AquaTechnica 5(2): 80-90(2023)
ISSN 2737-6095
DOI https://doi.org/10.33936/at.v5i2.5233
https://doi.org/10.5281/zenodo.7987022

Effect of larval density and diet on the growth and survival of *Perna perna* mussels in recirculation system (RAS)

Efecto de la densidad larval y la dieta sobre el crecimiento y supervivencia del mejillón *Perna perna* en sistemas de recirculación (RAS)

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Original article | Artículo original

Keywords
Brown mussel, closed systems, larviculture, stocking density, hatchery.

ABSTRACT | This study evaluated the effect of initial larval stocking density and microalgae concentration diet on the growth and the number of competent larvae (number of live larvae at the end of the experiment retained on a sieve with mesh size of 210 µm) of *Perna perna* in a water recirculation system (RAS). The experiment was conducted using a completely randomized design and factorial scheme. The first factor was the food concentration, that was composed of a mix of *Chaetoceros müelleri* (CM) and *Isochrysis galbana* (ISO), in a proportion of 30:70 respectively, at three levels (low: between 1.26 to 2.19, medium: between 2.36 to 3.94 and high: 5.03 to 7.29 x 10^4 cells mL^{-1}). The second factor being the larval culture density, also at three levels (20, 80, and 200 larvae mL^{-1}), with 3 replications in each treatment. Larvae fed with the diet with low and high concentrations did not reach competence (210 µm) in 16 days. However, on day 16, larvae fed with the medium concentration (2.36 to 3.94 x 10^4 cells mL^{-1}), reached in average percentage of competent larvae 21.8% ± 7.45, 27.4% ± 9.91 and 0.80% ± 0.26 at the densities 20, 80 and 200 larvae mL^{-1}, respectively.

Palabras clave
Mejillón marrón, sistemas cerrados, larvicultura, densidad de siembra, hatchery

RESUMEN | Este estudio evaluó el efecto de la densidad inicial de larvas y la concentración de microalgas en la dieta sobre el crecimiento y el número de larvas competentes (número de larvas vivas al final del experimento retenidas en un tamiz con un tamaño de malla de 210 µm) de *Perna perna* en un sistema de recirculación de agua (RAS). El experimento se llevó a cabo utilizando un diseño completamente al azar y un esquema factorial. El primer factor fue la concentración del alimento, que estuvo compuesto por una mezcla de *Chaetoceros müelleri* (CM) e *Isochrysis galbana* (ISO), en una proporción de 30:70, respectivamente, en tres niveles (bajo: entre 1,26 a 2,19, medio: entre 2,36 a 3,94 y alta: 5,03 a 7,29 x 10^4 células mL^{-1}). El segundo factor fue la densidad del cultivo larvario, también a tres niveles (20, 80 y 200 larvas mL^{-1}), con 3 repeticiones en cada tratamiento. Las larvas alimentadas con la dieta con bajas y altas concentraciones no alcanzaron la competencia (210 µm) en 16 días. Sin embargo, en el día 16, las larvas alimentadas con la concentración media (2,36 a 3,94 x 10^4 células mL^{-1}), alcanzaron en porcentaje promedio de larvas competentes 21,8% ± 7,45, 27,4% ± 9,91 y 0,80% ± 0,26 a las densidades 20, 80 y 200 larvas mL-1, respectivamente.

INTRODUCTION

One of the most important issues in the cultivation of mussels, a centuries-old activity practiced around the world (Ferreira, Magalhães 2004), is obtaining young forms (seeds) for development of the population in each cycle.

In the case of Brazil, the main species used for the cultivation of mytilids is *Perna perna* (Linneaus 1758). The state of Santa Catarina is responsible for the highest national production, totalling 14,078 tons (EPAGRI 2022). Production of this species occurs mainly through obtaining seeds based on natural capture in artificial collectors or collection from natural stocks (Suplicy 2017), both of which present limitations.

Production based on extracting seeds from natural stocks (Aarab *et al*. 2013, Ferreira, Magalhães 2004, Lazo, Pita 2012, Suplicy 2008) has constraints associated with the reduction of stocks in the short term, making them

Recibido: 06-10-2022 | **Aprobado:** 17-05-2023 | **Versión final:** 28-05-2023

unfeasible economically, ecologically (Marshall *et al*. 2010) and legally, since the activity is regulated by rigorous legislation (Domínguez *et al*. 2010).

Production based on harvesting from the natural environment through artificial collectors is subject to variability in the number of individuals collected throughout the year (Licet *et al*. 2011, Mesquita *et al*. 2001, Stakowian *et al*. 2020). Another issue is the competitive interaction with other species that are also harvested in artificial collectors (Tureck *et al*. 2020); for example, the invasive species *Mytilus galloprovincialis* (Lamarck 1819) that is present in the cultivations in Santa Catarina (Belz *et al*. 2020). In addition, the collectors occupy a considerable portion of the cultivated area (Ferreira, Magalhães 2004, Suplicy 2008). Therefore, the production of seeds in the laboratory allows for a reliable supply throughout the year (Helm *et al*. 2004), and may be characterized as a non-extractive activity.

The production of *P. perna* larvae in the laboratory has been conducted and described in static systems (Aarab *et al*. 2013, Beduschi *et al*. 2009) and in a continuous-flow system (FTS) (Turini *et al*. 2014), showing positive results for production chain of the species. However, it is necessary to improve cultivation techniques in order to achieve the efficiency found in other species of molluscs (i.e., oysters, pectinids) of economic interest produced in the same locality.

The implementation of water recirculation systems (RAS) presents an opportunity for the cultivation of larvae in a more efficient way, as it uses a smaller physical space and has low water consumption, in addition to allowing the control of the water temperature, the maintenance of stable water quality and beneficial microbiota (Blancheton *et al*. 2013, Kamermans *et al*. 2016, Ramos *et al*. 2021). These factors favor the well-being of the larvae and, therefore, are most promising for the larval cultivation of molluscs. However, the RAS demands considerable investment and operational capital (Kubitza 2006, Masser *et al*. 1992) and there are still few studies involving the cultivation of marine mollusc species, with applications of this system conducted only on an experimental basis (Joaquim *et al*. 2016, Magnesen, Jacobsen 2012, Merino *et al*. 2009, Pfeiffer, Rusch 2000, Xiongfei *et al*. 2005, Zohar *et al*. 2005).

There are some factors of great importance for the successful cultivation of larvae in RAS, whose objective is to maximize growth and survival without overloading the cultivation system and degrading water quality (Magnesen *et al*. 2006). Larval stocking density is a determining factor related to inter-individual competition for food, in addition to influencing the risk of contamination associated with a greater accumulation of organic material, bacteria and ammonia (Lagos *et al*. 2015). Another important factor is the availability of food, which, together with the correct temperature control, results in larvae being able to settle in a shorter time (Magnesen *et al*. 2006, Rico-Villa *et al*. 2008, 2009).

To promote the development of techniques for the production of *P. perna* mussel larvae in the laboratory, the present study aims to evaluate the growth and yield (number of competent larvae) of *P. perna* larvae submitted to three initial larval densities, and at three food concentrations in a water recirculation system.

MATERIAL AND METHODS

Obtaining the larvae

The experiment was carried out at the Marine Molluscs Laboratory (LMM) of the Federal University of Santa Catarina (UFSC), located at the Prof. “Elpídio Beltrame” Mariculture Station (CCA/UFSC).

To obtain D larvae, 70 mussels of the *P. perna* species, with an average height of 7.7 ± 2.1 cm, collected in the municipality of Porto Belo (S 27º08'632 W 48º32'665), were induced to spawn using the technique described by Turini *et al*. (2014). After fertilization, the embryos were incubated in a static tank with 15,000 L seawater until they reached the "D" larval stage. Upon reaching this stage, larvae were screened onto a sieve with a mesh size of 35 µm, quantified and stocked in the RAS system tanks according to experimental larval density treatments.

Experimental design

The experiment was carried out using a completely randomized design, with three replications, in a factorial scheme, with a total utilization of 27 experimental units (EUs). These EUs were distributed on three shelves, containing 9 larviculture tanks on each shelf, kept in a closed environment. The first factor was the concentration of food at three levels [low (LC): between 1.26 to 2.19 x 10^4 cells mL^{-1}; medium (MC): between 2.36 at 3.94 x 10^4 cells mL^{-1} and high concentration (HC): between 5.03 to 7.29 x 10^4 cells mL^{-1}; Table 1] and the second factor was larval density, also at three levels (20, 80 and 200 larvae mL^{-1}).

Structures and management

The RAS used in this study was described by Ramos *et al*. (2022, see Figs 1, 2 for details). Microalgae were supplied to the EUs through silicone tubes and a peristaltic dosing pump (Seko PR Seko-4- 4 L h^{-1}), accordingly with diet concentration (LC, MC, or HC). Marine water and food were delivered to the tanks through a specialized inlet connector that prevented the larvae from escaping if the water level of the tank raised above the desired level. The filtered marine water flowing into the EUs was adjusted to the flow of 200 mL min^{-1}.
The recirculation system caused the water that left the EUs containing plenty of food, faeces, pseudofaeces, and other suspension compounds to return to the receiving tank, where it was pumped through to the Skimmer (SK). Soon after, the treated water returned to the distribution tank being pumped through the UV filter, and then the heat pump, returning to the EU.

Management of the culture tanks was carried out every three days and consisted of cleaning the structures and sieving larvae (Ramos *et al*. 2022). The water filters were removed and cleaned using a water jet. The SK was set for automatic cleaning every 2 h. The larvae in the system were fed a mixed diet of the diatom *Chaetoceros müelleri* (CM) and the flagellate microalgae *Isochrysis galbana* (IG), in a proportion of 30:70 respectively, offered according to the number of cells by larvae per day as described by Helm *et al*. (2004; Feeding Strategy 2; Table 1).

Microalgae stocks were cultivated in 100-liter plastic bags enriched with F/2 medium for IG and F/2 + silicate solution for CM. The cultures were cultivated at 24 ± 2 ° C, with a salinity of 35 ± 1, and a photoperiod of 24 hours light.

Table 1. Microalgal concentrations of microalgae on diets used in the RAS.

Days after fertilization	**Microalgae Concentration (10^6 cells $larvae^{-1}$ day^{-1})**	**Microalgae Concentration dosed continuosly into EU (x10^4 cells mL^{-1})**		
		LC	**MC**	**HC**
1	4	1.26	2.36	5.03
2	4	1.26	2.36	5.03
3	4	1.26	2.36	5.03
4	4	1.26	2.36	5.03
5	4	1.26	2.36	5.03
6	6	1.48	2.44	5.26
7	6	1.48	2.44	5.26
8	6	1.48	2.44	5.26
9	7	1.57	3.40	5.42
10	7	1.57	3.40	5.42
11	7	1.57	3.40	5.42
12	8	2.19	3.94	7.29
13	8	2.19	3.94	7.29
14	8	2.19	3.94	7.29
15	8	2.19	3.94	7.29
16	8	2.19	3.94	7.29

CM = *Chaetoceros müelleri;* IG = *Isochrysis galbana;* LC = low microalgae concentration; MC = medium microalgae concentration; HC = high microalgae concentration.

Data collection

To assess larval growth, a sample of larvae from each experimental unit was removed on the fourth, tenth, thirteenth and sixteenth days of cultivation and fixed in 4% formalin. Subsequently, the length of 30 larvae (the longest anteroposterior dimension of the shell) was determined using a LEICA® optical microscope, a Sedgwick-Rafter camera, and the LAS EZ 2.0.0 software.

The number of competent larvae was defined at the end of the experiment (16th culture day) by the identification of eyed larvae with a foot (pediveliger) retained on a sieve with a mesh size of 210 µm. The competent larvae were fixed in 4% formalin and quantified with the aid of an optical microscope and a Sedgwick-Rafter chamber.

Physical and chemical parameters of the culture water temperature (°C), salinity (KASVI®, model K52-100), pH (ALFAKIT®, model AT-350), dissolved oxygen (O.D.; mg L^{-1} and %) were measured daily with the aid of a multiparameter probe (model YSI 556MPS).

Daily microalgae counts were performed at the inlet and outlet of water from the cultivation units with the aid of a Neubauer chamber and an optical microscope, calculating the difference between the algae concentration at the inlet and at the outlet, to evaluate food consumption in each unit.

Statistical analysis

The shell height and number of competent larvae were analyzed using a two-factor analysis of variance (microalgae concentration and culture density) and when there was a difference between treatments, their means were compared using Tukey's test at a significance level of 5%. The analyses were performed using the computer package SAS® (2003).

RESULTS

The average temperature of the water in the culture tanks during the experiment was 27.8 ± 0.4°C and salinity of 35. The average pH was 7.8 ± 0.12 and the dissolved oxygen was 5.8 ± 0.2 mg L^{-1} with saturation of 90.2 ± 2.9%.

Throughout the larviculture period, in all experimental units, the presence of microalgae was observed at the exit of the larviculture tanks in lower concentrations (LC) than at their entrance (Table 2). The consumption of microalgae in the diet of *P. perna* larvae in the RAS system suggests that, regardless of the concentration and stocking density used, consumption varies according to the days of cultivation. It is suggested, however, that consumption remains constant in tanks with 20 and 80 larvae mL^{-1} under the medium feed concentration regime (MC), although the highest consumption initially occured in tanks with 200 larvae mL^{-1} under the high feed system (HC) of microalgae in the first 11 days, with an abrupt drop in the final days.

Table 2. Concentration of microalgae at the entrance of larviculture tanks (diets provided) and at their exit, throughout the days of cultivation at different cultivation densities.

Microalgae concentration	Position of sample	Days after fertilization											
		1-5			6-8			9-11			12-16		
		Initial density storage (larvae mL^{-1})											
		20	80	200	20	80	200	20	80	200	20	80	200
		Microalgae concentration (x10^4 cells mL^{-1})											
Lower (LC)	entrance		1.26			1.48			1.57			2.19	
	exit	1.00	0.80	0.50	0.60	0.60	0.50	1.40	1.20	1.30	1.60	1.50	1.80
Medium microalgae (MC)	entrance		2.36			2.44			3.40			3.94	
	exit	1.70	1.60	1.30	1.50	1.20	1.90	1.70	1.40	1.90	2.40	2.00	3.50
High microalgae (HC)	entrance		5.03			5.26			5.42			7.29	
	exit	4.10	3.50	2.00	4.30	3.60	4.40	4.60	5.00	4.60	7.10	7.00	6.60

In relation to growth, the larvae fed at LC and HC did not reach the stage of pediveliger larvae (larvae retained in a sieve with a mesh size of 210 µm). The mean percentage of survival of larvae, when fed with the MC of

microalgae, was 21.8% ± 7.45, 27.4% ± 9.91 and 0.80% ± 0.26, respectively, in the densities of cultivation of 20, 80 and 200 larvae mL^{-1}, with yields at densities of 20 and 80 larvae mL^{-1} being significantly higher than at 200 larvae mL^{-1} ($P < 0.05$).

Initially, the shell length of the cultivated larvae did not differ among the stocking densities evaluated. Only after the 13th day of cultivation, was the mean shell height of larvae cultivated at a density of 20 larvae mL^{-1} higher than those cultivated at a density of 200 larvae mL^{-1} ($P = 0.0092$). On the 16th day of cultivation, the mean shell length of larvae cultivated at a density of 200 was lower ($P < 0.05$) than that observed at other cultivation densities (Figure 1). Evaluating the larval performance in relation to the concentration of food provided, it was observed that, on the 13th and 16th days, only the larvae fed with the MC of microalgae had a higher average shell length when compared to larvae fed with the low or high concentration ($P < 0.01$).

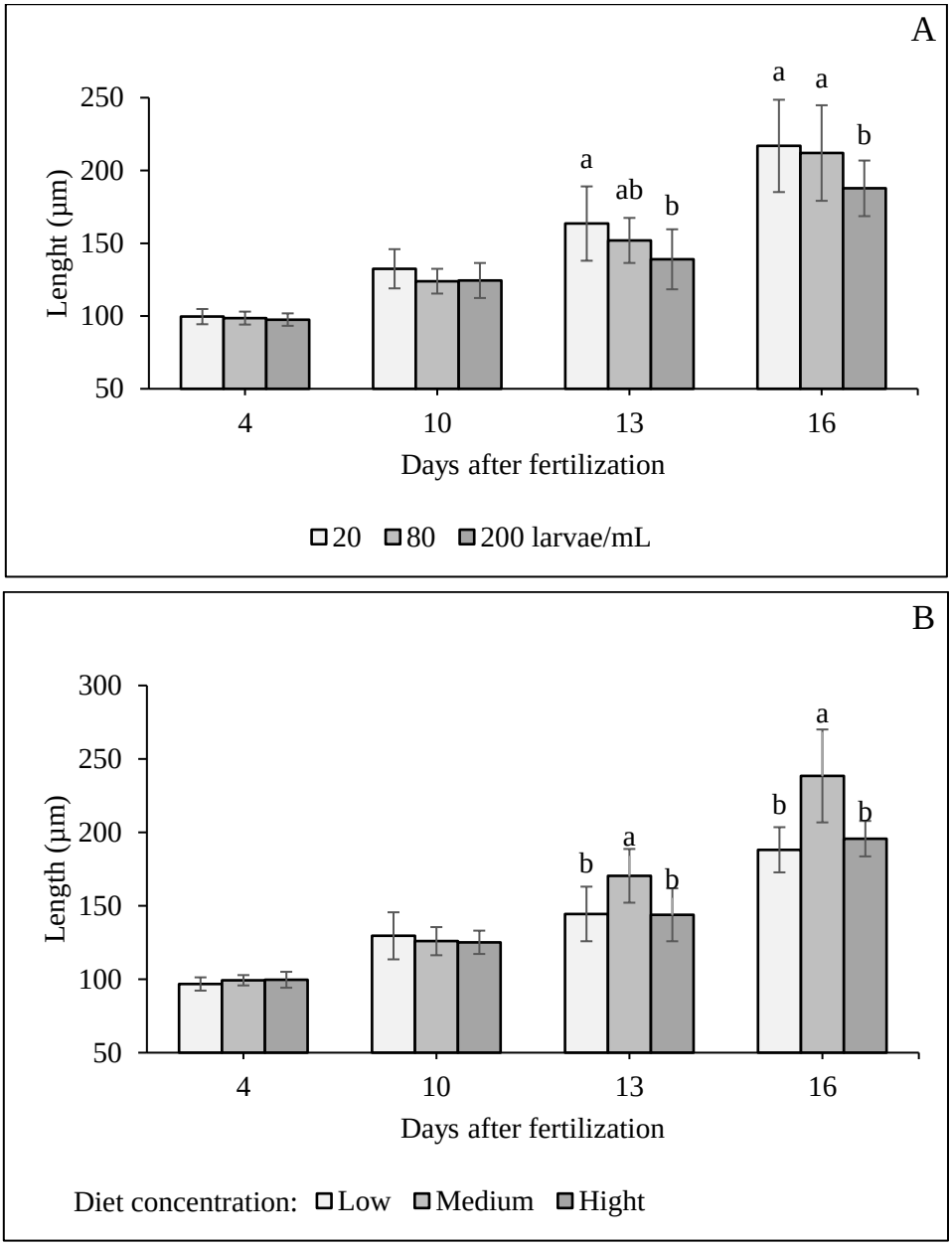

Figure 1 - Average larval shell length (µm) at initial stocking densities (larvae mL^{-1}; Fig. A) and at diet concentrations (Fig. B). Bars indicate ± standard deviation (n = 90). Low = between 1.26 to 2.19 x 10^4 cells mL^{-1}; Medium = between 2.36 to 3.94 x 10^4 cells mL^{-1}; Hight microalgae concentration = 5.03 to 7.29 x 10^4 cells mL^{-1}. Different letters mean significant differences. See Table 1 for details.

DISCUSSION

The development of larval production of bivalves in RAS proved to be efficient for the yield of *P. perna* larvae able to settle, and to be a viable alternative for obtaining young forms in a more sustainable way than in static models.

The production of *Mytilus edulis* (Linneaus 1758) larvae (Galley *et al*. 2010, Pechenik *et al*. 1990, Sprung, 1984), as well as *Perna viridis* (Linneaus 1758) larvae (Laxmilatha *et al*. 2011), was studied in static larval culture systems. For the mussel *P. perna*, studies using static larval culture systems (Aarab *et al*. 2013, Beduschi *et al*. 2009) and continuous flow systems (Turini *et al*. 2014) were carried out with yields (competent larvae) of approximately 10% (Turini *et al*. 2014) and survival rates above 80% (Aarab *et al*. 2013).

In comparison with other mytilid species, the survival rates of competent larvae of *P. perna* in this study were similar to those found for *Mytilus galloprovinciallis* (Lamarck 1819) when fed under the same diet regime (Pettersen *et al*. 2010).

The system used for the larval production of *P. perna* in RAS allowed for greater density of production due to the maintenance and stability of water quality by filters and the removal of organic particles by skimmers, in addition to requiring a lower volume of water (Harbach, Palm 2020, Kubitza 2006). *Argopecten purpuratus* (Lamarck 1819) larvae cultivated in RAS presented higher growth rates compared to those in static systems, mainly due to the reduction in the frequency of handling the animals, greater availability of food throughout the day, as well as higher temperatures and stable water quality (Merino *et al*. 2009). In the case of *Pecten maximus* (Linneaus 1758), survival of competent larvae, survival and daily larval growth rates are similar between FTS and RAS, although a decrease in dissolved oxygen has been noted in RAS, which may be related to the increase of bacterial population and in respiration rate of both larvae and bacteria (Magnesen, Jacobsen, 2012). In the present study, dissolved oxygen was stable (90.2 ± 2.9%), reducing the chances of a mass mortality event.

We found that at densities of 20 or 80 larvae mL^{-1} with feeding at MC of microalgae, the survival of competent larvae rates of *P. perna* (27.4% ± 9.91% and 21.8% ± 7.45%, RAS) were higher than in static systems. However, cultivation in a continuous water flow system has even higher survival of competent larvae at the same densities of larval cultivation (77.23% at density 20 and 33.5% at density of 80 larvae mL^{-1} (Turini *et al*. 2014).

The application of RAS for the cultivation of the sand mollusk *Venerupis corrugata* resulted in a mean survival of competent larvae rate (Joaquim *et al*. 2016) similar to that obtained in the present study (28.44% ± 16.15%). However, unlike the present study, there were no differences between the densities tested (10, 40 and 200 larvae mL^{-1}), demonstrating that the effect of larval density in *P. perna* is more important.

The system used in the larval culture in this study was based on the functioning of the cultivation units developed by King *et al*. (2005) for larviculture of the mytilid *Perna canaliculus* (Gmelin 1791). The species presented higher growth rate and expected survival of competent larvae (Ragg *et al*. 2010) compared to *P. perna* in the present study. Also, other bivalve species such as *Panopea zelandica* (Quoy, Gaimard 1835) showed, in this system, greater survival of competent larvae (76%) at lower densities of 100 larvae mL^{-1} (Le *et al*. 2017), while with small changes in the water flow applied to cultivation units, it is possible to obtain survival of competent larvae up to 89.34 ± 18.43% in the cultivation of *Crassostrea gigas* (Thunberg 1793) at a density of 160 larvae mL^{-1} (Fig. 2, Ramos *et al*. 2022).

High cultivation densities (200 larvae mL^{-1}) in the present study proved to be unviable for the larval cultivation of *P. perna*, showing a survival of competent larvae of 0.8% ± 0.26, as well as lower growth. Turini *et al*. (2014), obtained low larval survival rates of *P. perna* of 7.25% when cultivated in a FTS, at a density of 150 larvae mL^{-1}. The high density in small-volume culture systems affects the performance of bivalve larvae (Joaquim *et al*. 2016, Reiner 2011) and implies changes in the feeding behavior and possible concentration of metabolites not removed from the culture system (Turini *et al*. 2014).

The concentration of microalgae in the cultivation of *P. perna* larvae exerted a decisive influence on both growth and yield. Only the MC (2.36 to 3.94 cells mL^{-1}) of the larval diet regimen resulted in a viable larvae culture. Rico-Villa *et al*. (2009) report a similar result, in which the maximum growth rate for *C. gigas* in a continuous flow system occurred when the larvae were submitted to a concentration between 2 and 4 x 10^4 cells mL^{-1}. This fact suggests that the smaller amount of algae needed in recirculation systems may be associated with continuous feeding that guarantees the maintenance of adequate nutritional value for the larvae (Magnesen *et al*. 2006), which does not occur in the static system where there is a high feed concentration, decreasing throughout the day after each water change.

However, lower food concentrations (between 1.26 and 2.19 x 10^4 cells mL^{-1}) suggest an inability to achieve a positive energy correlation in order to maintain larval growth (Wacker, Von Elert 2002). Concomitantly, excessive food supplied to bivalves may be accompanied by a reduction in utilization and consequent saturation of the clearance activity (Cheng *et al*. 2020, Pascoe *et al*. 2009), as evidenced in the lower rates of algal consumption at higher concentrations of microalgae. Results with pectinid *Pecten maximus* larvae in a continuous flow system demonstrated a negative correlation between yield and algae concentration (Magnesen *et al*. 2006).

Another factor associated with the high concentrations of unused microalgae in the larval cultivation of *P. perna* was related to the accumulation of organic matter, which clogs the filters, generating possible overflows of tanks. In addition, there are harmful effects resulting from the growth of bacteria and/or compounds dissolved from algae cultures (Magnesen *et al*. 2006, Nicolas *et al*. 2004). This fact indicates that the monitoring of microbiota in larval cultures should be considered in RAS.

CONCLUSIONS

The results demonstrate that it is possible to produce *P. perna* mussel larvae in RAS. The densities of 20 and 80 larvae mL^{-1} associated with the MC (between 2.36 and 3.94 x 10^4 cells mL^{-1}) showed higher survival of competent larvae (21 and 27%, respectively, for 20 and 80 larvae mL^{-1}) and should be adopted in preference to the other conditions evaluated.

Declaration of conflict of interest

Nothing to declare.

Ethics approval

According to Brazilian law, authorization for the use of invertebrates, including mussel, is not required in the conduct of scientific experiments.

ACKNOWLEDGEMENTS

The authors would like to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support, financial coding 001.

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