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Simultaneous Growth of *Chaetoceros muelleri* and Bacteria in Batch Cultures

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Abstract

The relationship between bacterial load and the microalga *Chaetoceros muelleri* was analyzed in a scale-up experiment. The microalga was grown during five days in a 0.4-L Erlenmeyer flask, 2-L Fernbach flask, 18-L Carboy and 400-L column, during which the cell density of *C. muelleri*, the abundance of heterotrophic bacteria, *Vibrio* spp., and total bacteria were determined. The highest specific growth rates (μ) of *C. muelleri* occurred during the first day of culture (0.88 to 2.29 d^{-1}). Highest cell density was recorded on the fifth day at the 2-L (7.62×10^6 cells·mL^{-1}) and 18-L (6.32×10^6 cells·mL^{-1}), coinciding with the maximum counts of heterotrophic bacteria (16.55×10^5 and $>30 \times 10^5$ CFU·mL^{-1}, respectively). There was a high correlation (0.80, 0.75, 0.85; $p < 0.05$) between microalgal cell density and total bacteria in the first three culture volumes and a low correlation (0.27; $p = 0.34$) at 400-L column. The highest mean concentration of total bacteria (884.13×10^5 cells·mL^{-1}) during the five days occurred at 18-L Carboy. The concentration of total bacteria at all levels was always higher than that of heterotrophic bacteria. The average ratio of heterotrophic to total bacteria was higher in the 2-L (0.0108) and 18-L (0.0172) cultures. The high biomass of *C. muelleri* and the presence of *Vibrio* spp. at the 18-L and 400-L levels indicate that it is necessary to establish programs to prevent diseases and economic losses caused by pathogenic bacteria in penaeid shrimp farming.

Keywords

Chaetoceros muelleri, Bacterial Load

1. Introduction

An alternative approach to reduce the problem of contamination by pathogenic bacteria in the farming of mol-

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luscs, fish and crustaceans is the use of inert artificial diets, which in turn reduces production costs. In the case of shrimp farming, alternative feeds have been used to prevent the presence of pathogens, increase larval survival and reduce economic losses [1] [2]; however, the growth and survival rates obtained have not been entirely satisfactory and microalgae continue being the main source of food during the first stage of the development of commercially farmed species.

Compared with artificial diets, microalgae have high nutritional values and are easily ingested because of their size [3] [4]. One of the most used in aquaculture centers is *Chaetoceros muelleri*. This species is rich in essential nutrients and is the first live food offered during the early development stages of crustaceans [5]. Microalgal cultures, however, are non-axenic and may contain pathogenic bacteria such as *Vibrio* spp., and can be transferred to larval cultures during feeding. Studies on the diseases of commercial penaeid shrimp and bivalves indicate that the presence of pathogenic agents in cultivated organisms is due to the quality and quantity of the feed, high stocking densities, low water quality and stress, among others [6]-[8]. These authors find that pathogenic and probiotic vibrios cause stress and can alter the metabolic routes of larvae, juveniles and adults of the abalone *Haliotis rufescens*.

Several microbiological studies have focused on obtaining higher yields and survival rates in the culture of invertebrates, but bacterial load in microalgal cultures has been little studied and insufficient attention has been paid to microalgae as food and main source of contamination in culture systems. The purpose of this paper is to evaluate the abundance of *Vibrio* bacteria that cause diseases in cultured organisms, as well as the relationship between *C. muelleri* and heterotrophic and total bacteria during scale-up of the microalgal culture.

2. Materials and Methods

2.1. Microalgae

The marine diatom *Chaetoceros muelleri* was obtained from the collection of microalgae at the Institute of Oceanological Research of the Autonomous University of Baja California. This species was cultured at a temperature of $20°C \pm 1°C$ using f/2 medium [9]. To prepare the culture medium, seawater was filtered through a series of three 1-µm Cuno filters and irradiated with ultraviolet light. The growth of *C. muelleri* was evaluated over five days (in triplicate sample) at four levels of the scale-up process: 0.4 L (Erlenmeyer flask), 2 L (Fernbach flask), 18 L (Carboy), and 400 L (column). In the first two assays cases, the culture medium and flasks were sterilized by autoclaving at $121°C$ and 1.05 kg·cm^{-2} for 15 minutes. At 18-L Carboy, seawater was filtered and irradiated with ultraviolet lamps, treated with sodium hypochlorite at a rate of 0.30 mL·L^{-1}, and left to rest for 24 hours without aeration, after which sodium thiosulfate was added (0.15 mL·L^{-1}) to neutralize the chlorine and aeration was introduced for 2 hours. At 400-L column, filtered seawater was only subjected to ultraviolet irradiation. Depending on the culture volume, the cultures were inoculated under aseptic conditions with an initial mean density of 0.2×10^5, 0.3×10^6, 1.1×10^6, and 0.3×10^6 cells mL^{-1}, respectively, and they were maintained under constant photoperiod with light intensities of 80, 150, 240, and 1100 µmol quanta $\text{m}^{-2}\text{·s}^{-1}$, respectively, using 75-W fluorescent lamps in the first three assays and a 500-W lamp in the last. Irradiance was measured with a QSL-100 scalar PAR irradiance sensor (Biospherical Instruments). At 24-hour intervals after inoculation, the Erlenmeyer and Fernbach cultures were stirred manually to prevent cellular sedimentation, while the Carboy and column cultures were supplied aeration (5 and 25 L·min^{-1}). These culture conditions were maintained for five days. Cell density was quantified daily (in duplicate samples) at each level using a particle counter (Beckman Coulter Multisizer 3). Controls without microalgae were done for the Fernbach and Carboy cultures using aliquots of 100 and 200 mL, respectively. The aliquots were centrifuged twice (1644 x g, 15 minutes), and then passed through GF/F fiberglass filters to obtain a medium containing only bacteria associated with the culture and the f/2 nutrients.

2.2. Heterotrophic and *Vibrio* Bacteria

Heterotrophic and *Vibrio* bacteria were enumerated daily (in duplicate samples) by the spread plate method using Zobell Marine Agar and Thiosulfate Citrate Bile Sucrose (TCBS) agar, respectively. Both media were inoculated with 0.1 mL of serially diluted sterile aged seawater to guarantee from 30 to 300 colony forming units CFU·mL^{-1}. The plates were incubated at $25°C$ and the colonies were counted five days after inoculation for heterotrophs and after 48 and 72 hours for *Vibrio* spp. using a Quebec colony counter (values expressed as CFU·mL^{-1}).

2.3. Direct Counts of Total Bacteria

Total bacteria were enumerated daily (in duplicate samples) by epifluorescence microscopy. For this, 5 mL of the microalgal culture was fixed with 0.25 mL of 40% formaldehyde (final concentration of 2%) and filtered using black polycarbonate filters (0.22 μm pore size) and GF/F fiberglass filters as support. The samples were stained with DAPI (4', 6-diamidino-2-phenylindole) at a final concentration of 2 μg·mL^{-1} for 5 minutes in darkness [10].

The bacterial counts were performed on an Axiovert 200 (Carl Zeiss) microscope, with a 50-W lamp and UV excitation wavelength. Images were taken (25 - 40 bacterial cells per field) with an Olympus Dp70 camera adapted to the microscope. The results are expressed as number of bacteria per milliliter.

3. Results

3.1. Microalgal Growth

The growth of *Chaetoceros muelleri* during the scale-up process is shown in **Figure 1**. The exponential phase was observed on the first day in all the cultures except at 0.4-L Erlenmeyer and the death phase did not occur in any. At the Erlenmeyer, the mean initial cell density was $0.2 \times 10^5 \pm 0.0008$ cells·mL^{-1}, with a lag phase of one day, followed by the exponential growth phase that lasted two days, with a specific growth rate (μ) of 2.29 and 3.3 per day. The slow growth phase occurred on Days 3 to 5 and was characterized by a reduction in specific growth rate (0.51 - 0.04) and, consequently, an increase in doubling time from 1.36 to 17.35 days. At 2-, 18-, and 400-L of culture, the highest specific growth rates (μ) were 1.41, 0.88, and 1.25, respectively, and they were recorded 24 hours after initiating the experiment. The highest biomasses ($7.62 \times 10^6 \pm 0.5$ and $6.32 \times 10^6 \pm 0.1$ cells·mL^{-1}) were recorded in the Fernbach and Carboy cultures, respectively, on Day 5. Cell density at 400-L column ($2.57 \times 10^6 \pm 0.02$ cells·mL^{-1}) was higher than that found at 0.4-L Erlenmeyer ($2.12 \times 10^6 \pm 0.04$ cells·mL^{-1}) but lower than that found in the Fernbach and Carboy cultures.

3.2. Bacterial Growth

Mean growth of heterotrophic bacteria in *C. muelleri* cultures is shown in **Table 1**. At 0.4-L Erlenmeyer, the concentration of bacteria increased from 0.02×10^5 to 1.74×10^5 CFU·mL^{-1} after five days, a similar pattern to that of microalgal growth (from 0.2×10^5 to 21.2×10^5 cells·mL^{-1} after five days) (**Figure 1**). The bacteria to microalga ratio were 0.101 at the beginning of the culture and 0.082 on Day 5 (**Table 1**).

Bacterial growth at 2-L Fernbach was similar to that at 0.4-L Erlenmeyer, the concentration of heterotrophic bacteria increasing from 0.15×10^5 CFU·mL^{-1} on the day of inoculation (Day 0) to 16.55×10^5 CFU·mL^{-1} on Day 5, with a bacteria to microalga ratio of 0.046 and 0.217 for the respective days. The inoculum size (0.3×10^6
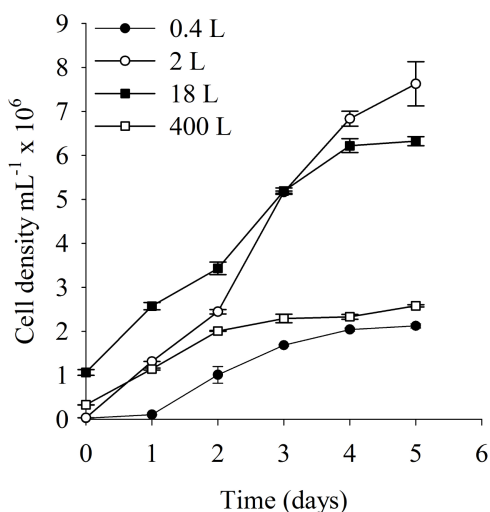

Figure 1. Average growth of *Chaetoceros muelleri* in different volumes of cultures using f/2 medium. The vertical bars indicate standard error (n = 3).

Table 1. Average concentration of bacteria in *Chaetoceros muelleri* culture.

Time	V	HB	HB:M	DSB	HB:DS	*Vibrio*
(Day)	(L)	(CFU·mL^{-1} × 10^5)		(Cell·mL^{-1} × 10^5)		CFU
0	0.4	0.02	0.101	7.01	0.0032	0
	2	0.15	0.046	45.24	0.0033	0
	18	3.43	0.322	104.49	0.0328	0
	400	1.19	0.362	139.03	0.0085	0
1	0.4	0.20	0.192	19.29	0.0102	0
	2	0.17	0.013	74.49	0.0023	0
	18	>3.00	0.117	423.53	0.0071	0
	400	2.69	0.235	271.91	0.0099	0
2	0.4	0.23	0.023	24.62	0.0095	0
	2	3.07	0.125	96.74	0.0317	0
	18	>3.00	0.087	576.03	0.0052	10
	400	>3.00	0.149	574.08	0.0052	0
3	0.4	0.20	0.012	44.37	0.0044	0
	2	1.45	0.028	231.63	0.0062	0
	18	19.53	0.376	1028.69	0.0190	0
	400	>3.00	0.131	956.10	0.0031	30
4	0.4	1.24	0.061	191.88	0.0006	0
	2	1.75	0.026	328.99	0.0053	0
	18	26.38	0.424	1053.07	0.0250	0
	400	9.60	0.412	480.25	0.0200	25
5	0.4	1.74	0.082	125.40	0.0138	0
	2	16.55	0.217	1036.37	0.1600	0
	18	>30.00	0.474	2118.95	0.0142	0
	400	1.55	0.060	307.04	0.0050	0

V = Volume Per Liter; HB = Heterotrophic Bacteria; CFU = Colony Formers Units mL^{-1} × 10^5; HB:M = Ratio Hetrotrophic Bacteria Microalga; DSB = Dapi Stain Bacteria; HB:DS = Ratio Heterotrophic Bacteria Dapi Stain.

cells·mL^{-1}) was one order of magnitude higher than at 0.4-L Erlenmeyer, and consequently there was a greater concentration of both bacteria and microalgae.

Mean initial concentration of heterotrophic bacteria at 18-L Carboy was 3.43×10^5 CFU·mL^{-1}, increasing to $>30 \times 10^5$ CFU·mL^{-1} on the fifth day, with a bacteria to microalga ratio of 0.322 and 0.474 for Days 0 and 5, respectively. The concentration of heterotrophic bacteria was one order of magnitude higher than that recorded at 2-L Fernbach and two orders of magnitude higher than at 0.4-L Erlenmeyer.

The concentration of heterotrophic bacteria at 400-L column was 1.19×10^5 CFU·mL^{-1} on Day zero. It increased to 9.60×10^5 CFU·mL^{-1} on Day 4 and decreased to 1.55×10^5 CFU·mL^{-1} on Day 5, the latter concentration being lower than that observed at the 18-L level. The bacteria to microalga ratio were 0.362 and 0.412 for Days 0 and 4, respectively.

3.3. Direct Bacterial Counts

Mean initial concentration of bacteria at 0.4-L Erlenmeyer was 7.01×10^5 cells·mL^{-1}, increasing to a maximum of 191.88×10^5 cells·mL^{-1} on day 4 of the culture (**Table 1**). In the Fernbach and bottle cultures, the abundance of bacteria increased from the day of inoculation (45.24 and 104.49×10^5 cells·mL^{-1}, respectively) up to 5 (1036.37 and 2118.95×10^5 cells·mL^{-1}, respectively). At 400-L column, the maximum concentration (956.10×10^5 cells mL^{-1}) was recorded on Day 3. The highest mean concentration of bacteria (884.13×10^5 cells·mL^{-1}) occurred at 18-L Carboy, and it was two times higher than at 400-L column (454.73×10^5 cells·mL^{-1}) and three times higher than at 2-L Fernbach (302.24×10^5 cells·mL^{-1}).

The concentrations of total bacteria in all the cultures were always higher than those of heterotrophic bacteria. The mean heterotrophic to total bacteria ratios during the five days of culture were higher in the Fernbach (0.0108) and Carboy (0.0172) assays than in the Erlenmeyer (0.0079) and column (0.0086) assays cultures.

3.4. Abundance of *Vibrio* spp.

The highest mean concentration of *Vibrio* spp. during the culture of *C. muelleri* occurred at 18-L Carboy on day 2 (10 CFU·mL^{-1}), and on days 3 (30 CFU·mL^{-1}) and 4 (25 CFU·mL^{-1}) at 400-L column (**Table 1**).

4. Discussion

4.1. Microalgal Growth

Cell densities of *C. muelleri* were similar to the values reported by other authors [11]-[14]. In our experiment, the conditioning phase was observed only at 0.4-L Erlenmeyer because the cells in the inoculum were in the late exponential phase and continued to reproduce once the culture conditions were reestablished in the f/2 medium [15]. The conditioning phase was not observed in the subsequent culture volumes because the cells were always in exponential phase when they were transferred (**Figure 1**). The specific growth rate of *C. muelleri* in the 0.4-L Erlenmeyer and 2-L Fernbach cultures during the first three days of culture was always higher than that observed at 18-L Carboy and 400-L column because of the presence of fewer heterotrophic bacteria in the first two culture volumes. This indicates that the cells in exponential growth produced less exudates and detritus than those cultured at 18-L and 400-L. On the other hand, secondary metabolites such as amino acids, carbohydrates, and vitamins required for bacterial growth are generally produced during the stationary phase. The highest density of bacterioplankton occurs after the maximum production of phytoplankton [16] [17], and under *in vitro* conditions the maximum abundance of bacteria occurs after the maximum production of chlorophyll *a* [18]. Our results therefore indicate that as the culture volume increases, the abundance of bacteria increases; hence, bacteria can be introduced into systems used for aquaculture production of crustacean and mollusc larvae when *C. muelleri* is used as food. Even though greater microalgal biomass is commonly found in the last days of culture, its use as food is not recommended because of the increased abundance of heterotrophic and *Vibrio* bacteria. These results suggest that the quality of water for the cultivation of *C. muelleri* should be improved to obtain greater biomass that can be used to feed aquatic organisms for a longer time.

4.2. Bacterial Growth

The culture of *C. muelleri* was not axenic since the bacterial population increased over time and during the scale-up process. Also other authors have indicated that it is difficult to maintain microalgal cultures free of bacteria because of their epiphytic relationship [19] [20]. In the present study, we observed bacteria adhering to the surface of *C. muelleri* (**Figure 2**), as pointed by [21] [22]. Our total bacterial counts obtained by epifluorescence microscopy were higher than the results obtained by the plate count method. Similar results were obtained by [23] and [24] recording only 1% of bacteria recovered by viable techniques. An explanation for the difference in the number of bacteria enumerated by both methods is that bacteria can be found in microalgal cultures that cannot grow in conventional culture media.

The maximum values of heterotrophic bacteria in all the cultures (**Table 1**) were recorded on day 5 of the experiment. Similarly, Lizárraga-Partida *et al*. [25] recorded concentrations from 1×10^4 to 1×10^7 CFU·mL^{-1} in cultured microalgae used to feed *Litopenaeus vannamei* larvae. In general, a direct relationship was observed between the concentration of heterotrophic bacteria and cell density of *C. muelleri*, since in the first three culture
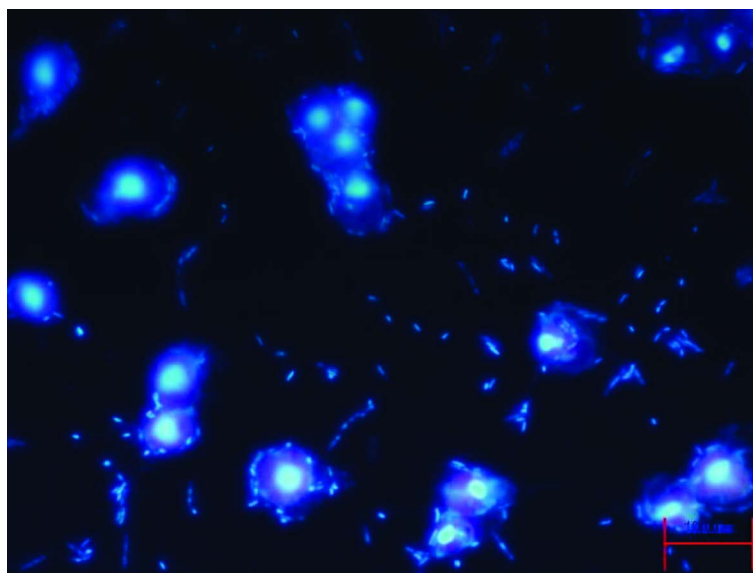
Figure 2. Free and attachment bacteria on the surface of *Chaetoceros muelleri*.

volumes, the concentration of heterotrophic bacteria increased as microalgal biomass increased, whereas at 400-L column both cell density and the number of heterotrophic bacteria decreased. The abundance of heterotrophic bacteria suggests a symbiotic relationship with microalgae, possibly because of the exudates released by the latter. According to [26] [27], optimum microalgal growth usually occurs in the presence of specific bacteria. This bacteria-alga relationship is important because phytoplankton excretes a wide variety of organic compounds including peptides, amino acids, carbohydrates, and lipopolysaccharides, which serve as a source of carbon and nitrogen to bacteria attached to the surfaces of algae. In turn, bacteria can provide growth factors and vitamins, or remineralize nutrients [28].

The decrease in the growth rate of *C. muelleri* at 400-L column can be associated with the decrease in bacterial biomass. The high correlation values (0.80, 0.75, 0.85; p < 0.05) obtained by direct counts for *C. muelleri* and total bacteria in the first three culture volumes indicate some type of dependency between these microorganisms. The low correlation coefficient (0.27, p = 0.34) found at 400-L column can be attributed to environmental variables like temperature, due to use incandescent light which modify bacterial growth.

4.3. *Vibrio* spp.

Vibrio bacteria were not detected at 0.4 L or 2 L due to seawater treatment (filtration, ultraviolet radiation) and because the culture medium was sterilized in autoclave. Similar results were obtained by [29]. The concentration of *Vibrio* spp. recorded on the second day at 18-L, however, was lower than that reported for seawater [30] because the water used for the culture medium was treated with sodium hypochlorite. Unlike the results in smaller cultures, the high concentrations of *Vibrio* spp. at 400-L column were due to the treatment of the culture water, which consisted of only filtration and ultraviolet radiation. This indicates that as the culture volume increases, it becomes more difficult to control the bacteria and maintain the innocuousness.

The high biomasses of *C. muelleri* and the presence of *Vibrio* bacteria detected in this study indicate that it is necessary to establish prevention and control systems in aquaculture laboratories since there have been worldwide reports of diseases attributed to *Vibrio* spp. in fish [31] and penaeid shrimp cultures [32], which affect massive cultures with subsequent economic losses. Though not all bacterial species found in cultures are pathogenic, they can act as secondary invaders.

Acknowledgements

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