Crude protein (N × 6.25) was determined by the Kjeldahl method after acid digestion using an auto-Kjeldahl System (Gerhardt, Germany).

### Lipid and ash contents analysis

The lipid content of *Artemia* was measured by ether extraction (A.O.A.C 2002). For this, two grams of dry samples from each replicate was weighted and homogenized well and transferred to 98% diethyl ether for 12 hours. To calculate the ash content, two grams of dry samples from each replicate were weighed and placed in an electric furnace for 6 hours at 550°C (A.O.A.C 2002).

### Total Carbohydrate content

The Carbohydrate content of the samples was measured according to Hedge and Hofreiter 1962 method. Briefly 100 mg of *Artemia* is hydrolyzed to simple sugar by addition of 5 ml of 2.5 N HCl and incubated in boiling water bath for 3 hours. The solution was neutralized by solid $Na_2CO_3$ until the effervescence ceases. The volume was made up to 100 ml using distilled water and centrifuged at 5000 rpm for 5 min. The supernatant was collected and 0.5 ml of it was diluted with equal volume of distilled water. Then 4 ml of 0.2% anthrone reagent was added to the reaction mixture and heated in boiling water bath for 8 min. The sample was cooled rapidly, and the optical density was read at 630 nm. The standard solution was prepared at different concentrations of 100 $\mu g.ml^{-1}$ glucose (Hedge and Hofreiter 1962).

### Total carotenoids

The adult *Artemia* from each treatment were transferred into the tubes containing 1.5 mL of pure ethanol and kept in the dark at 5 °C for 24 hours. Total carotenoids ($\mu gmg^{-1}$) for each treatment were measured using a spectrophotometer at 450 nm based on the following formula (Britton 1995):

Total Carotenoids (μg/mg) = $1\times10^4$ (OD450 / 2.62%) × (V/W)

Where OD is the optical density (at 450 nm of 1.0 cm cuvette path), V is the volume of fluid in the cuvette (1 mL), W is the weight (mg), and 2.62% is the absorption coefficient of 1.0% beta-carotene at 450 nm.

### Bacterial load of the culture media

The bacterial load of the culture water at different feeding treatments were determined on days 4, 8, 12 and 16 by sampling 1.0 mL from each replicate, diluted in five sequential intervals. Then, the resulting cultures of the last dilution were pour-plated in plate count agar and incubated at 37 °C for 48 h. Finally, the numbers of aerobic bacterial colonies were counted and calculated using the following equation expressed as log10 CFU $mL^{-1}$ (Vahdat et al. 2018).

CFU = colony count × inverted dilution factor

### Analysis of digestive enzymes

The whole body of *Artemia* was rinsed well with distilled water and homogenized by Polytron PT 1300 D homogenizer (15,000 rpm, 3 x 30 sec) (Switzerland) in ice-cold 50 mM Tris-HCl buffer, pH 7.5 (1:3 weight to volume). The homogenate was centrifuged at 10,000 g for 20 min at 4 ºC and the supernatant was collected (Chong et al. 2002) and aliquoted and stored at -80ºC for enzyme analysis (Chong et al. 2002).

Lipase activity was determined by hydrolysis of p-nitrophenyl myristate to p-nitrophenol and myristate (lijima et al. 1998). The total proteolytic activity of the samples was assayed in quadruplet using 2% azocasein prepared in 50mMTris-HCl, pH 7.5 as a substrate García-Carreño and Haard (1993). The alpha-amylase activity was determined by incubating the enzyme extract with 1% starch solution prepared in 20 mM sodium phosphate buffer and 6 mM sodium chloride (pH 6.9) at 25 °C; the method designed by Worthington (1991).

### Static Analyzing

Prior to analysis, Kolmogorov-Smirnov and Bartlett's tests were applied to verify the normality and homogeneity of variances (Sokal and Rohlf 1981). The results obtained from *Artemia* rearing were analyzed by one-way analysis of variance (ANOVA) by SPSS software (V 22). Tukey test was used in order to determine the significant differences between means (P<0.05). Charts were drawn by excel by Office software (V 2016).

## Result

The Raffinate used in this study contained 14.56%, 0.83%, 18.43%, 66.10%, and 95.78 %, respectively of protein, lipid, ash, carbohydrate (dry matter), and moisture (liquid) contents (A.O.A.C 2002).

### Growth and Survival *A. franciscana*

The survival rate of *A. franciscana* fed at the concentration of 1.25% raffinate and 10% algae, showed about 79% at day 20 (p<0.05), on the other hand, a concentration of raffinate with algae more than 5% proved to be appropriate for A. franciscana. Although the concentration of 1.25% raffinate and 10% algae (T7) revealed survival rates were better than other treatments (Fig. 1). Animals fed by 0.625% and 1.25% raffinate concentrations with 10% algae did reach above the size of 10 mm in total length totally, but control became gradually less than 10 mm until day 20 (p<0.05). An increase in raffinate to 1.25% and 10% algae led to a significant increase in total length in *A. franciscana* about 12 mm (figure 2).

### Reproductive Characteristics and Lifespan of *A. franciscana*

*A. franciscana* were fed by 1.25% raffinate and 10% algae had significantly offspring and nauplii of more than 1600 and 1290 by individuals respectively (P<0.05) (Table 2). The number of cysts and percentage of encysted in *A. franciscana* fed by raffinate at 0.625% and 5% algae showed higher values amounting to 328 and 27.17% respectively (P<0.05). At a concentration of 1.25% raffinate and 10% algae observed increasing brood (12 broods) and offspring per brood (134) that were amounting to 1.5-fold more than the control group significantly (P<0.05). post-reproductive showed no significant differences between treatments (P>0.05). Other reproductive characteristics such as reproductive period (57 days), offspring per female (53.42), and lifespan (66 days) showed higher values in diet with a concentration of 1.25% raffinate and 10% algae (T7) significantly (P<0.05). pre-reproductive period (21.60 days) showed higher in control groups significantly (P<0.05).

### Table 2 Should Be Here

The average daily offspring production per female per day by *A. franciscana* was highest in the first and second weeks and fifth and sixth weeks at all treatments (especially between the 2nd to 5th weeks) and showed a downward trend from the sixth week onwards (Fig. 3).

### Body biochemical composition and total carotenoid content of *Artemia*

The protein content of 63.72% in the control group was almost 1.3-fold higher than in T3 (2.5 % raffinate and 1.25 % algae) significantly (P < 0.05) (Table 3). The total amount of lipid showed significant differences (P < 0.05) among all treatments so the highest and lowest contents, respectively, were recorded in 0.625 % raffinate and 10% algae (19.59%), and control (11.77%) treatments. Differences (P > 0.05) were found in the amounts of total carbohydrates among treatments with values higher than 18% for diets containing 0.625% and 1.25 % raffinate with 2.5% algae (T2 and T3) significantly (P < 0.05). Total carotenoids 63.20 µg/mg were significantly found in the control treatment, amounting to 1.6 fold higher than groups containing 0.625% and 1.25 % raffinate with 2.5% algae (T2 and T3)(P<0.05). The lowest amount of ash was shown in control group (13.03%) significantly (P < 0.05).

### Table 3 Should Be Here

### Bacterial load in the culture media

On days 4, 8, and 12, the bacterial load (17.46, 13.89, and 11.35 × 105 CFU mL−1 respectively) in the *Artemia* culture of 1.25% raffinate with 2.5% algae was higher than others significantly (P < 0.05). Meanwhile, the control group and 1.25% raffinate with 10% algae (4.05 and 4.69 × 105 CFU mL−1 respectively) on

day 20 showed lower values significantly between other groups (P < 0.05) (Fig 4).

The results showed that the alkaline protease activity in *A. franciscana* increased significantly in treatments fed by raffinate and 10% algae compared to the control group amounting 2-fold (P > 0.05) (Table 4). The amylase activity in *A. franciscana* was significantly higher in groups that increased algae, and raffinate levels (46.89 U for T7) compared to other experimental treatments (P > 0.05) (Table 4). The activity of lipase enzyme in *A. franciscana* was higher in T7 (0.48U) compared to all treatments significantly (P < 0.05). Also, all groups that fed by raffinate, showed about 2-fold more lipase activity total (Table 4).

**Table 4 Should Be Here**

**Mass Culture of *Artemia***

*Artemia* fed by 1.25% raffinate and 10% algae produced more biomass (2518 g) compared to all other treatments (P < 0.05). All groups of *Artemia* that were fed by raffinate in two levels (with 3 levels of algae) generated more biomass in weeks 3 and 4 significantly (P < 0.05) except for treatment 2 (P > 0.05) (Fig 5).

Based on the results, the persence of raffinate and probiotic bacteria in *Artemia* culture made decreasing FCR in *Artemia*. The lowest FCR was found in a diet containing 1.25% raffinate with 10% algae (0.16) and the highest FCR was in control and 0.625% raffinate with 2.5% algae (P < 0.05)(fig 6).

Water quality variables showed no different significances for nitrogen compounds (total nitrogen, nitrite, and nitrate) in time. Total organic carbon increased in time in different treatments. meanwhile, with raising algae along with raffinate the TOC was increased. As for the TDS, the maximal values were observed in 2.5% and 5% algae with 0.625% and 1.25% raffinate in the last weeks, respectively (Fig 7).

## Discussion

**Growth, Survival and Biomass Production**

Studies conducted in the past have shown that bacterial growth induced by carbohydrate supplementation not only enhances water quality but also boosts the production of desired aquaculture animals (Avnimelech 1999; Crab et al. 2009b; Hari et al. 2004; Nootong et al. 2011). Therefore, in our study, the impact of bacterial growth stimulation on *Artemia* performance using various diets was examined. The findings demonstrate that *Artemia* performed better when raffinate (a source of carbohydrates) was added to the treatments, as measured by body length and higher survival rates. This suggests that *Artemia* may have benefited from bacteria that were grown on carbohydrates as a source of nutrition. Apart from promoting *Artemia* growth by providing extra nutrients, bacteria are also thought to aid in the breakdown of food by producing enzymes (Erasmus et al. 1997; Intriago and Jones 1993). For the control group's treatment, the C/N ratio was here at about 3:5, which is significantly below the range that would be expected (C/N ratio of > 10; Avnimelech 1999) Both as a source of additional food for *Artemia* and for the growth of heterotrophic bacterial communities. In light of this, a biofloc based on the development of probiotic bacterial communities may form or progress in experimental diets with a high C/N ratio. On days 4, 8, and 12 with a significant difference from the other treatments, the highest bacterial growth was found in 2.5% algae and 1.25% raffinate (C/N: 11). Intriago and Jones (1993) and Toi et al. (2013) commented that the bacteria (e.g., Flexibacter strain Inp3) was proven to be a proper food source for the growth and survival of *Artemia* and, in addition, could greatly help in better digestion of dietary algae in the gut of *Artemia*. Nevertheless, Seixas et al. (2009) reported that bacteria are not considered as high-energy food sources for *Artemia* compared to microalgae. Under standard feeding regime, generally slow growth and poor biomass production of *Artemia* were obtained. Also, biofloc formation in conditions of high C/N ratio (Asaduzzaman et al. 2008) might prevent uptake of bacteria by *Artemia*. At high densities bacteria tend to form bioflocs (Avnimelech 1999), which could easily be observed by visual examination of the culture vials. Due to their size, in the range from 8-56 microns (Avnimelech 2011), most of them were uptake by *Artemia*. In contrast, stimulation of probiotic bacterial growth conditions at a high C/N ratio did not negatively affect *Artemia* performance at deficient algae supply (around 2.5 to 10% algae in diets); higher body length and total biomass production were obtained when 5% and 10% algae with two levels of raffinate were compensated by providing carbohydrate as bacterial substrate at both C/N ratios. In the present study, the *Artemia* survival rate was strongly influenced by the diet type. The *Artemia*-fed 25% algae + 75% yeast (control diet) showed lower survival (75.33%) at the end of day 20, in comparison to the 10% algae + 1.25% raffinate diet. Vahdat et al. (2018), Dwivedi et al. (1980) and Basil et al. (1989) reported that separate uses of organic manures or agricultural waste in the diet of *Artemia* reduced nauplii survival rates to less than 10–50% till adult stage, but in present study 63–78% survival was achieved. The survival values detected here are even relatively higher than those reported by other workers possibly due to different cultural environments, initial stocking density, and feeding conditions. For example, the survival rates after 11 days of culture (88.8%) for *A. franciscana* fed experimental diet 7 containing 10% algae + 1.25% raffinate obtained in the current study was relatively higher compared to the data (72, 79, and 73.5%) reported by Naegel (1999), who fed *A. franciscana* with Nestum (a baby food), enriched Nestum, and the microalgae Chaetoceros sp., respectively, for 11 days. In other studies Anh et al. (2009) reported 52–54% survival by feeding *Artemia* with swine manure supplemented with soybean powder and rice bran and similarly, Maldonado-Montiel et al. (2003) reported 50% survival in *Artemia* by using poultry manure in ponds. Vahdat et al. (2018) reported 6-46% survival by feeding *Artemia* with algae and in different vermicompost powder (C/N: 3) levels. High survival in the current study proved that feed C/N ratio and feed content have a significant role in the production of *Artemia* biomass.

**Reproductive performance and longevity**

Under cultural conditions, the first-reproduction age of *Artemia* is thought to be a deciding factor in *Artemia* population dynamics. When the level of algae (5% and 10%) and raffinate (from 0.625% to 1.25%) increased, *A. franciscana* significantly was reduced a shorter time (by 27% in T7) to reach maturity. For three strains of *Artemia* (Tuticorin, USA, Belgian), diets containing organic manures (with varying percentages of incorporation) were used. These diets included cabbage leaves, cow, swine, and chicken manures (Basil et al. 1995). Vahdat et al. (2018) showed a higher level of vermicompost powder in *Artemia* culture required a longer time (by 20%) to reach maturity. A number of cysts production and percentage of encysted in 5% algae with 0.625% raffinate (C/N: 10.5) showed a higher value amounting to 25% more than the control treatment, but there was no difference in the percentage of encysted along with control.

Totally, in feeding diets, the percentage of encysted was less than the control group (except for 5%algae +0.625% raffinate). Vahdat et al. (2018) showed that with increasing vermicompost powder in the *Artemia* diet, the percentage of encysted enhanced from 21% (control) to 86 (100% vermicompost). Also, Vahdat and Oroujlou (2021) reported that soy meal can enhance the percentage of encysted by about 18% compared to the control, but there were no data about the number of cysts produced in the previous study.

Also, a diet with 10% algae and 1.25% raffinate resulted in a 1.15-fold increase of females' reproductive period herein (from 50 to 57 days). increasing of algae with raffinate in diets up to 10% and 1.25% enhanced 57% and 52%, respectively, of the total number of offspring and number of nauplii compared to those received standard diet. Yet, some indices namely the number of broods, offspring per brood, and total life span of females did differ significantly between the groups with increasing raffinate from 0.625% to 1.25% along with algae (2.5% to 10%); nevertheless, all these indices diminished markedly at control group. The effects of diets on the reproductive abilities of three strains of *Artemia* (from Tuticorin, the USA, and Belgium) revealed that the best performance among the three strains was observed in mixed diets (cabbage leaf cow, swine, and chicken manures each at a rate of 25%), which were followed by 20 percent of each of the cabbage leaves, cow, swine, and chicken manures, and 40 percent of the chicken manures (Basil et al. 1995). According to Vahdat et al. (2018), it appears that VCL powder is usable up to 25% only for *A. franciscana* reared and it cannot be fed to *A. franciscana* as a suitable food source for long-term periods. Vahdat and Oroujlou (2021) reported that wheat bran could enhance reproductive properties compared to rice bran and soy meal equivalent to the standard feeding group.

In the present investigation, the average daily offspring production by *A. franciscana* was highest in the first and second and fifth, and sixth weeks at all treatments (especially between the 1st to 2th weeks) and showed a downward trend from the sixth week onwards. Such a trend was also observed by Vahdat et al. (2018) and Anh et al. (2009), where in all groups displayed ascending patterns in fecundity from the 2nd to 4th weeks, which turned into descending modes from the 5th to 11th weeks. The highest fecundities of *Artemia* in their study were recorded, respectively, for 5% and 10% algae with 1.25% raffinate treatments.

### Water quality of AWL tank

Water quality showed salinity 80 ppt in general. Because it restricts the population growth of its predators, higher conditions (salinity greater than 100 ppt), as noted by Dhont and Lavens (1996), are advantageous for the development of *Artemia*. The ideal temperature range for several *Artemia* populations is between 19 and 25 $^{0}$C (Dhont and Lavens 1996). Probably the most important factor in aquaculture is the dissolved oxygen level (Boyd 1990). Only when oxygen levels are below 2 g/l does the production of *Artemia* biomass become inhibited (Dhont and Lavens 1996). The critical levels of dissolved oxygen for *Artemia* range between 0.7 and 1.3 mg/l at salinities between 75 and 320 g/l, according to Mitchell and Geddes (1977). This work's average oxygen content was around 7 mg/l. According to Dhont and Lavens (1996), *Artemia* can tolerate a pH between 6 and 8, which is between 7 and 8 in this work. The two main nitrogen compounds that are thought to pose a risk to crustacean growth are nitrite and ammonia (Mevel and Chamroux 1981). Dhont and Lavens (1996) point out that *Artemia* larvae can tolerate much higher concentrations of nitrite and ammonia than those attained in the current study.

### FCR in AWL tanks

*Artemia* is grown using a variety of production methods, from those that are intensive in fast flow channels to those that are extensive. The Philippines and Thailand have harvested up to 500 kg of biomass wet weight per ha/month from the latter(Jumalon et al. 1987; Sorgeloos et al. 1986; Tackaert and Sorgeloos 1993). In this study, the treatment of 10% algae+1.25% raffinate resulted in an FCR of 0.16 at 4 weeks with a production of 2518 g of adult *Artemia* per tank (1000 liters), while the control treatment showed an FCR of 0.24 with 1690 g *Artemia* biomass production per tank. The mixed diet of algae, rice bran, probiotic bacteria, and raffinate (with C/N around 10) contributed to a much higher yield of *Artemia* biomass (1.7 and 2.5/kg/m3 in the system without water exchange (Tobias et al. 1979; Dobbelier et al. 1980; Dhont and Lavens 1996; Dhont and Van Stappen 2003). The bacterial population that grew in the tanks may also be a source of additional nutrients (Gorospe and Nakamura 1996; Milligan et al. 1980) and probiotic bacteria (Vahdat et al. 2022) for the *Artemia*. Moreover, the FCR of our system was extremely low (0.16-0.24), considering that an FCR of 3– 7 in different temperatures in extensive systems (Vanhaecke and Sorgeloos 1989), and 1 for inert diets, is regarded as normal (Dhont and Lavens 1996). The cost-effectiveness of the product at any scale of production would greatly benefit from this fact.

### Chemical composition of *Artemia*

In this study, the experimental dry diets contained significantly lower protein (13.98% up to 15.97%), and higher lipid levels (21.18% up to 23.49%) compared to the control diet. But the amount of protein was high in the *A. franciscana* fed all feeding treatments, proving the capacity of *Artemia* in converting plant protein to animal protein. The lipid content in the *Artemia*-fed diets containing different levels of lipid was between 15.6%–19.6% showing a significantly increasing trend with elevations in the dietary levels of lipid reflecting the dietary fat content. These data are following the previous studies reporting the effects of diet types on the biochemical compositions of *Artemia*. For example, Simpson and Ronsivalli (1987) reported that 15 days of growing *Artemia* with rice bran and whey powder resulted in protein contents of 50 and 61 points, lipids of 9 and 7 points, ash of 9 and 9 points, and carbohydrates of 24 and 17 points, respectively. Vahdat et al. (2022) mentioned dry algae in *Artemia* diets along with probiotic bacteria did not change protein in the *A. franciscana* body. Additionally, Naegel. (1999) found that *A. franciscana* grown with the alga Chaetoceros, Nestum (powdered baby food), and enriched Nestum, respectively, had protein levels of 56.15, 43.01, and 41.01 percent, and lipid contents of 29.95, 16.50.0, and 20.03.00 percent. Vahdat and Oroujlou (2021) reported that diets containing wheat bran and rice bran could increase protein and lipid in the *Artemia* body. Different levels of vermicompost in an *Artemia* diet decreased protein, and increased lipid in *Artemia* (Vahdat et al. 2018). Total carotenoids in the body of *Artemia* showed a significant decreasing trend in the control group. It seems that decreasing algae in dry diets could potentially involve decreasing the body's carotenoids. Vahdat et al. (2022) demonstrated that *Artemia*, like other crustaceans, receives carotenoid resources through dietary intake, most of which is mobilized to the gonads and egg production. Existing of *D. salina* in the control group as an important rich source of beta-carotene (12–14% of the dry weight) (Ben-Amotz et al. 2009) could be influenced by *Artemia* carotenoids. As Vahdat et al. (2018) reported feeding *A. franciscana* with 100% algae and 75%algae+25% vermicompost increased carotenoids of 45.90 µg

mg−1 to 47.73 µg mg−1 at the adult stage. Also, Vahdat and Oroujlou (2021) mentioned that agricultural products (like soy meal, rice bran, and wheat bran) could decrease total carotenoids up to 10-fold less than standard feeding (46.27 µg mg−1, containing 25% algae). It is noteworthy here that decreasing algae in diets with probiotic bacteria (as a source of carotenoids) could hold total carotenoids in *Artemia* like standard situations.

### Digestive Enzymes

Numerous factors, including age and food quality, have an impact on the activity of digestive enzymes. When using a suitable live or dry feed to promote the growth and survival of *Artemia*, knowledge of these factors may serve as a guide. The Lacto*Bacillus* spp. and *Bacillus* spp. are among those most frequently used in aquaculture. This bacterium can secrete a variety of extracellular enzymes and is naturally present in the *Artemia* culture medium (Moriarty 1997) like proteases (bacitracin and subtilin) (Maget-Dana and Peypoux 1994; Sanders et al. 2003). Because these bacteria can stimulate the digestive system, the host's ability to grow is ultimately improved by better digestion. In particular, *Bacillus coagulans, Bacillus subtilis, LactoBacillus rhamnosus*, *LactoBacillus plantarum*, and *Bacillus licheniformis* have been successfully applied as probiotics for rainbow trout (Raida et al. 2003; Bagheri et al. 2008, Merrifield et al. 2010) and sea bass larvae (*Dicentrarchus labrax*, L.) (Touraki et al. 2012). This product is being used in *Artemia* for the first time. Both proteins and carbohydrates can be broken down by *B. subtilis* and *B. licheniformis*. In accordance with study of Ahmadnia Motlagh et al. (2012), the probiotic bacteria used in diets need at least 10 days to stimulate the release of the digestive enzymes' protease and amylase. It will be easier to formulate a feed with greater digestive enzyme efficiency if aware of how different feed ingredients affect enzyme activity (Deguara et al. 2003). Whether the stimulation of the digestive system or the activity of the bacteria in the digestive tract was to blame for the increase in enzyme activity is still unknown. Probiotic bacteria may improve *Artemia*'s ability to use dietary carbohydrates. Chinese shrimp (*P. chinensis*) (Wang and Xu 2006), *Rutilus rutilus* (Skrodenyt_e-Arbac iauskien 2007), *Sparus aurata* (Suzer et al. 2008), and *Penaeus vannamei* (Wang 2007) have been used as a model to show that bacteria can produce extracellular digestive enzymes.

Life stage, the quantity and chemical makeup of food, and nutritional needs all have an impact on the activities of the digestive enzymes. The presence of high levels of bacteria and algae (1.25% + 10%) in the diets for 20 days significantly increased protease activity in *Artemia* compared to other groups (Vahdat et al. 2022). The treatments fed diets containing raffinate (0.625% and 1.25%) along with 10% algae exhibited higher significance in alkaline protease (2.2-fold), amylase (1.5-fold), and lipase (2.18-fold) compared to control, that showed C/N: 9.5 with adding probiotic bacteria powder could improve digestion enzymes which increased growth rate, survival rate, biomass and FCR in A. franciscana. Avella et al. (2010) investigated this in a study with a combination of three *Bacillus* strains, *B. pumilus*, *B. subtilis*, and *B. licheniformis* were included in the diet of gilthead sea bream (*Sparus aurata*) larvae, the standard length and body weight were noticeably increased. Moreover, in another study on common carp, *Bacillus* sp used as a probiotic led to an increase in fat, starch, and protein in the diets (Wang and Xu 2006). It has been demonstrated that *Bacillus* bacteria can boost the protease, amylase, and lipase activity in *Penaeus vannamei* (Wang 2007). Ahmadnia Motlagh et al. (2012) reported that protease and amylase in *A. urmiana* at day 15 enhanced when probiotics (*Bacillus subtilis* and *B. licheniformis* with a ratio of 1:1) increased from 104 to 106 CFU/g feed, while lipase activity did not show significant difference compared to control. In the present study, the protease activity significantly increased with an increase in dietary algae (from 2.5% up to 10%) and raffinate levels. Vahdat et al. (2022) reported that 10% of algae with 1.25% of bacteria in the diet of *Artemia* resulted in increased alkaline protease activity that could be due to the induction of probiotics for enzyme secretion. Kolkovski (2001) hypothesized that the type and concentration of bacteria and algae in the feed may have an impact on the protease activity.

## Conclusion

the results of current research revealed that the levels of algae and raffinate examined can be accounted for as a suitable food source for the rearing of *A. franciscana* in long-term periods (the whole life cycle) and batch culture (4 weeks for biomass). However, it is possible to use raffinate in the diet of *Artemia* as the greater alternative levels rendered sharp enhancement in growth, biomass, and reproductive performances of *Artemia* (C/N up to 20). According to the findings of this study, a diet based on 5% and 10% algae with 1.25% raffinate is recommended in small-scale cultures. Also, this study demonstrates that bacteria (mainly probiotic bacteria) can be used as a food source for *Artemia*, especially when the algal supply is limited. The nutritional quality of the in-situ-produced bacteria might depend on the standing C/N ratio and/or the carbon source supplied (by raffinate). The main difference between the diets described in this study and other production systems is in the approach to the waste products and discharges of no waste products during the entire growth cycle. Based on the findings of this study, further research is suggested on the effects of raffinate level (as a source of carbohydrate for manipulating C/N) in *Artemia* rearing earthen ponds (in long- and/or short-term applications). Such studies can also provide a more comprehensive judgment on the efficiency of raffinate and probiotics bacteria powder application in semi-natural environments of *Artemia* culture ponds, controlled systems, and even biofloc aquaculture systems.

## Declarations

### Ethical Approval

not applicable

### Competing interests

not applicable

### Authors' contributions

not applicable

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**Availability of data and materials**

The datasets and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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## Tables

Table 1- Different percentages of supplemental dietary combinations of algae and raffinate

| Experimental diets | Rice barn (%) | Algae (%)* | Raffinate (%) | Probiotic Bacteria** (%) | C/N ratio |
|---|---|---|---|---|---|
| T1 | Algae 25% + yeast 75% (Control Group) | | | | 3.5 |
| T2 | 96.775 | 2.5 | 0.625 | 0.1 | 11 |
| T3 | 96.150 | 2.5 | 1.25 | 0.1 | 11 |
| T4 | 94.275 | 5 | 0.625 | 0.1 | 10.5 |
| T5 | 93.650 | 5 | 1.25 | 0.1 | 10.5 |
| T6 | 89.275 | 10 | 0.625 | 0.1 | 9.5 |
| T7 | 88.650 | 10 | 1.25 | 0.1 | 9.5 |

| Experimental diets | Protein (%) | Lipid (%) | Carbohydrate (%) | Ash (%) |
|---|---|---|---|---|
| T1 | 41.24 | 10.18 | 44.64 | 3.93 |
| T2 | 14.09 | 20.35 | 49.32 | 16.24 |
| T3 | 13.98 | 20.23 | 48.70 | 17.09 |
| T4 | 14.73 | 20.86 | 47.76 | 16.65 |
| T5 | 14.65 | 20.73 | 48.08 | 16.57 |
| T6 | 16.05 | 21.18 | 45.27 | 17.50 |
| T7 | 15.97 | 21.13 | 45.58 | 17.32 |

*The algae used, was *Dunaliella salina*.

**The freeze-dried of *Bacillus Coagulans, Bacillus Subtilis, Bacillus Licheniformis, Lactobacillus Rhamnosus, Enterococcus Faecium* and *Lactobacillus Plantarum* with same ratio and total count $6\times10^9$ CFU/g.

Table 2- Mean (±SD) of various reproductive and lifespan characteristics for *A. franciscana* under exposure of different concentrations of raffinate

| Characteristics | T1 | T2 | T3 | T4 | T5 | T6 | T7 |
|---|---|---|---|---|---|---|---|
| Total Number of Offspring | $922.96\pm53.91^{a}$ | $1028.13\pm43.99^{ab}$ | $1310.43\pm68.37^{cd}$ | $1194.43\pm51.36^{bc}$ | $1532.00\pm69.55^{de}$ | $1366.76\pm45.32^{cde}$ | $1602.63\pm61.65^{e}$ |
| Number of Nauplii | $675.83\pm44.97^{a}$ | $821.56\pm36.89^{ab}$ | $991.73\pm50.46^{bc}$ | $865.73\pm40.11^{ab}$ | $1258.90\pm58.02^{de}$ | $1075.23\pm47.17^{cd}$ | $1292.36\pm52.51^{e}$ |
| Number of Cysts | $247.13\pm23.04^{ab}$ | $206.56\pm17.42^{a}$ | $318.70\pm29.76^{ab}$ | $328.70\pm31.07^{b}$ | $273.10\pm33.72^{ab}$ | $291.53\pm24.26^{ab}$ | $310.26\pm25.49^{ab}$ |
| Percentage of Encysted | $27.19\pm2.17^{b}$ | $20.02\pm1.36^{ab}$ | $24.08\pm1.58^{ab}$ | $27.07\pm1.90^{b}$ | $17.38\pm1.85^{a}$ | $21.68\pm1.69^{ab}$ | $19.30\pm1.35^{ab}$ |
| Number of Brood | $9.73\pm0.38^{a}$ | $8.86\pm0.27^{a}$ | $11.53\pm0.39^{b}$ | $11.26\pm0.30^{b}$ | $11.53\pm0.49^{b}$ | $11.54\pm0.33^{b}$ | $12.03\pm0.24^{b}$ |
| Offspring Per Brood | $94.76\pm4.07^{a}$ | $116.96\pm4.51^{bc}$ | $113.07\pm3.66^{ab}$ | $106.12\pm3.86^{ab}$ | $136.44\pm5.93^{c}$ | $119.53\pm3.63^{bc}$ | $134.87\pm6.20^{c}$ |
| Reproductive Period | $50.80\pm0.89^{ab}$ | $48.30\pm0.85^{a}$ | $53.63\pm0.77^{bc}$ | $55.00\pm0.82^{bc}$ | $56.46\pm0.88^{c}$ | $53.36\pm1.93^{bc}$ | $57.83\pm0.80^{c}$ |
| Post-Reproductive Period | $3.03\pm0.37^{a}$ | $3.96\pm0.68^{a}$ | $4.03\pm0.57^{a}$ | $4.16\pm0.37^{a}$ | $6.30\pm1.97^{a}$ | $4.26\pm0.41^{a}$ | $4.26\pm0.42^{a}$ |
| Pre-Reproductive Period | $21.60\pm0.22^{d}$ | $19.73\pm0.16^{c}$ | $17.56\pm0.22^{b}$ | $17.73\pm0.23^{b}$ | $16.26\pm0.18^{ab}$ | $17.70\pm0.31^{b}$ | $15.76\pm0.46^{a}$ |
| Offspring Per Female | $30.76\pm1.79^{a}$ | $34.27\pm1.46^{ab}$ | $43.68\pm2.27^{cd}$ | $39.81\pm1.71^{bc}$ | $51.06\pm2.31^{de}$ | $45.55\pm1.51^{cde}$ | $53.42\pm2.05^{e}$ |
| Life-Span | $58.43\pm0.62^{ab}$ | $57.00\pm0.40^{a}$ | $60.23\pm0.48^{b}$ | $62.90\pm0.50^{c}$ | $63.96\pm0.55^{c}$ | $64.36\pm0.46^{c}$ | $66.86\pm0.47^{d}$ |

Values within the same row not sharing common letters are significantly different ($P<0.05$).

Table 3- Proximate composition of whole body (expressed in percent dry weight) and total carotenoids (µg mg−1) of *Artemia franciscana* fed diets containing different levels of raffinate and algae

| | T1 | T2 | T3 | T4 | T5 | T6 | T7 |
|---|---|---|---|---|---|---|---|
| Lipid | 11.77±0.08$^{a}$ | 15.61±0.16$^{b}$ | 16.02±0.12$^{b}$ | 18.25±0.41$^{c}$ | 19.13±0.45$^{cd}$ | 19.59±0.02$^{d}$ | 19.21±0.58$^{cd}$ |
| Ash | 13.03±0.55$^{a}$ | 15.61±0.33$^{b}$ | 16.38±0.22$^{b}$ | 17.25±0.96$^{bc}$ | 18.84±0.24$^{c}$ | 18.58±0.29$^{c}$ | 18.52±0.32$^{c}$ |
| Protein | 63.72±0.35g | 50.11±0.35$^{b}$ | 48.57±0.06$^{a}$ | 54.88±0.11$^{d}$ | 53.17±0.33$^{c}$ | 61.18±0.44$^{f}$ | 59.50±0.13$^{e}$ |
| Carbohydrate | 11.25±0.99$^{b}$ | 18.44±0.16$^{c}$ | 18.79±0.16$^{c}$ | 9.37±1.49$^{b}$ | 8.65±1.02$^{b}$ | 1.08±0.16$^{a}$ | 2.54±0.41$^{a}$ |
| Carotenoids | 63.20±0.39$^{d}$ | 39.69±0.33$^{a}$ | 40.51±0.50$^{a}$ | 43.88±0.28$^{b}$ | 44.73±0.07$^{b}$ | 50.12±0.63$^{c}$ | 51.33±0.54$^{c}$ |

Values within the same row not sharing common letters are significantly different (P<0.05).

Table 4- Digestive enzymes activities (U activity min ¹mg ¹ protein) in A. franciscana with 6 replicates (Means ± SD).

| | T1 | T2 | T3 | T4 | T5 | T6 | T7 |
|---|---|---|---|---|---|---|---|
| Alkaline protease | 0.82±0.14$^{b}$ | 0.29±0.04$^{a}$ | 0.64±0.17$^{b}$ | 0.87±0.07$^{b}$ | 1.30±0.09$^{c}$ | 1.65±0.08$^{d}$ | 1.81±0.07$^{d}$ |
| Amylase activity | 30.35±1.97$^{a}$ | 32.63±0.21$^{a}$ | 31.92±0.88$^{a}$ | 36.64±1.58$^{b}$ | 32.13±0.02$^{a}$ | 40.83±1.02$^{c}$ | 46.89±1.47$^{d}$ |
| Lipase activity | 0.22±0.00$^{a}$ | 0.43±0.02$^{bc}$ | 0.43±0.01$^{bc}$ | 0.41±0.01$^{b}$ | 0.42±0.00$^{b}$ | 0.46±0.03$^{cd}$ | 0.48±0.01$^{d}$ |

Values within the same row not sharing common letters are significantly different (P<0.05).

## Figures

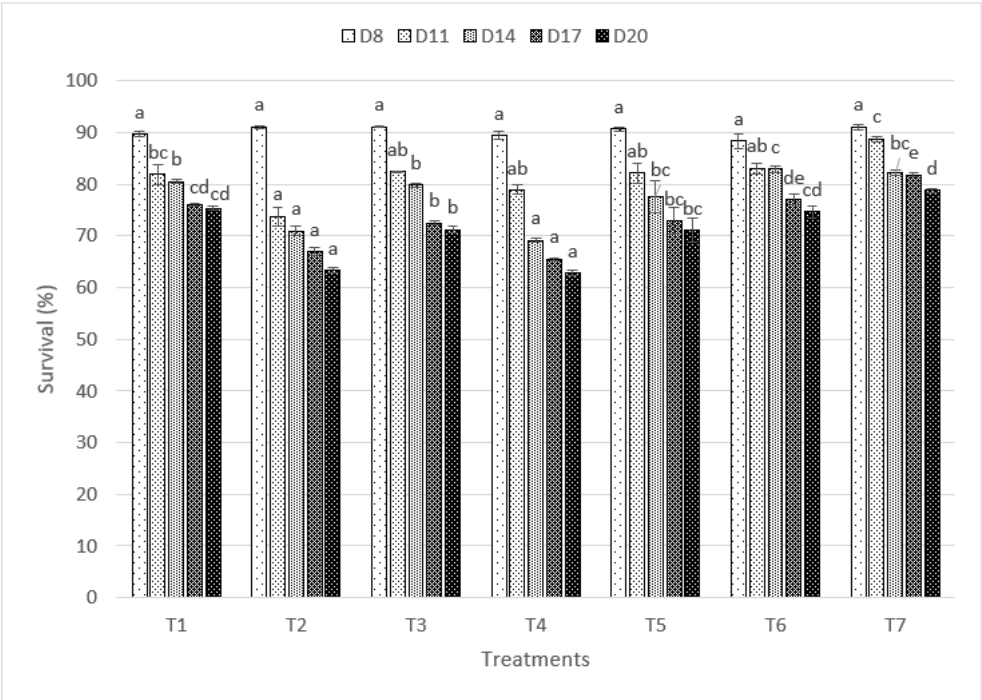


**Figure 1**

Survival of *A. franciscana* cultured at different concentrations of raffinate

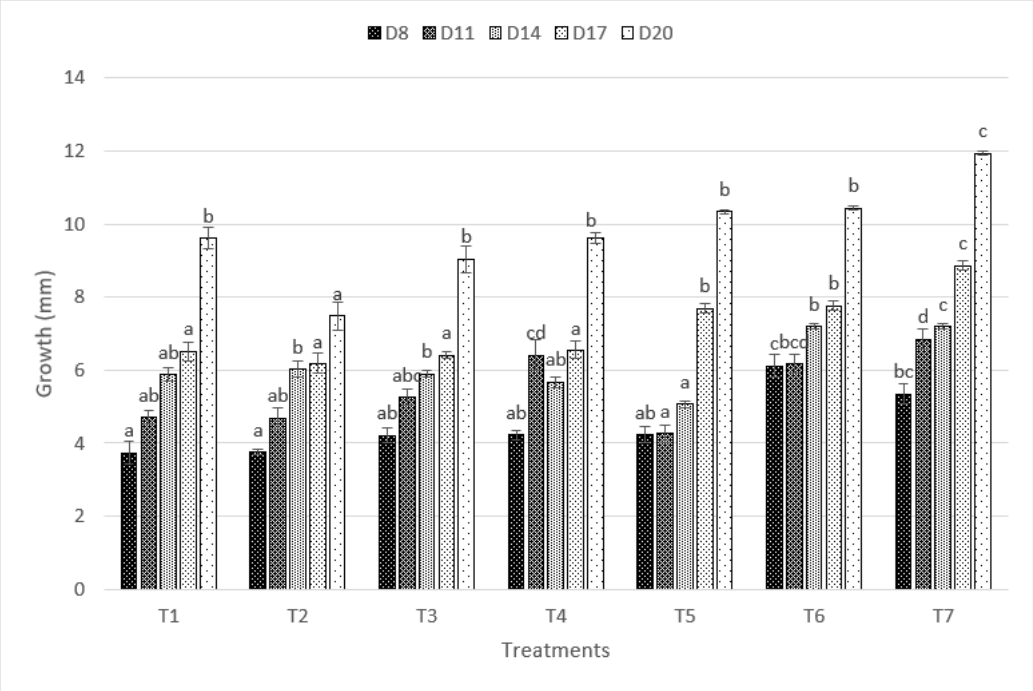


**Figure 2**

Growth of *A. franciscana* cultured at different concentrations of raffinate

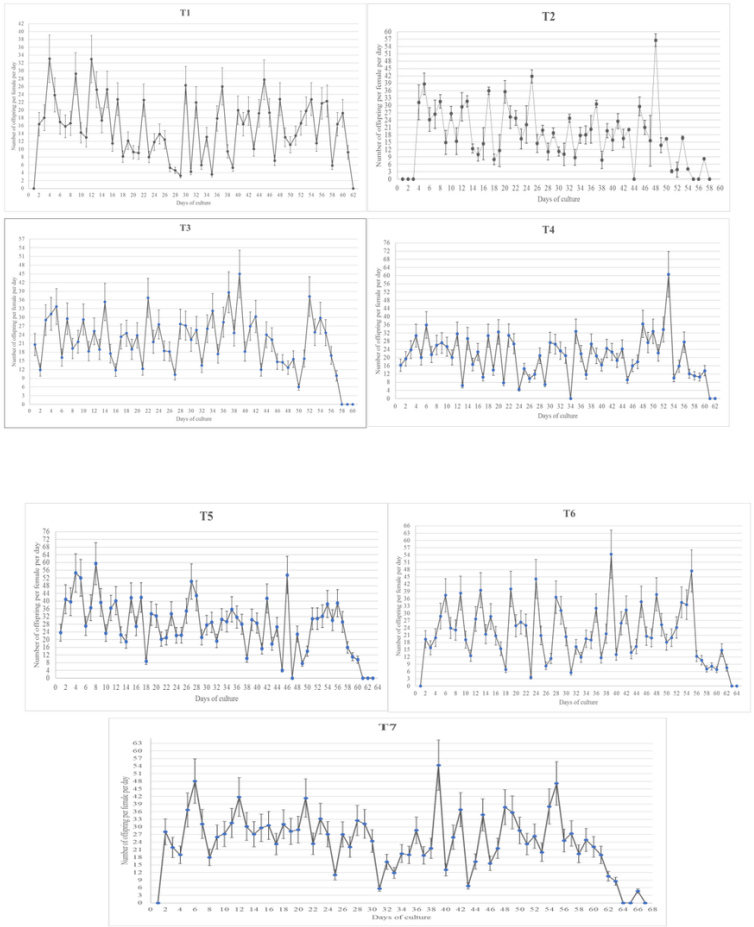

**Figure 3**

Average (mean ± SE) daily offspring production per female over the experimental period in *Artemia franciscana* at feeding regimes containing different concentration of raffinate and algae [SE = SD/√n; n = 30 females]

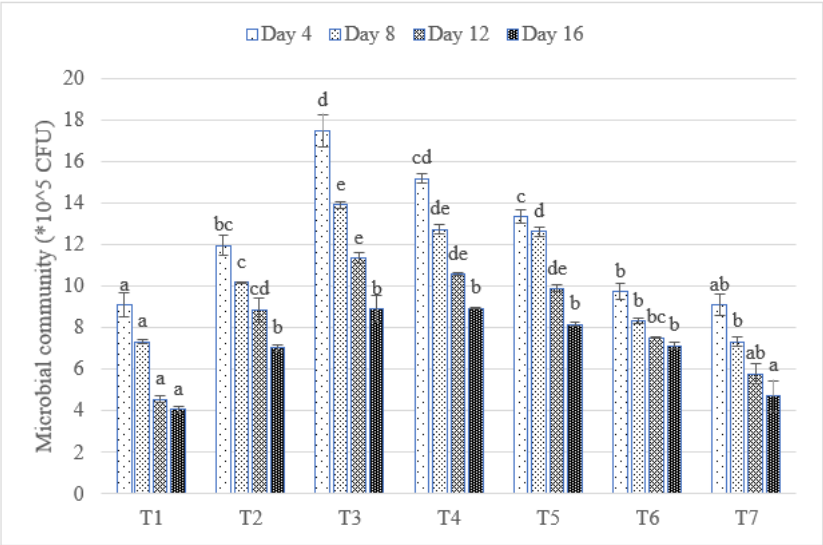


**Figure 4**

Total bacterial load (n ×$10^5$ CFU $mL^{-1}$; mean ± SE) in the culture medium of *Artemia franciscana* at different treatments on rearing period

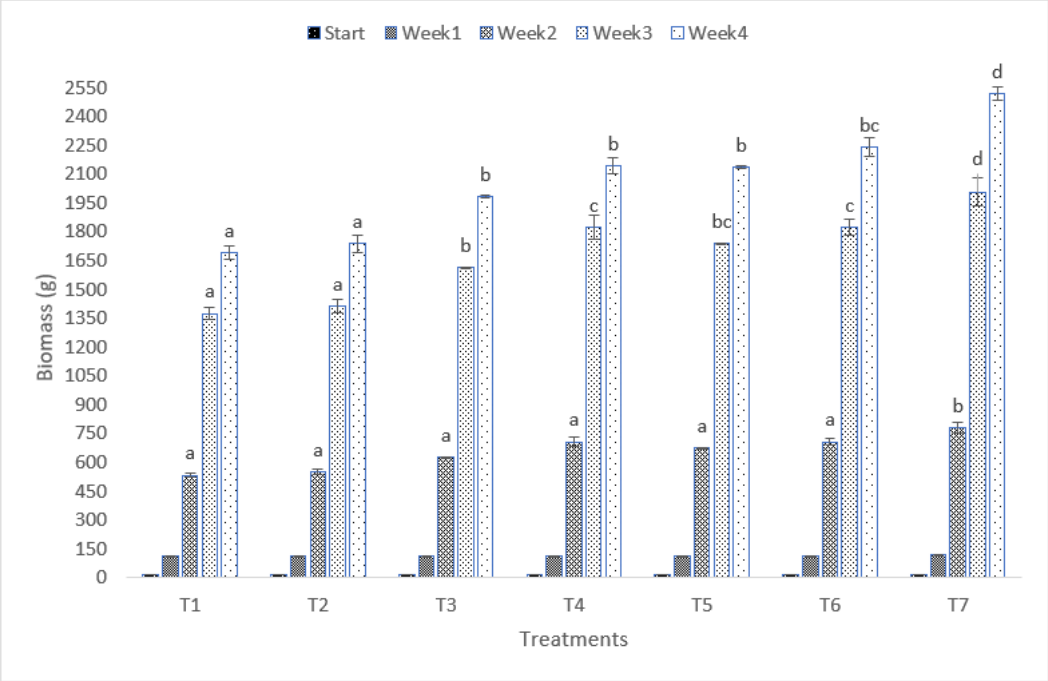


**Figure 5**

Biomass (g) production of *A. franciscana* cultured at different treatments in AWL tanks for 4 weeks.

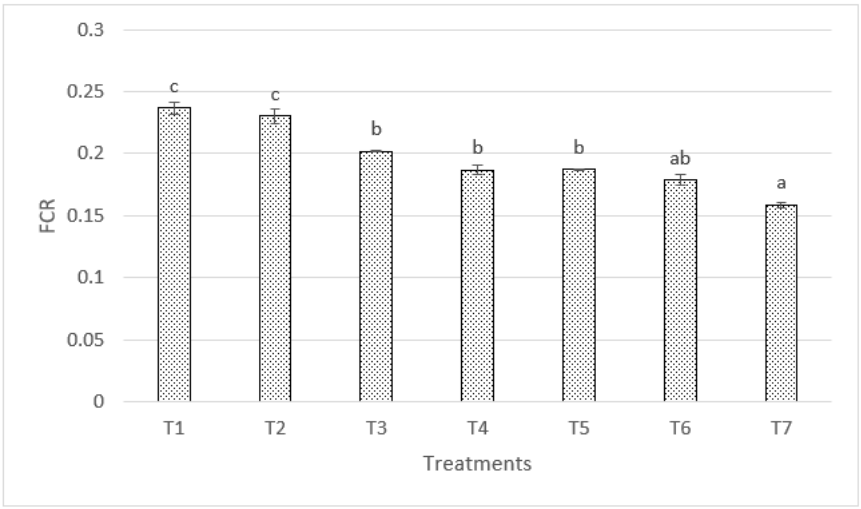


**Figure 6**

FCR for *Artemia franciscana* fed by different diets in AWL tanks after 4 weeks.

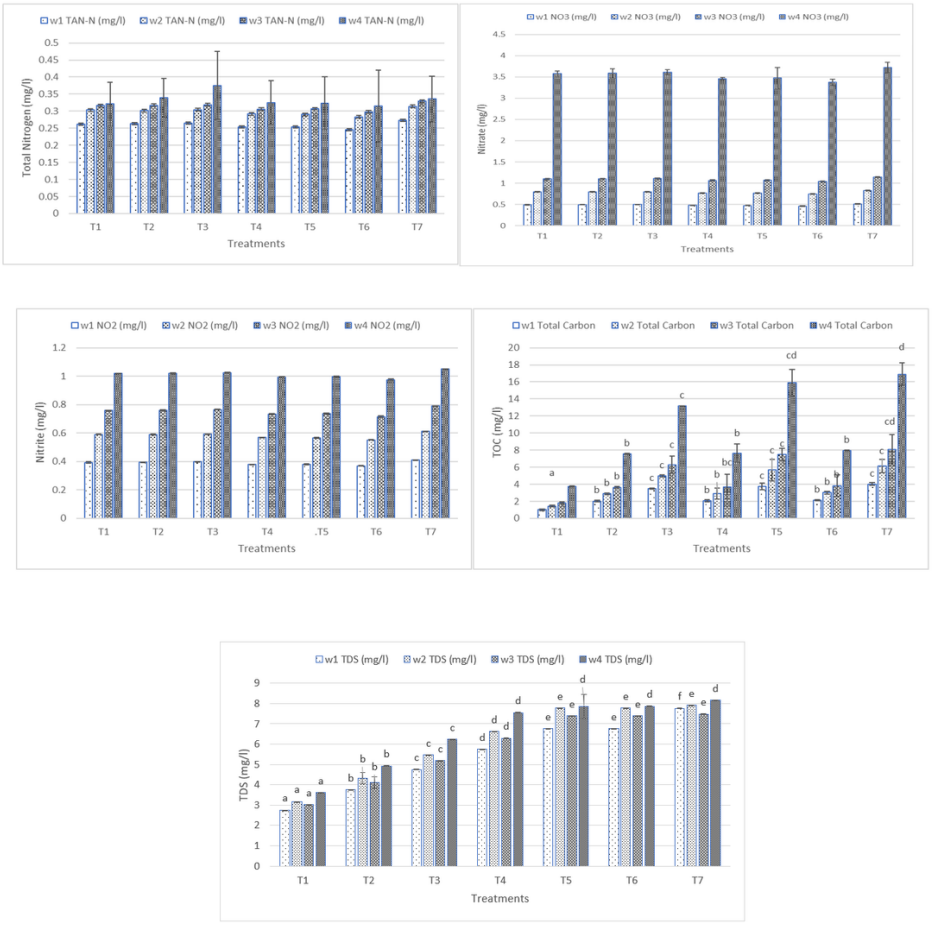


**Figure 7**

Mean (±SD) weekly water chemical (total nitrogen, nitrite, nitrate, total organic carbon and TDS) fluctuations over the entire culture period (four weeks).