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Porphyridium cruentum (red microalga) added in Biofloc system to growth and pigmentation source of *Heros severus.*

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Abstract
Inside aquarist industry, colored organisms are one of the most important criteria in the market. Since fish cannot synthesize pigments *de novo*, they must be supplied as additives in diet or culture. Therefore, in this study, it was compared the survival, growth, and pigment content of *H. severus* using *P. cruentum* as a carbohydrate and pigment source in a Biofloc system. The culture was carried out in 60 L containers with 20 juvenile of *H. severus* for 90 days. The following experimental treatments were used: Control and *P. cruentum* (freeze-dried, powder, and frozen). The Frozen treatment showed the best results in growth (11.29 cm and 124.87g), 100% survival, and a pigment concentration of 4.20 µg. Therefore, *P. creuntum* can be considered as a natural source of low-cost pigments for organism commercialization.

Keywords: *Porphyridium cruentum*, *Heros severus*, Biofloc, pigments, growth.

1. Introduction
In recent years, ornamental aquaculture has become a key activity for the economy of some regions around the world, whose import in different countries has an estimated value of one billion dollars and a total industry estimate of $18 to 20 billion dollars per year [1], becoming an important component of world trade due to the increase in international demand and the export market. In Mexico, around 20.8 million of freshwater ornamental fish are produced annually, of which around 61 varieties of 19 species of ornamental fish of national, Asian, and South African origin are cultivated [2].
This interest is because there are attractive and colorful species that can be kept as pets in little aquaria to enjoy their beauty, making them a very popular hobby [3, 4]. Among the cultivated species it can be found the peach fish (*Heros severus*), a freshwater fish with great potential for aquarium life: not aggressive behavior, good adaptation, and easy reproduction [5, 6]. In the aquarist industry, the color of organisms is one of the most important criteria for their commercialization, which is a quality criterion that can determine the price of various species in the market, however, fish cannot synthesize pigments *de novo* and must be supplied as additives [7]. This characteristic has generated an increase in studies dedicated to finding natural sources of pigments that increase the pigmentation of these organisms.
Among the natural sources of pigments, we find the microalgae *Porphyridium cruentum*, which is one of the marine microalgae that has currently called attention to use for this purpose [8]. Among the great ecological value of this microalgae in the ecosystem, as a primary producer, it can fix carbon, reducing the greenhouse level gases in the atmosphere [9]. Likewise, it plays an important role in the trophic chain since it constitutes the principal food for the larval stages of many fishes, crustaceans, and mollusks, both in free life and in culture systems. Also, it is rich in different bioactive substances, which have a high value of direct use as pigments, since they are used as a natural colorant for food and cosmetics, or as a fluorescent probe in fluorometric techniques [10, 8]. On the other hand, recent studies have demonstrated their hepatoprotective, anti-inflammatory, and antioxidant properties. Also, was used in therapies for the treatment of some cancerous tumors and leukemia treatment [8, 11].

Likewise, the accelerated growth of the ornamental aquaculture industry caused a negative impact on the environment because their water effluents discharge organic compounds and pollute the other water ecosystems that receive this wastewater [12]. To reduce these environmental damages and optimize fish production, the use of Biofloc technology has been developed, which consists of the creation of microbial flocs produced by their carbon-nitrogen ratio content and only 0.5 to 1% water change per day [13] and strong water movement by air supply [14, 15]. Many authors considered that the use of low protein diets and external carbohydrate source as cassava, macroalga, and moringa meal or liquid molasses, that allows the growth of bacterial community that use these sources for they metabolism and uptake toxic nitrogen compounds transforming them in non-toxic substances, solving the problem of nutrient saturation in the culture system [14-18].

It should be noted that this microalgae, *P. creuntum*, is not used in ornamental aquaculture industry and commercial species; however, it may represent a viable alternative for its production due to its high contribution of carotenoids that, in addition to providing pigmentation to the skin and tissue of organisms, provide nutrients that are vital for growth, serve as antioxidants, support reproductive metabolism, the immune system, and reduce stress effects [19], giving to *P. cruentum* microalgae a great option value to be used for pigmentation in the ornamental and aquaculture industry.

Among the most commonly used techniques for obtaining these carotenoids are sun and oven drying. However, it requires temperatures between 40° to 80°C for a long period, which is a negative condition to prevent sample and bioactive and nutritional compounds polluted [20]. Another technique is freeze-drying, which consists of dehydrating the cells at low temperatures and under vacuum, eliminating the possibility of degradation [21]. This method is one of the most advanced for drying products with high nutritional value in the food industry since it preserves their nutritional qualities, as well as flavor, aroma, and color [22].

Therefore, the present work aims to use *P. cruentum* microalgae preserved with different techniques (Powder, Freeze-dried, and Frozen) to produce Biofloc and increase the coloration and development of *H. severus*.

2. Materials and methods

The experimental work was carried out at the Live Food Production and Biofloc Laboratory at Universidad Autónoma Metropolitana, Unidad Xochimilco.

2.1 Culture of *Porphyridium cruentum*

The culture started from an isolated sample of *P. cruentum* preserved in agar, which was inoculated with 500 mL of water (45 PSU) in a 1 L container, with aeration and continuous light at 21°C and fertilized every third day with 0.5 mL of Triple17 fertilizer (17% N-P-K, VIGORO Excelso® brand) and a commercial multivitamin with vitamins a, b1, b2, b6, b12, c, d2; nicotinamide; calcium; phosphorus; iron; magnesium; zinc; manganese; potassium and soy lecithin (Simivitamins®). The culture was stepped at 5, 10, 20 L, each time the culture reached a concentration of 500 x 10^6 cells mL^{-1}, to be filtered through 10 µm sieve for drying with different techniques.

2.2 Experimental design

Juveniles of *H. severus* were acclimatized for four weeks in 25 L aquaria with a 200 W thermostat to maintain the temperature at 27±2°C and pH of 6.5. They were fed with WARDLEYS® brand flake, which was free of pigments. A rock and sponge filter were placed to maintain the cleanliness of the aquarium and air stone to maintain high oxygen levels and move all water column. Organisms of 2.69±0.37 were transferred to a container with a volume of 60 L of water and a density of 20 organisms per container with the same physical and chemical parameters [23].

Every 15 days, a 100 mL sample was taken from each culture vessel to determine the concentration of nitrite (NO_2- mgL^{-1}), nitrate (NO_3- mgL^{-1}), ammonium (NH_4-mgL^{-1}), phosphate (PO_4^{-3}) using a HANNA® Multiparameter Model No. HI83325. In addition, 1 L of sample was taken from each vessel and placed in an Imhoff Cone to determine the amount of settleable solids and thus not to exceed 15 mL per liter [24].

Four experimental treatments were carried out with the following carbon sources: a) Control (coffee); b) *P. cruentum* Lyophilized; c) *P. cruentum* Powder; and d) *P. cruentum* Frozen. The experimental culture was carried out for 90 days. Each treatment was carried out in triplicate.

2.3 Biofloc production

For floc formation in the culture system, all treatments were added with 0.01% of the total fish biomass, maintaining a C:N ratio of 20:1 [25].

2.4 Fish feeding

A commercial diet, brand EL PEDREGAL®, was used for all treatments with 45% protein, 15% fat, and 3.0% fiber, which was considered to be free of pigments. The feed was supplied with 5% of the total biomass of the organisms, divided into two rations according to Emerenciano *et al*. formulas [25].

2.5 Biometric parameters

Every 15 days it was measured the standard length and weight of the organisms. The standard-length was measured with the aim of a digital Vernier Calliper® model TM-52012 with a precision of 0.01mm. The weight of each organism was determined with a digital scale Nimbus® with a precision of 0.01g.

Gain value (G) was obtained using the following formula:

G = Final value – initial value

Absolute growth rate (AGR) value was obtained using the formula:

$$AGR = \frac{\text{Finnal value} - \text{Initial value}}{\text{Total culture days}}$$

Instantaneous growth rate (IGR) value was obtained using the formula:

$$IGR = \frac{\text{LN (Finnal value)} - \text{LN (Initial value)}}{\text{Total culture days}} \text{ x } 100$$

For survival index (SI) the following formula (Wang *et al.*) [28] was used:

$$SI\% = \frac{\text{Total survival organisms}}{\text{Total Innitial organisms}} \text{ x } 100$$

2.6 Biofloc zooplankton composition

Every 15 days a sample of 100 mL was taken, which was left to sediment for 30 minutes and a sample of 1 mL was taken

with Leica ICC50 HD optical microscope connected to a Image® Pro-Plus 7.0 program. Taxonomic groups were identified with specialized literature [26].

2.7 Pigment extraction

One gram of fish muscular tissue was taken from six organisms from each experimental container and placed in a 10 mL plastic container with anhydrous sodium sulfate (2.5 g). Subsequently, the sample was homogenized, and 5 mL of chloroform was added. The samples were storage for 48 hrs at 0°C. The supernatant was taken (5 mL) and analyzed in Spectronic 20, Genesys at 500 nm and contrasted with the control and the blank [27].

Tissue carotenoid content value (µg wet weight^{-1}) was calculated using the following formula:

$$\text{Carotenoids content} = \frac{\text{Maximum absorbance wavelength}}{(0.25 * \text{sample weight (g)})} * 10$$

Where:

10= Dilution factor

0.25= Extinction Coefficient

2.8. Statistical analysis

A database was made with all values in Excel 2019 to obtain descriptive statistics, as well as the growth curves. To compare growth and carotenoid concentration between experimental groups, a one-way ANOVA analysis was made. Founding significant differences ($p<0.05$), a multiple means test was made using Tukey's test technique.

3. Results

3.1 Physical and chemical water parameters

Regarding chemical parameters evaluation, the Powder treatment obtained the lowest range of NH_4^+, followed by the Frozen treatment with 0.60 mgL^{-1} and 0.63 mgL^{-1}, respectively, as for NO_2^-, NO_3^- and PO_4^{3-}, the highest range was the Lyophilized treatment with 24 mgL^{-1}, 92.27 mgL^{-1} and 6.60 mgL^{-1} respectively. However, it had the lowest value for pH with 7.87. Table 1 shows the mean value of chemical parameter throughout the experimental treatments.

Table 1: Chemical parameters mean values of culture medium of experimental treatments.

Variable (mgL^{-1})	Experimental treatment			
	Control	Powder	Frozen	Lyophilized
NH_4^+	0.63	0.60	0.63	0.65
NO_2^-	19.00	10.00	19.00	24.00
NO_3^-	85.07	81.57	85.07	92.27
PO_4^{3-}	5.77	2.97	5.77	6.60
pH	8.10	8.23	8.10	7.87

3.2 Survival

The highest treatment survival value at the end of the experiment was Frozen treatment with 100%., followed by Powder treatment with a value of 88%. Lyophilized treatment with 83%. Meanwhile, the Control treatment obtained only 80% of survival.

3.3 Biometric parameters

The mean values (±D.S.) of standard length and initial and final weight, as well as AGR, IGR, and gain, are shown in Table 2. The Frozen treatment obtained the highest values of standard length (11.29±0.43 cm), AGR (0.09 cm day^{-1}), IGR (1.58% day^{-1}) and gain (8.60 cm). The Control treatment obtained the lowest values of final length (6.26±0.38 cm), AGR (0.06 cm day^{-1}), IGR (1.40% day^{-1}), and gain (3.20 cm day^{-1}). With respect weight values, the Frozen treatment obtained the highest values with 124.87±0.50 g, AGR (1.36 g day^{-1}), IGR (5.04% day^{-1}), and gain (75.57 g). All values presented significant differences among treatments ($P<0.001$).

Table 2: Mean values of biometric parameters from *H. severus* at four experimental treatments.

Biometric variable	Control	Powder	Frozen	Lyophilized
Initial length (cm)	2.69±0.41	2.69±0.40	2.69±0.35	2.69±0.32
Final length (cm)	6.26±0.38	10.07±0.49	11.29±0.43	11.07±0.36
AGR (cm día^{-1})	0.06	0.08	0.09	0.09
IGR (% día^{-1})	1.40	1.45	1.58	1.55
Gain (cm)	3.20	7.38	8.60	8.38
Initial weight (g)	1.27±0.57	1.27±0.57	1.27±0.45	1.27±0.38
Final weight (g)	23.71±0.90	97.55±0.48	124.87±0.50	122.42±0.70
AGR (g día^{-1})	0.22	1.06	1.36	1.33
IGR (% día^{-1})	4.36	4.77	5.04	5.02
Gaian (g)	13.32	58.76	75.57	74.06

At Fig.1, the growth curves of four experimental treatments with respect standard length.

Fig. 2 shows the weight growth curve of the organisms at four experimental treatments.

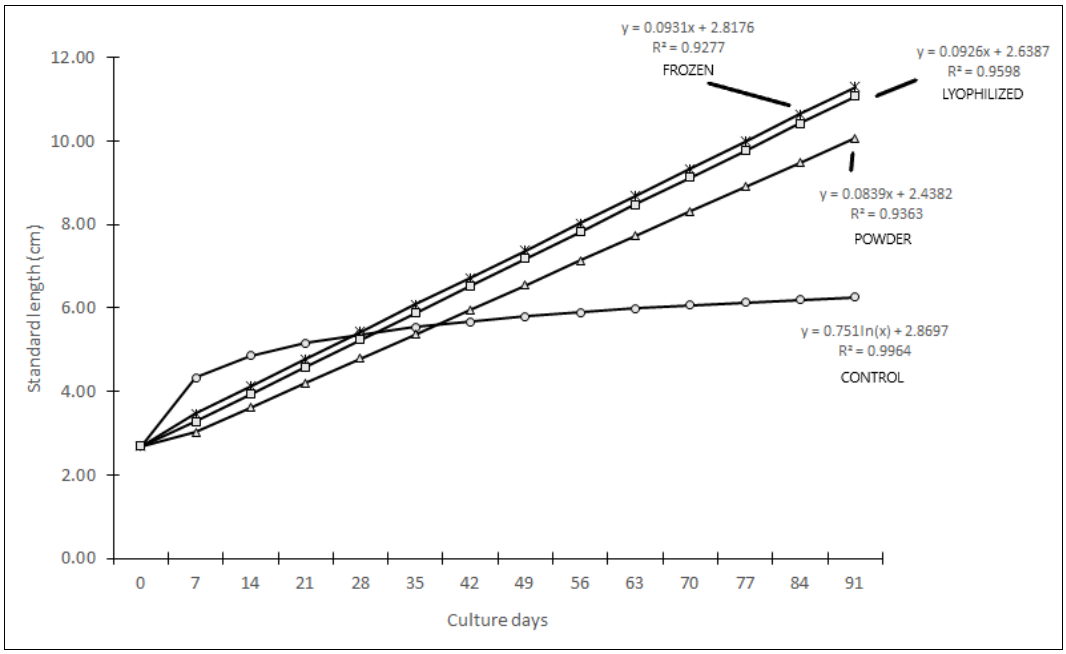

Fig 1: Standard length growth curves (cm) of *H. severus* fishes at four experimental treatments.

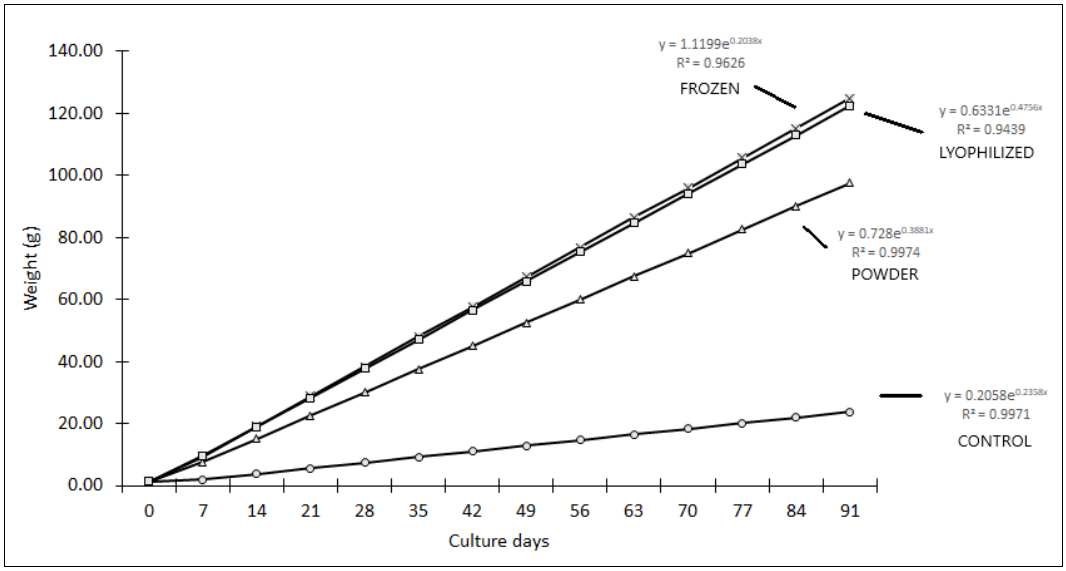

Fig 2: Weight growth curves (g) from *H. severus* organisms at four experimental treatments.

3.4 Zooplanktonic Biofloc composition

In table 3 it is showing the composition of microorganisms observed in the Biofloc throughout the experiment. Among the genera that always were present in the four experimental treatments were *Centropyxis* sp., *Paramecium* sp., and *Vorticella* sp. However, the Control treatment had a richer Biofloc structure than the other treatments. At the fourth week of the acclimatization period, all treatments have rotifers presence, for the sixth week, the Powder treatment already had nematodes presence.

Table 3: Microorganisms present in the culture medium of the four experimental treatments.

Experimental treatment	Presence of zooplankton genus
Control	*Arcella, Centropyxis, Tokophyra, Coleps, Paramecium, Philodina Brachionus, Lecane, Aelosoma, Vorticella.*
Powder	*Lecane, Vorticella, Centropyxis, Paranema, Paramecium, Nematodos*
Frozen	*Coleps, Centropyxis, Paramecium, Philodina, Tokophyra, Vorticella.*
Lyophilized	*Coleps, Lecane, Philodina, Centropyxis, Tokophyra, Paramecium.*

3.5 Pigmentation of fish

The Frozen treatment showed differences in carotenoid content and optical density with respect the other experimental treatments. This treatment obtained the highest value with 4.20µg and 0.105 A respectively. The lowest values were showed at Control treatment with 0.013 A and 0.52µg (Table 4). Fish pigmentation was significantly different for all treatments ($P< 0.001$).

Table 4: Optical density and carotenoid content values of *H. severus* of experimental treatments.

Experimental treatment	Optical density (500 nm)	Carotenoid content (µg)
Control	0.013	0.52
Powder	0.048	1.91
Frozen	0.105	4.20
Lyophilized	0.035	1.39

4. Discussion

Biofloc is an increasingly used technology in ornamental fish culture, because it offers numerous benefits like the improvement of water quality caused by microbial adhesion and their use as a natural biological filter, helping to maintain optimal oxygen levels in the culture system, reducing ammonia and nitrite levels [14, 18]. In this context, with respect to the culture of organisms in Biofloc in this experiment, the system demonstrated the ability to maintain physical and chemical parameters, especially ammonium, within the optimal range as mentioned by Veras *et al.* [6], where the optimal value for this species is 1.02±0.46 mg L^{-1}. In this study, a minimum average of 0.60 mg L^{-1} was observed for the Powder treatment, while the maximum value, which corresponds to the Lyophilized treatment, obtained an average of 0.65 mg L^{-1}. This low ammonium levels content can be reflected in the survival of the organisms because all treatments show survival rates above 85%. Only in Lyophilized treatment show 15% of mortality.

In an experimental study with *Astronotus ocellatus* (Oscar fish) in a Biofloc system [29], in which was added a pigment-rich diet, it was obtained a survival up 85%, like that reported in this experiment. However, in another study discussing the addition of pigment substrates like *Capsicum annuum*, *Rhodococcus* sp., and *Tagetes erecta* in *Pterophyllum scalare* (Angel fish) [27], which were not placed in a Biofloc system, they obtained survivals of 77.8, 75.0, and 72.2%, respectively. Therefore, the use of Biofloc technology in aquaculture is highly profitable since, as mentioned above, it improves water quality and increase the survival of organisms, as mentioned by Avilés *et al.* [30].

On the other hand, a close relationship is established between fish growth and diet digestibility, especially the protein fraction as the primary factor in their diet, since it is one of the most important nutrients for this process [31, 32]. However, some studies report a positive effect of pigments on fish growth, like Pérez-Escalante [33] study which obtain a positive effect in the diet with Jamaican pigments (*Hibiscus sabdariffa*) on growth, weight gain and survival in *Carassius auratus* (Goldfish). They observed that these increase as the dose of anthocyanins in the diet is added. In the present study, the protein content supplied by the feed was 45%, it is important to mention that the protein content of *P. cruentum* as an additive for the formation of Biofloc is 39% [34] and according to the obtained results, it is observed that the treatments rich in carotenoids supplied by the microalgae were more efficient in size and weight than the Control treatment, since the highest growth is shown in the Frozen treatment with a standard length of 11.29 cm with lower differences of 0.22 and 1.22 cm in the Lyophilized and Powder treatment.

According to Tacón [35], the IGR is a sign of nutritional status, as a good sign of protein quality, and when standard length and weight growth were compared of *H. severus* organisms, it is found that Castro-Castellón *et al.* [29], obtained better results in size in *A. ocellatus* with a pigmented diet in a Biofloc system than the Control diet, which obtained values of AGR of 0.03 cm day^{-1}, IGR of 0.89% day^{-1}, and gain of 3.96 cm, it should be noted that both diets were between 47-55% protein content. In this study, the Frozen treatment was the one that obtained the highest AGR, IGR, and gain, with 0.09 cm day^{-1}, 1.58% $day^{-1,}$ and 8.60 cm, respectively.

Castro-Mejía *et al.* [36], studied *Amatitlania nigrofasciata* (Convict cichlid) growth efficiency fed with 0. 8mm shrimp pellet, TetraColor®, carrot, and beet, in a Biofloc system and observed that the diets with carrot showed highest values of size and weight with an AGR, IGR and gain of 0.05 cm day^{-1}, 4.55% day^{-1} and 2.24 cm respectively. These results were compared with those obtained in the present study, which were higher. However, it is important to note that the carrot diet used by Castro-Mejía *et al.* [36], had a protein content of 30%, which represents 15% less compared to the diet used in this study.

Regarding the efficiency in weight of the organisms, Aviles-López *et al.* [30], used two diets for *Danio rerio* (Zebrafish) and *A. ocellatus.* The diets consisted in *Daphnia* sp. enriched with microalgae and bacteria; the other diet consisted with trout pellets (45% protein) and Biofloc produced with coffee as carbon source. Live feed diet obtained higher values with a AGR (0.03 g day^{-1}), a IGR (2.02% day^{-1}) and a gain (4.51g) for *D. rerio,* and a AGR (0.08g day^{-1}), IGR (2.70% day^{-1}) and gain (10.75g) for *A. ocellatus*. However, in both cases, the results were lower than those found in the present study, since the treatment with the Frozen treatment obtained a higher AGR, IGR, and gain with 1.35g day^{-1}, 5.04% $day^{-1,}$ and 75.57g, respectively.

All experimental treatments showed, in early culture stages, more abundant communities of rotifers, but Powder treatment presented nematodes communities. According to the observations of Perez [37], rotifers can break the flocs, consume bacteria, and generate mucilage which contributes to the formation of new ones. On the other hand, nematodes, recognized for their high crude protein and essential fatty acid content, which play a crucial role in the Biofloc system, providing a rich and continuous source of live food for the organisms, as was mentioned by Monroy-Dosta *et al.* [38]. Therefore, not only the quantity, but also the quality of the protein supplied in fish culture is essential, as it positively influences the growth of the organisms, as indicated by Schulz *et al.* [39]. Moreover, the inclusion in the diet of natural additives rich in pigments not only contributes to the coloration of fish but also plays a crucial role in their growth, as shown in the present study, supporting the statement of Amar *et al.* [40].

Color in ornamental fishes constitutes a fundamental characteristic for evaluating their quality and in recent years there has been growing interest in finding natural sources of pigments [41]. In this context, the use of microalgae biomass has been investigated for its potential as a color increase agent [42, 43, 44].

Frozen treatment showed higher pigmentation with a total carotenoid content of 4.20µg. When these results were compared with those obtained by Alishahi *et al.* [5], which added *Dunaliella salina* microalgae powder as a supplement

in diets for *H. severus* achieved a skin carotenoid concentration of 1.0 to 1.2µg, but a higher concentration is highlighted in the present study. Likewise, in a study with addition of *Spirulina platensis* in *A. ocellatus* diet to evaluate pigmentation [45], similar results were obtained with a total carotenoid concentration in the skin of the fish of 4.72µg. It is important to note that the efficiency of carotenoids on the physiological response, and thus on fish coloration and growth, is species-specific, due to variability in the use of metabolic pathways and the transformation of these compounds into pigment tissues [5].

The use of microalgae like *P. cruentum* has great importance in ornamental fish industry, because it can synthesize valuable bioactive substances such as phycoerythrin, astaxanthin, and carrageenan, as well as extracellular polysaccharides and PUFA's during its growth process. It also accumulates mainly arachidonic (ARA, 20:4n-6) and eicosapentaenoic (EPA, 20:5n-3) acids [46]. These fatty acids allow *P. cruentum* their antioxidant and immunomodulatory function [47]. However, there are certain limitations that must be considered such as the variability in its nutritional composition, because it depends of their culture methods. However, the use of microalga *P. cruentuem* biomass for ornamental fish pigmentation seems promising because the facility to obtain it and their subsequent freezing method, was economically profitable, besides the fact that it can be a good substitute for synthetic pigments which only represent an expensive pigment (between 15-20% dietary cost) and possibility a toxic factor to cultured aquatic organisms [49].

5. Conclusions

Positive results were obtained with the use of *P. crentum* microalgae, principally at Frozen treatment, because it shows the highest carotenoids amount, better coloration, growth, and weight, providing them with a greater wellbeing. Those characteristics were use as important criteria for the commercialization. *P. creuntum* can be considered as a viable option in aquaculture industry to use it as a natural additive.

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