Effect of different C/N ratios on *Artemia* biomass culture

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Abstract: This study was conducted with aim to determine the best C/N ratio for optimum growth performance of *Artemia* under laboratory conditions. The experiment consisted of four C/N ratios corresponding to 5, 10, 15 and 20 (C-treatments) and a blank treatment used as control; three replicates were involved for each. The C/N ratios were regulated by daily adding molasses into the *Artemia* culture medium based on TAN concentration, whereas none of molasses was added into the blank treatment. *Artemia* nauplii (Instar I) were reared in 1.5 L plastic bottle containing 1 L seawater at salinity of 30‰, stocking density was 300 ind./L and maintaining in a room temperature condition with continuously aeration supporting. In the first two days of culturing, *Artemia* were fed with microalgae *Chaetoceros* sp., and from the 3rd day onwards to the end of experimental period, *Artemia* feed (30% of protein and 9% of lipid) was offered as food for the culture. Molasses were added to C-treatments from 3rd day of the culture and since then was daily regulated basing on TAN measurement in the culture medium. After 42 days of culturing, the result showed that the best survival (48 %) and biomass production (4.9 g/L) were obtained in C/N=5 medium, lowest was at control (29.1 %; 3.1 g/L, respectively) and *Artemia* cultured in C-treatments tend to have better survival as well as biomass production compared to the control. The best growth in length at DAH7 (4.42 mm) and at DAH14 (8.36 mm) was recorded in C/N=10 but there was no significant difference comparing to others.

Index term: *Artemia*, biomass, biofloc, C/N ratio, molasses.

I. INTRODUCTION

Artemia is one of the best live foods among zooplankton which distinguishes by their high nutritional content and small in size of nauplius (about 0.4 mm). Most former studies agreed that they are really necessary for the large amount of marine finfish and shellfish species, especially in their earliest stages (Sorgeloos *et al.*, 2001). From the last two decades, the demand on *Artemia* products for hatcheries was fast increasing. The annual requirement on *Artemia* cysts on the world market has increased from a few tons in the middle 1970s to over 2,000 tons in the early 2000s. However, the demand on *Artemia* cysts is sometimes over the supply. So that to match the requirement on this products, *Artemia* have been cultured in some countries on the world. In Vietnam, *Artemia* was introduced in salt field of Vinh Chau-Bac Lieu since 1989 by College of Aquaculture and Fisheries of Can Tho University and has become a favorite species for local culture nowadays. In traditional methods, *Artemia* are cultured in ponds at highly saline water from 80-120‰ and fed with microalgae so called "green water" which are stimulated to massive grow by chicken manure. Moreover, rice bran is also used as a supplemental feed with the amount of 60 kg/ha/day and 200-300 kg of chicken manure in dry weight (DW)/ha/week (Baert *et al.*,1997; Anh, 2009). Meanwhile, the accumulation of nitrogen (N) and phosphorus (P) in bottom mud of *Artemia* ponds after each crop was about 0.6-1.7 mg/g and 0.2-0.9 mg/g, respectively; together with those accumulated during rainy season by feeding shrimp/fishes leading to algal bloom which caused failure in the next year *Artemia* crop (Nguyen Van Hoa *et al.*, 2010). According to Ebeling *et al.* (2006) there are three ways to reduce the accumulation of organic matter in aquaculture pond: (1) the process of nitrogen fixation by algae (photosynthesis), (2) autotrophic bacteria catalytic converting ammonia to nitrate (bio-filter), and (3) heterotrophic bacteria using directly nitrogen from ammonia to increase their population through supplemental carbon (biofloc technology). Nowadays, biofloc technology is widely applied in aquaculture including *Artemia* biomass culture (Sui *et al.*, 2013; Ronald *et al.*, 2013). Biofloc consists of plankton, bacteria, microalgae, and protozoa often accounting for 35-50% of protein content, 0.6-12% of fat and 21-32% of the ash. The main benefit of bio-floc is reducing ammonia in farming environments that limit the use of the bio-filter system (improving water quality and increasing nutrient use efficiency), reducing the need for water exchange, and to maintain bio-farming system in flocs people often use cheap carbon sources (low cost) to balance the C/N ratio (Avnimelech, 2007). Biofloc has poly-β-hydroxybutyrate that may against pathogenic bacteria in aquaculture (Avnimelech, 2012). Additionally, bio-flocs can improve growth performance and feed utilization of the cultured shrimp (Xu *et al.*, 2012), or fish (Avnimelech, 2007), probably through providing a supplemental food source and enhancing feed digestion. Moreover, bacteria are small in size (few microns); hence bacteria can also be a feed source for *Artemia* (Dobbleleir *et al.*, 1980; Sorgeloos, 1980). Therefore, applying biofloc technology in *Artemia* culture is a potential solution to improve productivity, reduce production costs, increase profits, especially protect environment, and increase the sustainability for farming process (Sui *et al.*, 2013; Ronald *et al.*, 2013). However, the previous studies did not point out at the ad libitum feeding regime which C/N ratios can manipulate bacterial growth and *Artemia* takes the most advantage from these heterotrophic bacteria. For this reason, the different C/N ratios were applied in *Artemia* biomass culture to determine which C/N ratio resulted optimum growth of *Artemia*.

II. MATERIALS AND METHODS

Experimental setup

This experiment was conducted with 5 treatments: T1 (blank/control), T2, T3, T4 and T5 (C-treatments), molasses was daily added into T2 to T5 to get C/N ratio at 5, 10, 15 and 20, respectively. For the control treatment, the culture was zero molasses addition. *Artemia* nauplii (Instar I) were reared in 1.5 L plastic bottle containing 1 L of seawater with salinity at 30 ‰, density 300 ind./L, pH about 7.5-8.6 and rearing in room temperature (28 ^{o}C), with continuously aeration supporting.

Total ammonium nitrogen (TAN) was measured every 3 days and based on the TAN value and C constituent in analyzed molasses, the amount of molasses was added to reach tested C/N ratios. There were three batches of culturing with different generations (parent, 1st generation or F1 and 2nd generation or F2) for each treatment. When *Artemia* reproduced naupllii around 500 nauplii/L, the biomass was harvested and the nauplii were reset-up for the next biomass culturing (300 nauplii/L) and so on.

Preparation of *Artemia* nauplii: 01 g of *Artemia franciscana* Vinh Chau was added into the 1.5 L conical plastic bottle containing 1 L of seawater at 30‰, the optimal conditions for hatching were maintained such as pH about 7.5-8.6 temperature at 28 ^{o}C and continuously aeration supporting (Sorgeloos *et al.*, 1986). After 24h of incubation, freshly hatched *Artemia* nauplii were harvested to set up the experiment.

Food preparation and feeding: *Artemia* were offered microalgae *Chaetoceros* sp., during the first two days of culturing and from the 3rd onwards to the end of experimental period, *Artemia* were fed with *Artemia* feed containing 30% of protein and 9% of lipid following feeding regime by Hoa (1993). *Artemia* feed was soaked in seawater (salinity 30‰) for 15 minutes, then the solution was filtered through the 50 µm net before feeding. The total amount of daily feed was divided in four parts, and distributed to *Artemia* in four times per day (at 8:00, 11:00, 14:00 and 17:00). The amount of feed was adjusted by day based on the demand of *Artemia* through the observation of feed in water (water color) and in digestive track of *Artemia*.

Data collection and analysis

Water quality: pH and temperature were daily recorded at 8:00 and 14:00 by pH meter and thermometer, respectively. TAN (NH_3/NH_4^+) concentration was recorded every 3 days and nitrite (NO_2^-) concentration was recorded weekly by test-kit (Sera; Germany).

Bacteria density was observed by measuring the optical density (OD) of particles in culture water at 550 nm in wavelength. Water samples for OD were collected after 4 hours of molasses adding, and then the water samples (2 samples/week) were ground by stirring homogenizer until the homogenous mixture was obtained. Those mixture liquids were put into cuvettes (4 ml) before measuring by spectrophotometer.

Survival at DAH7 and at DAH14 was counted and calculated following the below formula:

Survival rate (%) = final number of Artemia at DAH7 or DAH14/Initial number of Artemia at stock × 100.

Individual length of *Artemia* at DAH7 and at DAH14 was recorded by randomly measuring the individual length of 30 *Artemia* in each treatment, *Artemia* was measured from the head to the furca of *Artemia* under specific binocular (Olympus SZ51, Japan)

Fecundity: Number of cysts or nauplii per female were recorded from 30 randomize females in each the brook sac of each *Artemia* female was opened and counted all the number of cysts (yellow-brown in color) or nauplii (orange in color) under microscope (10×).

Final *Artemia* biomass production is a lump sum of three times biomass harvesting (in number and weight).

Statistical analysis

The data of biomass production, survival, OD were checked for normal distribution and homogeneity of variance by p-p plots and Levene´s test of Statistica 7.0 software for windows. If one of these assumptions could not be satisfied, data were transformed, prior to one-way analysis of variance (ANOVA), followed by Tukey´s honestly significant difference (HSD) test, employed at 0.05 probability level. A non-parametric, Kruskal-Wallis, test was used when the transformation could not be applied.

III. RESULTS AND DISCUSSION

Water quality

The water parameters of experimental culture were presented in the Table 1. The average temperature of each treatment at 7:00 AM and 14:00 PM was 27.8- 28.9 °C, respectively. According to Hoa *et al*. (2007), the temperature in this culture was in suitable range for the growth of *Artemia*. In the other hand, the pH of culture water in this study was in the range of 8.1 – 8.4, assuming as optimum range for the growth of *Artemia* (Hoa *et al.*, 2007).

Concerning to other abiotic parameters, sampling during experimental period indicated that average TAN content was lowest at T4 (0.25 mg/L) and highest found at control treatment (0.28 ± 0.21 mg/L), that might be related to the growth of bacteria when molasses were daily added to T2, T3, T4 and T5. Meanwhile, NO_2^- content ranged from 0.05 to 0.2 mg/L (7th day) and 1 to 2

mg/L (14th day) and NO2- content of control treatment was always lower than other treatments and that could also be the result of higher growth of bacteria in T2, T3 T4 and T5 because of bacteria is also feed source for *Artemia* (Dobbleleir *et al.*, 1980; Sorgeloos, 1980). However, Dhont *et al*. (1996) reported that *Artemia* have ability to resist high level of TAN and NO_2^- content and LC50 (lethal concentration of 50%) of *Artemia* on TAN and NO_2^- concentration 24 hours is 1000 mg/L and 320 mg/L, respectively. Therefore, TAN and NO_2^-concentration in this study was in suitable range for the growth of *Artemia*.

Optical density

According to Barman *et al*. (2015), the growth of bacteria can be observed by comparative daily OD checking, the higher final OD number is the higher bacterial cells in the water when compared to initial OD number. The optical density of culture water was presented in Table 1. The OD of water culture was in range 0.12-0.36, the OD was insignificantly increased in all treatments with carbon addition as compared to the control, except at the second recording (OD2), there was a significantly difference between T3 and control treatment (it also is presented in Table 1). So, the result of OD in this study indicated that bacteria increased their cells in all carbon treated culture. However, the OD of T5 was not observed because all *Artemia* in this treatment died before day 8th.

Table 1: Optical density (OD) of culture water (n=4). Dash is indicated for non-observation

Treatment	Average OD	OD2
T1 (control)	0.012 ± 0.005	0.010 ± 0.001^{a}
T2 (C/N5)	0.028 ± 0.019	0.015 ± 0.002^{ab}
T3 (C/N10)	0.034 ± 0.021	0.026 ± 0.010^{b}
T4 (C/N15)	0.036 ± 0.024	0.027 ± 0.007^{ab}
T5 (C/N20)	-	-

***Artemia* growth performance**

Survival of *Artemia* in this study is showed in Table 2. In first batch (parent generation), the survival of *Artemia* varied from 0% to 33%, the highest survival (33.2 %) was obtained in the control and lowest was T5 (all *Artemia* died before day 8th), however, there was not statistically significant found between treatments ($p > 0.05$). In the second batch (F1), the survival of *Artemia* in the study was in range 44- 67%, the C-treatment (T2, T3 and T4) indicated higher survivals (65-67%) than the control (only 44%) but again no significant was found between them. In the last batch (F2), lowest survival was obtained in the control (only 10%) and statistical significantly ($p < 0.05$; Table 2) to other C- treatments (ranging from 20-49%). In total, the average survival of *Artemia* of three batches was 29- 48% and significantly highest value was obtained in all C-treatments as compared to that obtained in the control ($p < 0.05$). In comparison between C/N ratios, the survival of *Artemia* was not significantly different among carbon added treatments for the first batch and second batch, except of C/N ratio 20 as all *Artemia* died at day 8th of culturing in the first batch. In the last batch, there was difference among C-treatments, the two C/N ratios 5 and 10 (T2, T3) gave better survival (39-49%) than at T4 (C/N=15), only 20% and this difference is statistical significantly ($p < 0.05$).

Regarding to the failure at day 8th in the C/N ration 20, the death of all *Artemia* may related to massive growth of bacteria, according to Toi *et al*. (2013a) the huge increase of bacteria affected on swimming activity, oxygen uptake and food utilization of *Artemia*, his study revealed that swimming activity and food utilized of *Artemia* reduced when the C/N ratio increased from 10 to 50. In this study the massive growth of bacteria may increase by the increase of C/N ratio, based on the OD results (Table 1) the OD increased 2-3 times in the treatments with C/N manipulating as compared to control treatment (without carbon adding). In addition, the OD increased when C/N ratio in the culture water was also increased (Table 1). The massive growth may be the cause of low survival of *Artemia* in T4 (3rd batch), and died off in T5 (1st batch) in this study. Toi *et al*. (2013b) assumed that the manipulation of C/N ratio can improve survival of *Artemia* when microalgae were used as a main food. This result is similar to our finding in the second and the third batch at C/N ratio of 5, 10 and 15 although *Artemia* feed was used.

Table 2: Survival (%) of *Artemia* in three batches culturing. From the 2nd batch to 3rd batch, the T5 was not set up because the parent generation died before getting maturity.

Treatment	Survival (%)			
	1st batch (P)	2nd batch (F1)	3rd batch (F2)	Average
T1 (control)	33.2 ± 7.9^{b}	44.0 ± 6.5^{a}	10.1 ± 2.2^{a}	29.1 ± 15.9^{a}
T2 (C/N5)	29.1 ± 5.7^{b}	65.3 ± 5.9^{a}	49.4 ± 6.0^{c}	48.0 ± 16.5^{b}
T3 (C/N10)	23.9 ± 5.1^{b}	67.4 ± 17.2^{a}	39.3 ± 8.0^{c}	43.6 ± 21.5^{b}
T4 (C/N15)	31.8 ± 7.7^{b}	65.8 ± 1.4^{a}	20.4 ± 3.9^{b}	39.3 ± 20.9^{b}
T5 (C/N20)	0^{a}	-	-	-

Artemia performance in term of individual length was showed in Table 3. At DAH7, the length of *Artemia* was in range 4.1-4.4 mm and it increased in all the culture where carbon was added, but the increase in length was not significant different as compared to those *Artemia* in the control treatment. After 14 day of culturing (DAH14), *Artemia* in control treatment was smaller than *Artemia* in the C-treatments (7.69 mm versus 8.11 to 8.36 mm; Table 4), except for *Artemia* in T5. The increase of C/N ratio was resulted in improved length of *Artemia*, but no significantly difference was found in length between treatments ($p > 0.05$). Toi *et al*. (2013) reported that the higher growth performance in term of length was obtained in the carbon added treatment, which

may result from bacteria grown in the culture medium when they used added carbon as nutrition source. As a consequence, *Artemia* benefitted from these bacteria that are believed to contribute with enzymes to breakdown of food and easier absorbed by target animal (Intriago and Jone, 1993) and it can be used as a direct food for *Artemia* (Hoa *et al.*, 2007).

Table 3. The individual length (mm) of *Artemia* of parent generation

Treatment	Individual length (mm)	
	DAH7 length	DAH14 length
T1 (control)	4.10 ± 0.61^{a}	7.69 ± 0.97^{a}
T2 (C/N = 5)	4.12 ± 0.58^{a}	8.11 ± 0.80^{a}
T3 (C/N = 10)	4.33 ± 0.86^{a}	8.27 ± 1.10^{a}
T4 (C/N = 15)	4.34 ± 1.06^{a}	8.36 ± 0.93^{a}
T5 (C/N = 20)	4.42 ± 0.87^{a}	-

Total biomass production

The result of *Artemia* biomass production was showed in Table 4. The biomass production in 1st batch, 2nd batch and third batch was harvested at DAH16, DAH14 and DAH12 of each batch, respectively. This result indicated that the use of offspring for re-stocking is not only shorter culture period to get harvesting but also save time and cost for biomass production. The biomass production in the first batch was in range 0.78 - 1.08 g/L, there was no significant between the carbon-treated treatment and the control treatment. It was clear that carbon addition in the first batch was not improved biomass production that might be *Artemia* were not adaptation to *Artemia* feed and bio-floc environment, and in later batches, *Artemia* had better adaptation to *Artemia* feed and bio-floc environment, therefore shorten time of culture. For the second batch, biomass production in the control treatment was lower than in C- treatments, the lowest biomass (1.48 g/L) was obtained in control and highest biomass was recorded in T2 (2.2 g/L). However, only significant different was found ($p < 0.05$) between T1 (control) and T2 (C/N ratio 5) while there were not significant different among others. In addition, biomass production was improved in all carbon-added treatments at the third batch, the highest biomass (1.66 g/L) was in T2 whereas lowest biomass was obtained in the control treatment. The difference in biomass production was significant higher in all carbon-added treatments than in control treatment except T4 (Table 4). The addition of carbon at C/N=5 resulted in highest biomass production and was significant different compared to that obtained in the C/N=15, but no significantly difference was found when compared to the biomass production in C/N=10.

In general, after three culture batches, total biomass production in three batches was in range 3.1 to 4.9 g/L indicated that addition of carbon in the culture resulted in higher biomass production than in the control treatment. Based total biomass harvesting, apparently that, the carbon addition in *Artemia* culture with C/N =5 gave best result in improving of biomass production, although not significantly ($p > 0.05$) to other ratios among C-treatments but was significant difference to those in the control ($p < 0.05$; Table 4). According to Toi *et al*. (2013a; 2013b) bacterial growth stimulated by the addition of carbon not only improves water quality but also increases the production of *Artemia*, the biomass production increased nearly double at DAH 14 in the culture where *Artemia* fed with *Tetraselmis* sp. and carbon added to get C/N ratio 10 and sucrose was used as the carbon source.

Table 4: Biomass production (g/L).

Treatment	Biomass production (g/L)			
	1st batch (P)	2nd batch (F1)	3rd batch (F2)	Total biomass
T1 (control)	1.08 ± 0.20^{a}	1.48 ± 0.25^{a}	0.56 ± 0.11^{a}	3.11 ± 0.33^{a}
T2 (C/N = 5)	1.02 ± 0.19^{a}	2.20 ± 0.24^{b}	1.66 ± 0.10^{c}	4.89 ± 0.43^{b}
T3 (C/N = 10)	0.78 ± 0.20^{a}	2.03 ± 0.31^{ab}	1.33 ± 0.29^{bc}	4.14 ± 0.40^{ab}
T4 (C/N = 15)	1.04 ± 0.26^{a}	1.89 ± 0.13^{ab}	0.96 ± 0.21^{ab}	3.90 ± 0.52^{ab}
T5 (C/N = 20)	0	-	-	-

In contrast, in present study the *Artemia* feed (30% protein; 9% lipid) and molasses was used, so that the experimental condition was in difference. According to Crab (2010) the nutritional value of biofloc is dependent on carbon source using, the crude protein of biofloc produce by glucose (40% DW) was higher than biofloc produced by starch (20% DW) and acetate (19% DW). Therefore, the biofloc produced in this study might have lower quality than that obtained from previous study. This reason may result lower improved biomass production in this study.

IV. CONCLUSIONS

After 42 days of culturing with three batches, there were improved in length of *Artemia* in the carbon-treated treatments but it was not significantly different compared to the control. However, significantly improved survival in all carbon-treated treatments was found, except for the culture with C/N ratio 20.

The improve biomass production was obtained in all carbon-treated treatments, except for C/N 20. No significantly difference was found in biomass production between carbon-treated treatments as compared to the control, but significantly higher biomass production than in control was found in T2 (C/N5).

The result of this study indicated that *Artemia* culture with carbon addition at C/N=5 gave the best survival and biomass production.

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