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The combined effect of stocking density and C/N ratio on growth performance and biomass production of *Artemia* reared in a biofloc system under laboratory culture conditions

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ABSTRACT
The combined effects of different densities of *Artemia* and C/N ratios reared in a biofloc system on growth and biomass production of *Artemia* were assessed under laboratory conditions. A 3×4 factorial experiment with three levels of *Artemia* density (500, 750, and 1000 nauplii/L) and three C/N ratios (5, 10, and 15) was randomly designed in four replicates. Newly-hatched *Artemia* nauplii were reared in 1.5 L bottles containing 1 L of seawater at 30‰. Molasses with a 38% carbon content was used as a carbon source to stimulate bacterial growth in the *Artemia* culture medium. The amount of molasses added was based on a concentration of total ammonia nitrogen (TAN) in the culture medium. Results showed that significant interaction effects between *Artemia* density and C/N ratios were not observed for growth, survival and biomass production. The manipulation of C/N at 5 and 10 promoted better growth of *Artemia* in terms of length compared to that obtained by *Artemia* in the higher C/N ratio. However, it was not clear whether there was an increase in growth performance in the culture where *Artemia* were reared at high densities. Moreover, the addition of molasses produced biomass in stocking density at 500 ind./L that was nearly similar to that obtained in the 750 and 1,000 ind./L.

INTRODUCTION

Artemia is a small crustacean (measuring 0.04 cm for nauplius, 1 cm for adult size) found in brine habitats ranging in salinity from 70‰ to 250‰. It is known as a highly nutritional live food for hatcheries in aquaculture, especially for the early stage of aquatic larvae **(Sorgeloos *et al*., 1986; Dhont and Sorgeloos 2002)**. This has led to an increased demand for *Artemia* products by aquaculture hatcheries over the years, sometimes resulting in an oversupply **(Sorgeloos, 2001)**. To meet such rising demand, *Artemia* has been introduced to integrated cultures in the saltpans of many countries around the world **(Toi *et al*., 2014)**. Besides, *Artemia* biomass (adult *Artemia* and nauplii) has a high nutrition value, containing 50–60% protein and high levels of essential amino acids and fatty acids **(Dhont & Sorgeloos 2002; Castro *et al*., 2009; Zadehmohseni *et al*., 2020)**, making it a perfect ingredient for substituting fishmeal protein in fish and shrimp diets **(Dhont & Sorgeloos, 2002; Anh *et al*., 2011)**.

In *Artemia* culture, excesses in organic matter, along with *Artemia* wastes, that accumulate during the culture period often pollute the water environment. Therefore, it is necessary to improve the culture environment in order to improve the biomass yield of *Artemia* as a whole. There are many ways to reduce pollution in aquaculture ponds, but the method of stimulating heterotrophic bacteria to grow in these ponds is considered to be the best way **(Avnimelech, 2012)**, since heterotrophic bacteria will convert the excess nitrogen from food and aquatic animal waste in their biomass or nutrients, which are then available for algae to take up. It is also known that the growth of heterotrophic bacteria is greatly influenced by the ratio of C/N in the medium, and heterotrophic bacteria experience better growth when C/N is greater than 10 **(Avnimelech, 1999)**.

Given the nature of biofloc technology (BFT), its application in *Artemia* culture helps to improve the pond environment and create a food source for *Artemia*, thanks to the resulting development of heterotrophic bacteria. When the environment is poor with food, *Artemia* use more bacteria, and with the application of BFT, *Artemia* biomass increases significantly compared to the culture without BFT. The filtering ability of *Artemia* is influenced, however, by the overgrowth of bacteria when a C/N at 50 is applied, leading to poor growth and survival in comparison to the application of a C/N at 10 **(Toi *et al.*, 2013a)**. Furthermore, stocking density is one of the main factors influencing growth, survival, productivity, and culture medium in *Artemia* culture **(Sy, 2012; Thong and Hoa, 2018)**. Survey on *Artemia* farming conducted by **Anh *et al.* (2014)** and **Vinh *et al.* (2020)**, they found that too low stocking density increased production costs and decreased the efficiency of the culture unit, whereas too high stocking density exceeds the capacity of the farm, causing interactions in *Artemia* populations through competition for food and living space, resulted in long-term stress on *Artemia*, slow growth and survival, and yield. As a result, the income of *Artemia* producers has decreased. Therefore, the aim of the current study is to determine the optimal C/N ratio and *Artemia* stocking density for maximum *Artemia* biomass production under laboratory conditions in order to provide a scientific foundation for future application on a larger scale.

MATERIALS AND METHODS

Experimental setup

The experimental system was conducted at the *Artemia* Laboratory Unit of Collegge Aquaculture & Fisheries, Can Tho University. A 3×4 factorial experiment involving three stocking densities of *Artemia* (500, 750, and 1,000 nauplii/L) combined with three C/N ratios (5, 10, and 15) was randomly designed in four replicates.

Artemia nauplii (new hatched nauplii) were reared in 1.5-L bottles containing 1 L of seawater at 30‰. Continuous aeration for each culture bottle and illumination were provided. Molasses (38% carbon) was used as the carbon source to stimulate the growth of heterotrophic bacteria in the *Artemia* culture medium. The amount of molasses added was based on a concentration of total ammonia nitrogen (TAN) in the culture medium. Sodium bicarbonate or baking soda ($NaHCO_3$) was added to the culture to maintain alkalinity of the water medium at 120 ± 10 mg $CaCO_3$/L. Water change was not applied

to this study, but new seawater was added to compensate for evaporation during of experiemt.

***Artemia* nauplii preparation**

Artemia franciscana Vinh Chau dry cysts were incubated at a density of 1 g/L in seawater of 30‰, and a temperature of 28 ± 0.5 °C; continuous illumination and aeration were also provided **(Sorgeloos *et al.*, 1986)**. After 24 h for incubation, the newly hatched *Artemia* nauplii were randomly collected for the experimental setup.

Food preparation and feeding

Chaetoceros sp., a suitable food for *Artemia*, was provided for *Artemia* in the first 2 days of culture, at a feeding rate recommended by **Coutteau *et al.* (1990)**. Then, *Artemia* were offered formulated feed (protein 30% and 9% lipid) at 4 times/day **(Han *et al.*, 2016)**, with the feeding ration increased per day according to the age of the *Artemia* **(De Los Santos *et al.*, 1980)**. Prior to being offered to *Artemia*, the feed was diluted in water for 15 minutes, and the feed solution was sieved through a 50-µm mesh size net.

Sample collection and data analysis

Physio-chemical parameters

Temperature and pH of culture water were measured daily at 8 am and 2 pm by pH meter and thermometer (Hanna), respectively. Total ammonia nitrogen (or TAN; NH_3/NH_4^+) and nitrite (NO_2^-) in the culture medium were tested for 3 days at 8 am via Sera test-kit (Germany).

Biological parameters

The growth of *Artemia*: thirty animals from each treatment were randomly collected. Before measuring for length, *Artemia* were preserved in Lugol's solution; they were then placed on a slide and measured under a specialized microscope using the eyepiece micrometer, from the head to the furca of *Artemia*. The following formula was used to calculate the individual length of *Artemia*:

$$L\ (mm) = A / 10 \times 1 / \gamma$$

where L is the length of *Artemia* (mm), A is the number of measured lines, and γ is magnification.

Artemia biomass production (g/L): *Artemia* biomass in each culture bottle was harvested at the end of the experiment by net; after harvesting, excess water was removed from the biomass.

Artemia survival was determined on day 7, 14, and 21. First, *Artemia* in each culture bottle were concentrated in the net, and live *Artemia* were then counted. *Artemia* survival in the treatment was calculated using the following formula:

$$S\ (\%) = Nt / No \times 100$$

where Nt is the final number of live *Artemia*, and No is the initial stocking density.

Statistical analysis

Datasets were calculated for standard deviation by two-way ANOVA, which was used to test the effect of C/N ratio, stocking density, and the interaction between these 2 factors on growth performance, survival, and biomass production of *Artemia*. In addition,

the Tukey's honestly significant difference post-hoc test (significance level $p < 0.05$) was applied using Statistica 6.0 software.

RESULTS

Physio-chemical parameters

The average temperature ranged from 28.1 ºC to 29.1 ºC and pH values were around 8.3. The concentration of TAN in the experiment ranged from 0.6 to 0.7 mg/L, and NO_2^- ranged from 0.3 to 0.8 mg/L.

Biological parameters

The survival rate of *Artemia* is presented in Table 1. On day 7, the survival rate ranged from 68.5% to 87%; it then gradually decreased, so that by day 21, the survival rate ranged from 31.7% to 48.0%, with the highest occurring in T4 (48.0%), and the lowest in T2 (31.7%). While the low C/N ratio produced higher survival in all stocking densities, there was no statistically significant difference between treatments, and there was no interaction between stocking density and C/N ratio on the survival of *Artemia* (Table1).

Table 1: Combined effects of stocking density and C/N ratio on survival of *Artemia*

Artemia density (Nauplii/L)	C/N ratio	Survival of *Artemia*		
		Day 7	Day 14	Day 21
500	5	79.3 ± 15.0[a]	69.7 ± 17.9[a]	43.3 ± 20.2[a]
500	10	70.3 ± 22.5[a]	43.7 ± 26.5[a]	31.7 ± 17.6[a]
500	15	68.5 ± 32.0[a]	55.8 ± 26.0[a]	30.0 ± 8.9[a]
750	5	85.3 ± 9.2[a]	60.8 ± 14.7[a]	48.0 ± 14.9[a]
750	10	68.8 ± 25.3[a]	54.8 ± 19.3[a]	42.3 ± 16.4[a]
750	15	71.5 ± 21.7[a]	44.0 ± 24.9[a]	40.5 ± 9.5[a]
1000	5	85.5 ± 4.5[a]	58.3 ± 9.8[a]	47.8 ± 7.4[a]
1000	10	83.5 ± 10.0[a]	63.3 ± 2.8[a]	46.8 ± 3.8[a]
1000	15	87.0 ± 11.0[a]	57.3 ± 14.2[a]	46.3 ± 5.9[a]
Effect of *Artemia* density				
500		73.2 ± 23.0[a]	53.6 ± 23.8[a]	35.0 ± 13.0[a]
750		75.2 ± 19.6[a]	53.2 ± 19.5[a]	43.5 ± 12.3[a]
1000		85.3 ± 8.3[a]	59.6 ± 9.6[a]	46.8 ± 5.5[a]
Effect of C/N ratio				
5		78.0 ± 15.9[a]	56.1 ± 20.9[a]	45.9 ± 13.4[a]
10		75.5 ± 18.7[a]	55.0 ± 17.1[a]	38.8 ± 12.1[a]
15		80.3 ± 20.3[a]	57.9 ± 17.4[a]	41.1 ± 8.9[a]
ANOVA: P-values				
Artemia density (1)		0.257099	0.627463	0.082188
C/N ratio (2)		0.779232	0.858400	0.492722
Interaction (1) x (2)		0.721494	0.319772	0.886239

Main effect means within the same column sharing different superscripts are significantly different ($p<0.05$).

Growth performance of *Artemia*

The growth performance of *Artemia* in terms of length is shown in Table 2. On day 7, the length of *Artemia* ranged from 4.4 mm to 4.9 mm. There was no significant difference when comparing between C/N ratios in the same stocking density, and while the length of *Artemia* was influenced by stocking density ($p<0.05$), no interaction was found between stocking density and C/N ratio as regards growth in length of *Artemia*.

Table 2: Combined effects of different stocking densities and C/N ratios on total length and biomass production of *Artemia*

Artemia density (Nauplii/L)	C/N ratio	Total length (mm)			*Artemia* biomass (g/L)
		Day 7	Day 14	Day 21	
500	5	4.8 ± 0.5^{c}	7.8 ± 0.6^{c}	8.3 ± 1.1^{b}	3.59 ± 0.62^{a}
500	10	4.7 ± 0.5^{abc}	7.5 ± 0.5^{abc}	8.0 ± 0.6^{b}	2.77 ± 0.66^{a}
500	15	4.7 ± 0.5^{abc}	7.7 ± 0.4^{bc}	7.9 ± 0.5^{ab}	2.66 ± 0.49^{a}
750	5	4.9 ± 0.4^{c}	7.7 ± 0.5^{bc}	7.9 ± 0.7^{a}	3.58 ± 1.05^{a}
750	10	4.8 ± 0.4^{c}	7.4 ± 0.4^{ab}	7.5 ± 0.7^{a}	2.80 ± 1.16^{a}
750	15	4.8 ± 0.3^{bc}	7.3 ± 0.6^{a}	7.6 ± 0.5^{a}	2.99 ± 0.61^{a}
1000	5	4.5 ± 0.3^{ab}	7.3 ± 0.5^{ab}	7.5 ± 0.7^{a}	4.72 ± 1.12^{a}
1000	10	4.5 ± 0.3^{ab}	7.2 ± 0.6^{a}	7.9 ± 0.5^{a}	3.94 ± 0.66^{a}
1000	15	4.4 ± 0.2^{a}	7.3 ± 0.5^{a}	7.2 ± 0.5^{a}	4.17 ± 0.93^{a}
Effect of *Artemia* density					
500		4.8 ± 0.5^{b}	7.7 ± 0.5^{c}	8.0 ± 0.8^{b}	3.01 ± 0.68^{a}
750		4.9 ± 0.4^{b}	7.4 ± 0.5^{b}	7.3 ± 0.7^{a}	3.12 ± 0.91^{a}
1000		4.5 ± 0.3^{a}	7.2 ± 0.5^{a}	7.1 ± 0.6^{a}	4.28 ± 0.87^{b}
Effect of C/N ratio					
5		4.8 ± 0.4^{a}	7.6 ± 0.6^{ab}	7.4 ± 0.8^{a}	3.77 ± 1.04^{a}
10		4.7 ± 0.4^{a}	7.3 ± 0.5^{a}	7.5 ± 0.7^{a}	3.17 ± 0.94^{a}
15		4.7 ± 0.4^{a}	7.4 ± 0.5^{b}	7.6 ± 0.9^{a}	3.47 ± 0.99^{a}
ANOVA: P-values					
Artemia density (1)		0.000000***	0.000001***	0.000000***	0.002239**
C/N ratio (2)		0.240444	0.003337**	0.172936	0.576555
Interaction (1) x (2)		0.964633	0.232043	0.029481*	0.419351

*Main effect means within the same column sharing different superscripts are significantly different ($p<0.05$).*** denotes $P < 0.001$; **: $P < 0.01$ and *: $P < 0.05$)*

On day 14, the average length ranged from 7.2 to 7.8 mm: the largest size of *Artemia* was in T1 (7.8 mm), and the smallest, in T8 (7.2 mm). The length of *Artemia* in the stocking density at 500 ind./L was significantly larger than that of *Artemia* in the higher stocking density, except for *Artemia* in treatment T3, which was not significantly higher than that obtained in the higher stocking density. At stocking densities of 500 and 750 ind./L, the size of *Artemia* in terms of individual lengths in all treatments with C/N at

5 was not significantly better than that in the treatments with C/N at 10 and 15. The length of *Artemia* in T4 with C/N at 5 was significantly better ($p<0.05$) than that of *Artemia* in treatment T6 with C/N at 15. Consequently, the C/N ratio displayed its effect on growth in the length of *Artemia* (Table 2) when compared in stocking densities. The application of C/N ratios 5 and 10 in stocking densities of 500 ind./L produced better growth in length when compared to *Artemia* in higher stocking densities. In general, stocking density (SD) had an effect on the length growth of *Artemia* as well as an interaction between the stocking density and the C/N ratio on day 21 (Table 2).

The *Artemia* biomass obtained in the current study ranged from 2.6 to 4.7 g/L (Table 2); while the highest biomass was in T7 (4.7 g/L) and lowest was in T3 (2.6 g/L), there was no significant difference ($p > 0.05$) among the treatments. However, the application of C/N in the lower stocking density produced a biomass that was nearly equal to that of the higher stocking density.

DISCUSSION

The suitable temperature for *Artemia* is reported to be in the range of 22–35 °C **(De Los Santos *et al.*, 1980)**, and the suitable pH for *Artemia* is from 7.0–9.0 **(Nguyen, 1993)**. So that, the temperatures and pH values in current studty were in the suitable range for the development of *Artemia*.

According to **Dhont and Lavens (1996),** *Artemia* is a species that can tolerate high levels of TAN and NO_2^-, with an LC_{50} of TAN and NO_2^- for *Artemia* of 1,000 mg/L and 320 mg/L, respectively. However, both of these parameters can be minimized by stimulating heterotrophic bacterial growth. **Hari *et al.* (2006)** demonstrated that the accumulation of TAN and NO_2^- in water was reduced by adding carbohydrate, so the low TAN and nitrite contents in the present experiment may be utilized by bacteria.

The quality of culture water is significantly improved with the application of biofloc technology in the aquaculture system: heterotrophic bacteria are stimulated by the addition of carbon to the culture system, when bacteria metabolize the nitrogen in the system as well as carbon to form new cells; this activity results in improved water quality **(Avnimelech, 1999; Hari *et al.*, 2006)**, and may be a factor in helping to improve *Artemia* survival. According to **Toi *et al.* (2013a)**, survival increased significantly with the application of C/N at 10 to *Artemia* biomass culture in poor feed conditions compared to the traditional *Artemia* culture system; however, survival decreased sharply with the application of C/N at 50 in the culture because of the massive growth of bacteria, as *Artemia* could not consume all of the feed and the bacteria, leading to overfeeding **(Toi *et al.*, 2013b)**.

Bacteria are considered to be a good food source for *Artemia*, which has also been demonstrated for bacteria that grow in culture water **(Intriago & Jones, 1993; Gorospe & Nakamura, 1996; Verschuere *et al.*, 2000; Toi *et al.*, 2013b)**; more specifically,

Artemia length improved significantly in either rich or poor conditions when carbon fertilized to achieve a C/N at 10 in the culture, compared with no carbon supplementation. However, when C/N is increased to 50, the growth performance of *Artemia* cultured in rich food conditions is not better than that of *Artemia* cultured without the addition of carbon, which is probably due to an overgrowth of bacteria, plus rich organic conditions **(Toi *et al*., 2013b)**. *Artemia* is a continuous filter feeder with non-selected food: they have the ability to filter particles less than 50 µm **(D'Agostino,1980)** in culture media, so when overfeeding occurs, the food passes more rapidly into the digestive tract—there is not enough time for digestion in the gut, leading to decreased *Artemia* growth. In addition, bacteria are considered to be the source of activating enzymes that help *Artemia* to better activate food items **(Intriago & Jones, 1993; Verschuere *et al*., 2000)**.

In the current experiment, *Artemia* were cultured under rich food conditions; consequently, stimulating bacteria at C/N levels between 5 and 10 did not clearly show a difference in improving the growth of *Artemia*. During the first two weeks, the growth in length of *Artemia* improved when a C/N at 5 was applied, but it was not so clear as regards an increase of growth performance afterwards.

Artemia biomass increased significantly with carbon supplementation to achieve the C/N 10 ratio in standard feeding treatments, and *Artemia* used heterotrophic bacteria when the environment was lacking of feed **(Toi *et al*., 2013a)**. Bacteria are not the only feed that can be supplied to *Artemia*, but they are a source of the activating enzymes that help *Artemia* to digest and better absorb food items **(Gorospe & Nakamura, 1996; Toi *et al*., 2014)**. In contrast, when raising the C/N to 50, the biomass yield decreased in comparison to the treatment without the addition of carbon. This is believed to be due to bacterial overgrowth and the addition of standard feeding causing an excess of food that, in turn, affect the digestion and absorption of food by *Artemia*. In the current experiment, C/N ratios of 5, 10, and 15 were used in the culture of *Artemia* with standard diets; this had no effect on biomass enhancement, possibly because the bacterial growth was not in excess of these ratios. These C/Ns have contributed to the feed of *Artemia* in amounts that are not excessive—which is appropriate for the growth of *Artemia*—and thus there is no significant difference in biomass yield between treatments.

CONCLUSION

The low C/N ratio produced higher survival in all stocking densities, In additionm, the application of C/N ratios 5 and 10 in stocking densities of 500 ind./L produced better growth in length when compared to *Artemia* in higher stocking densities. The application of C/N in the lower stocking density produced a biomass that was nearly equal to that of the higher stocking density.

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