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The effect of single-cell detritus produced by the gutweed (*Enteromorpha intestinalis*) on filter-feeder growth: *Artemia franciscana* as a case study

Ngo Thi Thu Thao * and Huynh Thanh Toi
College of Aquaculture & Fisheries, Can Tho Univerisity, Viet Nam.

*Corresponding Author: thuthao@ctu.edu.vn

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ABSTRACT

In this study, we evaluated the single-cell detritus (SCD) produced by *Enteromorpha intestinalis* (gutweed) in order to develop a suitable diet for *Artemia* (brine shrimp) as a way of reducing its dependence on microalgae. This involved examining the effects of the SCD diet on the survival rate, growth performance, and reproductive characteristics of *Artemia franciscana* under laboratory conditions. The *Artemia* were fed 05 different diets, each diet administered in three replications. Commercial shrimp feed was used as the control and the four test diets comprised shrimp feed replaced by SCD in proportions of 100, 75, 50, and 25%. The 100% shrimp food diet promoted the highest reproduction (90 offspring/female), but the 25% replacement also gave positive results (78 offspring/female). In terms of growth, the 25% replacement produced the greatest length (10.3 mm) on day 14. The findings indicate that the diet with 25% SCD should be further studied to determine its applicability to feeding *Artemia* or other filter feeders.

INTRODUCTION

With enhanced development of the aquaculture industry, the consumption of high-nutritional-value *Artemia* (brine shrimp) products is on the increase. The regions in which *Artemia* is cultured are expanding, but productivity is not increasing accordingly. In addition to green water (microalgae), *Artemia* can be fed rice bran, but the efficiency is only 20% (Nguyen *et al.*, 2007), with rice bran and soybean meal producing low-quality biomass and cysts (Sorgeloos *et al.*, 1986; Van *et al*., 2011). Research is being conducted to determine how to obtain high, stable productivity that is suitable for the development of the *Artemia* farming industry. Phytoplankton, and especially microalgae, is an essential food source that has long been used for larval fish and shellfish by farmers, but the price is now too high. It is thus necessary find a replacement food source that is low cost, simple to administer, and that ensures nutrient and digestibility requirements.

Single-cell detritus (SCD) may meet this need (Uchida, 1996; Camacho *et al.*, 2004; Ngo and Nguyen, 2017). Studies on SCD have been conducted on *Artemia* (Uchida *et al.*, 1997), the clam *Ruditapes decussatus* (Camacho *et al.*, 2004), and the oyster *Crassostrea belcheri* (Tanyaros and Chuseingjaw, 2016), indicating its potential for wider use in aquaculture practices. Recently, Ngo and Nguyen (2017) and Ngo *et al*. (2018) showed that SCD harvested from red seaweed (*Gracilaria tenuistipitata*) and fermented with yeast (*Saccharomyces cerevisiae*) (i.e., SCD-Y) could obtain a density of 301×10^4 particles/mL. The authors also found that a diet of 100% SCD-Y, or a combination of 50% SCD and 50% shrimp feed, promoted a relatively high survival rate and good growth performance in *Artemia*, and had a positive influence on the maturity of *Artemia franciscana*. However, the source of *G. tenuistipitata* is the central region of Vietnam, and sometimes it is in short supply. Gutweed (*Enteromorpha*) occurs commonly in the brackish waters of the Mekong Delta in Vietnam. This green seaweed has been found in several southern provinces, such as Soc Trang, Bac Lieu, and Ca Mau, and might be a potential source for SCD for feed.

Consequently, an evaluation of the effect of SCD from gutweed (*Enteromorpha intestinalis*) on the growth of *A. franciscana* was considered to be useful, in addition to determining the practices for handling feed sources for filter feeders, such as zooplankton, crustaceans, and bivalve mollusks, in laboratories and hatcheries. This study was thus aimed at evaluating various concentrations of SCD from *Enteromorpha* in *Artemia* feed in order to develop a suitable diet for *Artemia* in culture and to reducing the dependence on microalgae. A suitable feeding regime was investigated in order to evaluate the effects of a diet containing SCD on the survival rate, growth performance, and reproductive characteristics of *A. franciscana* under laboratory conditions.

MATERIALS AND METHODS

Preparation of SCD from *Enteromorpha intestinalis*

Samples of *Enteromorpha intestinalis* were collected from Soc Trang province, Vietnam, and transported to the laboratory. In the laboratory, the fresh gutweed was rinsed and all debris and biofouling organisms were removed. The samples were then dried indoors for 24 to 48 h, depending on the weather conditions. The dryness of the samples was evaluated by hand, and when dry, the gutweed was ground to a powder in a blender prior to preparation of the SCD (modified from Uchida *et al*., 1996, 1997 and Tanyaros *et al*., 2014).

The reverse osmosis technique of Tanyaros *et al*. (2014) was modified to produce the SCD, which included three steps: 1) the dried gutweed was homogenized using a food blender and then sieved through a 200-µm-mesh screen; 2) the sieved material was placed in a 500-mL Erlenmeyer flask with freshwater (1:25 ratio) and agitated using a shaker operated at 100 rpm for 2 h; and 3) 10^6 yeast cells/mL (for fermentation) and 70

mg/L glucose (nutritional solution for the yeast) were added and the sample was left for 48 h, after which the SCD was harvested. The SCD was then sieved through a 50-µm-mesh screen, after which it was stored at 4ºC prior to feeding to the *Artemia*.

Evaluating the effects of SCD-supplemented diets on the reproduction, growth, and survival rates in *Artemia*

Artemia nauplii were fed on microalgae (*Chaetoceros*) for the first two days of the experiment. From Day 3 onwards, five different diets were administered in three replications, as follows: Treatment 1 (control feed, CF) –100% commercial shrimp feed; Treatment 2 (S100)–100% SCD; Treatment 3 (S75) –75% SCD + 25% commercial feed; Treatment 4 (S50)–50% SCD + 50% commercial feed; and Treatment 5 (S25)–25% SCD + 75% commercial feed.

***Artemia* culture.** Twenty 10-L glass bottles were prepared by adding 2 L brine water at 80‰ to each bottle. Newly hatching *Artemia* were inoculated into the bottles at a density of 100 individuals/L. Aeration and illumination were supplied continuously during the culture period. The *Artemia* were fed three times per day at 8:00, 13:00, and 18:00 h. On Day 1, all the *Artemia* were fed with *Chaetoceros* at a density of 50,000 cells/mL. On day 2, 100,000 cells/mL were administered. From Day 3, the *Artemia* were fed on one of the five treatments described above. Commercial feed No. 0 for post-larvae shrimp contained 40–42% protein (Grobest, Viet Nam), and this was immersed in saltwater at 30‰ for 15 min and then filtered through a 50-µm-mesh sieve before being fed to the *Artemia*. The amount of feed used was that recommended by Nguyen (1993). After one week, the standard feed quantity for one *Artemia* was 0.061 mg/day. On Day 14, this was 0.2215 mg/individual/day and from Day 20, 0.3694 mg/individual/day. The water in each culture bottle was renewed by 20–30% every 2–3 days, depending on changes in the environmental factors.

Determination of total bacterial density. The bacterial density was determined on Days 1 and 9 by the method described in Huys (2002). Before culturing the bacteria on Trypticase soy agar (TSA) plates, the TSA plates were prepared using TSA + 1.5% salt (TSA^{+}). Sterile test tubes containing 9 mL saline (0.85%) were used to perform a serial dilution of each sample. After dilution, 100 µL of each sample was removed by sterile micropipette and transferred to the sterile TSA plate, where the inoculum was gradually spread over surface of the plate using a sterile spreader until dry. The bacteria-inoculated plates were incubated at 30ºC for 24–48 h. The total colony-forming units (CFUs) were counted as soon as they were visible. The number of CFUs needed to be between 20 and 200. The means and standard deviations of the CFUs were calculated and the bacterial density was calculated as follows:

$$\text{CFU/ mL} = \text{number of colonies} \times \text{dilution} \times 10 \quad (1)$$

Data collection

Environmental parameters. The pH values, NO_2^- and alkalinity (mg calcium carbonate [$CaCO_3$]/L) concentrations were measured in the *Artemia* culture media on Days 1, 7, 14, and 21 using SERA test kits (Germany). The temperature was recorded daily at 7:00 and 14:00 h. The salinity was maintained at around 80‰ during the culture period.

Growth performance of *Artemia.* Samples of 10 *Artemia* individuals from each culture plate were collected and fixed in Lugol solution in order to measure their total lengths. This was measured on Days 1, 3, 5, 7, 10, and 14 from the top of the head to the end of the tail using binoculars microscope fitted with a micrometer eyepiece. The following formula was applied:

$$L\ (mm) = A/10 \times 1/\gamma \quad (2)$$

where L = length of *Artemia* (mm); A = length under the binoculars; and γ = magnification (0.8–4 times).

Survival rate of *Artemia*. The number of live *Artemia* was counted on days 7, 14, and 21 in order to calculate the survival rate, and the following formula was applied:

$$SR\ (\%) = (N2 \times 100)/N1 \quad (3)$$

where N1 = initial number of *Artemia* specimens; and N2 = number of *Artemia* specimens at collecting time.

Reproduction in *Artemia*. When new mating (riding couples) occurred in the *Artemia* population, each couple was collected, recorded, and transferred into a new bottle. Fifteen couples (male + female) were collected to determine their individual lengths at first mating. Every three days from the first mating, 15 embryo-carrying females from each treatment were collected to determine their fecundity and reproductive mode, the brood sack being opened to count the number of embryos inside.

Statistical analysis

The mean value and standard deviation was calculated for all the datasets using Microsoft Excel 2010. Data analysis was performed using one-way analysis of variance (ANOVA) to compare the differences between the mean values of the treatments using the Duncan test in SPSS software (level of significance $P < 0.05$).

RESULTS & DISCUSSION

1. Environmental parameters of the *Artemia* culture medium

During the 21 days of the experiment, the pH fluctuated from 7.4 to 7.5 and the temperature at 7:00 AM and 14:00 PM fluctuated from 28.6 to 30.1ºC. According to Nguyen *et al.* (2007), *A. franciscana* grows well at 22–35ºC and pH values of 7.0–9.0, so the temperature in this study was suitable for the growth, survival, and reproduction of *Artemia*.

On Day 1 of the experiment, the NO_2^- concentration in the treatments was low. Overall, the NO_2^- concentration was the highest in the CF treatment, increasing from 0 ±

0 to 0.15 ± 0.07 mg/L, followed by treatment S25, with the remaining treatments having the lowest NO_2^- concentrations (Table 1). At the end of the experiment, the NO_2^- concentration in the CF treatment was 1.5 times higher than for S25, three times higher than for S75, and five times higher than for S100. This variation may have been caused by differences in the protein content of the feed and the different survival rates under the treatments, so the concentration of NO_2^- increased in the water and varied among the treatments. Despite this, the NO_2^- concentrations in the treatments were below the safety threshold for aquatic animals in general and *Artemia* in particular (Dhont and Lavens, 1996).

Table 1. NO_2^- concentrations (mg/L) for different feed treatments

Feed treatment	Culture period			
	Day 1	Day 7	Day 14	Day 21
100CF	0	0.08±0.04	0.10±0.00	0.15±0.07
100SCD	0	0	0.03±0.04	0.03±0.04
75SCD	0	0	0.05±0.00	0.05±0.00
50SCD	0	0	0.05±0.00	0.08±0.04
25SCD	0	0.05±0.00	0.08±0.04	0.10±0.00

Alkalinity was one of the factors that affected the growth and development of *Artemia*. On Day 21, the highest alkalinity was in S50 (152.2 mg $CaCO_3$/L) and the lowest was in CF and S100 (134.3 mg $CaCO_3$/L). Supporting the findings of Vo (2018), *Artemia* showed the best growth and development at alkalinity ranges of 120 to 180 mg $CaCO_3$/L, so the alkalinity in this experiment was appropriate for the growth, survival, and reproduction of *Artemia*.

2. Total bacterial density

The total bacterial density in the *Artemia* culture water on Day 1 was 5.20 logCFU/mL (Table 2). On Day 9, this was variable for all treatments. The total bacteria density was highest in the CF treatment (6.35 logCFU/mL), followed by S75 (6.33 logCFU/mL). This value was 1.14 times higher than for S100 (5.57 logCFU/mL) and 0.95 times higher than for S50 (6.02 logCFU/mL). Thus, only a small fluctuation in total bacteria density among the treatments was recorded (5.57–6.35 logCFU/mL) and the difference was not significant ($p > 0.05$). *Artemia*, with its non-selective feeding behavior, is capable of filtering suspended matter in water (organic matter, bacteria, SCD) in a particle size range of <50 μm. Changing the water on days 3, 5, and 7 probably caused the differences in total bacteria density.

Table 2. Total bacteria density (logCFU/mL) in the water medium during the experiment. Data with different letters in the same row indicate significant difference ($p < 0.05$)

Culture day	Treatment code				
	CF	S100	S75	S50	S25
1	5.20 ± 0.08^{a}	5.20 ± 0.08^{a}	5.20 ± 0.08^{a}	5.20 ± 0.08^{a}	5.20 ± 0.08^{a}
9	6.35 ± 0.56^{a}	5.57 ± 0.38^{a}	6.33 ± 0.30^{a}	6.02 ± 0.77^{a}	6.04 ± 0.40^{a}

3. Growth, survival rate, and reproduction of *Artemia*

3.1 Artemia growth

The newly hatching *Artemia* nauplii had an average length of 0.34 mm. *Artemia* length differed between treatments ($p < 0.05$) from Day 3, after which S25 showed the longest lengths, which was significant ($p < 0.05$). The shortest *Artemia*s resulted from S100. On Day 7, CF (4.28 mm), S75 (4.62 mm), and S50 (4.21 mm) showed no significant differences, but the difference was statistically significant for S100 (2.10 mm) and S75 (3.10 mm).

On Day 14, the *Artemia* length in all treatments increased significantly ($p < 0.05$), reaching the highest value in S25 (10.31 mm), followed by CF (9.44 mm), and the lowest value in S100 (5.69 mm). These were longer lengths than those found by Ngo (2018), who reported 6.67 mm on Day 14 from 100% commercial feed (= CF). This might be there was no mineral supplementation in the food used in that study, while the influence of environmental factors may have slowed the growth rate.

Table 3. *Artemia* length (mm) with different feed treatments. Data with different letters in the same column indicate significant difference ($p < 0.05$)

Culture day	Treatment code				
	CF	S100	S75	S50	S25
1	0.34 ± 0.05^{a}	0.34 ± 0.05^{a}	0.34 ± 0.05^{a}	0.34 ± 0.05^{a}	0.34 ± 0.05^{a}
3	1.12 ± 0.05^{ab}	1.01 ± 0.03^{a}	1.07 ± 0.03^{ab}	1.90 ± 0.02^{ab}	1.18 ± 0.01^{b}
5	3.02 ± 0.06^{b}	1.55 ± 0.13^{a}	2.40 ± 0.05^{b}	2.53 ± 0.18^{b}	3.75 ± 0.03^{c}
7	4.28 ± 0.19^{c}	2.10 ± 0.11^{a}	3.10 ± 0.09^{b}	4.21 ± 0.02^{c}	4.62 ± 0.12^{c}
10	5.44 ± 0.41^{bc}	2.94 ± 0.06^{a}	4.87 ± 0.02^{b}	5.78 ± 0.36^{c}	5.83 ± 0.39^{c}
14	9.44 ± 0.02^{d}	5.69 ± 0.07^{a}	7.18 ± 0.15^{b}	8.78 ± 0.04^{c}	10.31 ± 0.02^{e}

3.2 Artemia survival rate

On Day 7, the highest rate of *Artemia* survival occurred in CF (92.3%), followed by S25 (91%), with significant differences for S100, S75, and S50 ($p < 0.05$). On Day 14, the highest survival rate (85.5%) was recorded for S25. There was no significant difference for CF (84%) and S75 (84.3%) ($p > 0.05$) on Day 14, although these were statistically significant when compared to S100 and S50 ($p < 0.05$). The highest survival

rate presented for S25 (81.1%), albeit the values were still high for CF (79.8%), S75 (75.4%), and S50 (70.3%). The lowest survival rate presented for S100 (64.3%) on Day 21. These results were higher than those reported by Ngo and Huy (2017), who found 72% after 14 days using 50% SCD from *G. tenuistipitata* + 50% shrimp feed. Our results indicate that a diet mixed with 25% gutweed SCD + 75% commercial feed produced the highest survival rate of *Artemia*, supporting the use of this species for biomass culturing.

Table 4. *Artemia* survival rate for different feeding treatments (%). Data with different letters in the same column indicate significant difference ($p < 0.05$)

Treatment code	Culture period		
	Day 7	Day 14	Day 21
CF	92.33 ± 4.65^{c}	84.17 ± 5.36^{c}	79.75 ± 5.65^{c}
S100	74.75 ± 3.19^{a}	70.25 ± 3.78^{a}	64.33 ± 5.86^{a}
S75	88.17 ± 2.75^{bc}	84.33 ± 1.89^{c}	75.42 ± 3.41^{bc}
S50	82.50 ± 3.63^{b}	77.00 ± 3.13^{b}	70.33 ± 2.27^{ab}
S25	91.00 ± 2.75^{c}	85.50 ± 1.98^{c}	81.08 ± 1.88^{c}

3.3 Artemia reproduction

The individual length of the female on the first day of a riding couple was greater than the male. The length on the first day of mating was the highest for S25 (7.98 mm), and this was significantly different than for the other treatments ($p < 0.05$). Furthermore, S25 also produced the greatest growth (8.47 mm at Day 17).

Artemia reproduced 100% cysts. With CF, riding couples were observed on Day 9–earlier that for all treatments containing SCD. Also, the higher the SCD concentration, the later riding couples were observed.

Table 5 presents the results for the average fecundity of *Artemia* females, which fluctuated from 38.7 to 89.5 offspring/brood. The CF treatment gave the highest value (89.5 offspring/brood) and this was significantly different from the others ($p < 0.05$), followed by S25 (78.1 offspring/brood). The lowest fecundity was for S100 (38.7 offspring/brood). These results indicate that the 100% commercial feed (CF) was the best diet for growth, maturation, and reproduction. However, S25 and S50 showed similar results, meaning that those diets probably fulfilled the nutritional requirements of *Artemia*, especially during the maturation and reproduction stages.

Uchida *et al.*, (1997) prepared an algal suspension containing protoplasmic detritus [termed, single cell detritus (SCD)] from freeze-dried fronds of *Ulva* and its dietary value to *Artemia* nauplii was tested after size fractionation. The authors found that the fraction passing through a 100-µm mesh and containing SCD of 2–14 µm in diameter, contributed to the survival of *Artemia*. The bacterium *Pseudoalteromonas espejiana* strain AR06 FERM BP-5024 degraded *Ulva* forming new SCD and the dietary value of *Ulva* for *Artemia* growth. The protein content of the SCD was approximately doubled by the attaching of bacteria, suggesting the enhanced *Artemia* growth is attributable to the

combined effect of the SCD and the bacteria. According to Felix and Pradeepa (2011), SCD with high protein content crude is 35% and high nutritional value. Particles SCD can be produced in other sizes different, suitable to the nutritional needs of each species, can partially or completely replace microalgae as feed in the hatchery. Thao (2019) reported that green seaweeds (*Enteromorpha intestinalis)* after fermentation with yeast showed the increase in percentages of protein from 16.01% in raw material to 21.16 % in SCD product. Author also applied the diet with 50% shrimp feed and 50% SCD for feeding *Artemia* and showed the high survival rate and good growth performance, as well as positive effects on the reproductive characteristics of *Artemia franciscana*.

Table 5. Some reproductive parameters for *A. franciscana*. Data with different letters in the same column indicate significant difference ($p < 0.05$)

Treatment code	Embryos/female	Female length (mm)		Male length (mm)	
		Mating day	Day 17	Mating day	Day 17
CF	89.53±15.12^{d}	7.19±0.43b^{b}	10.07±0.48^{c}	6.44±0.40^{c}	9.05±0.41^{c}
S100	38.67±6.67^{a}	6.04±0.41^{a}	8.20±0.61^{a}	5.35±0.32^{a}	7.34±0.63^{a}
S75	54.93±8.78^{b}	7.29±0.46^{b}	8.98±0.61^{bc}	6.52±0.46^{bc}	8.04±0.76^{b}
S50	63.07±10.79^{bc}	7.50±0.41^{bc}	9.08±0.37^{b}	6.97±0.44^{c}	8.28±0.44^{bc}
S25	78.07±8.81^{cd}	7.98±0.80^{c}	9.59±0.48^{bc}	7.13±0.56^{c}	8.47±0.42^{bc}

Studies on SCD have been conducted on the bivalve mollusc such as clam *Ruditapes decussatus* (Camacho *et al*., 2004) and oyster *Crassostrea belcheri* (Tanyaros and Chuseingjaw, 2016), showing its potential use in aquaculture practices. Camacho *et al*. (2004) applied the sequential action of two enzymes: endoglucanases and cellulases and two bacteria (CECT 5255 and CECT 5256) transformed *Laminaria saccharina* meal into a suspension of algal cells and detritus of less than 20 μm in diameter. SCD from *L. saccharina* can replace between 80% and 90% of the live phytoplankton content in the feeding of *R. decussatus*, with growth rates equaling, and even surpassing, those resulting from live phytoplankton diets. Tanyaros and Chuseingjaw (2016) stated that the replacement of microalgae with SCD from *Porphyra haitanensis* was unsuitable for nursing oyster larvae. However, for juvenile oysters (shell width 1.85 ± 0.03 mm and shell length 1.78 ± 0.06 mm) substituting 75% of microalgae with SCD showed lower absolute shell growth, and lower daily yields and survival rates when compared to rates substituting 50% or lower substitution with SCD, or 100% microalgae ($P < 0.05$). Authors suggested that substituting 50% of the traditional microalgae with SCD produced from seaweed (*P. haitanensis*) can be used as a partial microalgae substitute for the nursery culture of the juvenile tropical oyster (*Crassostrea belcheri*). Our findings showed that a diet mixed with 25% gutweed SCD + 75% commercial feed obtained the highest survival rate of *Artemia,* also this combination probably fulfilled the nutritional requirements of *Artemia* for the maturation and reproduction stages.

CONCLUSION & RECOMMENDATION

The diet with 25% SCD from *E. intestinalis* + 75% commercial feed showed the best result in terms of growth rate, survival rate, and reproduction in *A. franciscana* after 21 days of culture.

Further research should be conducted into the nutritional composition of SCD and the ability to use SCD as a food for other filter-feeding species.

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