GROWTH, COMPOSITION AND BIOMASS YIELDS OF *Chaetoceros muelleri* MASS CULTURES WITH DIFFERENT ROUTINES AND TANK DEPTHS.

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RESUMEN

Se comparó la producción de los sistemas de cultivo multipasos de *Chaetoceros muelleri* de los laboratorios CD y AL. El primero mantiene los niveles intermedios a 20-22 ºC, con 7.9 mol/m^2/d de luz continua y los masivos se producen al exterior en estanques de 2.5 m^3 y 1 m de profundidad. El segundo mantiene ambos niveles al exterior y los masivos son de 3.3 m^3 y 0.5 m de profundidad. La concentración de los cultivos al interior de CD fue $0.77 \cdot 10^6$ cel/mL. Los de AL dieron $2.04 \cdot 10^6$ cel/mL en dos días. En dos días, los cultivos masivos de AL redituaron $1.38 \cdot 10^6$ cel/mL y los de CD $0.88 \cdot 10^6$ cel/mL, pero los de AL se cosecharon en un día en los meses más cálidos y en los fríos los de CD redituaron $0.72 \cdot 10^6$ cel/mL en tres días. La producción de biomasa fue mayor en los estanques de 0.5 m, con 40 g/m^3 en un día y 49 g/m^3 en dos días. En los de 1 m fue de 32.8-33.6 g/m^3 con las dos rutinas. Las proteínas que se cosecharon en los cultivos de AL (29.5 g/m^3) no variaron con las dos rutinas, pero la cosecha de lípidos y de carbohidratos fue menor después de un día (5.1-5.4 g/m^3 y 10.1 g/m^3 en dos días). En CD, los cultivos de tres días resultaron contaminados con bacterias y protozoarios, que causaron un aumento en las proteínas (26.4 g/m^3 y 78%). La alta concentración de lípidos en los cultivos de dos días (9.7 g/m^3 y 29%) indica senescencia celular, probablemente debida a la continua limitación por luz.

Palabras clave: cultivos masivos; crecimiento; producción de biomasa; composición; profundidad de estanques; *Chaetoceros muelleri.*

ABSTRACT

The production of the cultures of *Chaetoceros muelleri* of hatchery CD, that maintains the intermediate levels with temperatures of 20-22 ºC and 7.9 mol/m^2/d of continuous light was compared to that of hatchery AL, that grows the last steps outdoors. Its mass cultures are in 0.5-m deep tanks, those of CD are 1-m deep. The indoor cultures of *C. muelleri* in CD had mean concentrations of $0.77 \cdot 10^6$ cells/mL after three days, those of AL were $2.04 \cdot 10^6$ cells/mL after two days. Outdoors, the normal routine lasted two days in both hatcheries, but it yielded $0.88 \cdot 10^6$ cells/mL in CD and $1.38 \cdot 10^6$ cells/mL in AL. During warmer months, the cultures of AL were harvested after one day with concentrations of $1.84 \cdot 10^6$ cells/mL. During the colder periods those of CD lasted three days, with $0.77 \cdot 10^6$ cells/mL. Biomass production was higher in AL: the normal two days routine yielded 49 g/m^3 in AL and 32.8 g/m^3 in CD. In one day, the cultures of AL of the warmer months gave productions of 40 g/m^3 and those of three days in CD were 33.6 g/m^3. Protein yields were similar in AL for the cultures of one and two days (29.5 g/m^3). Lipids and carbohydrates were significantly lower after one day (5.1-5.4 g/m^3) than after two days (10.1 g/m^3 in both cases). In CD, the three days-old cultures were contaminated with bacteria and protozoa, that caused a high content of proteins (26.4 g/m^3 = 78% of the biomass). The high lipid content (9.7 g/m^3: 29%) after 2 days indicated an incipient cell senescence, probably caused by light limitation.

Key words: mass cultures; growth; biomass yield; biomass composition; tank depth; *Chaetoceros muelleri*

Depending on their stage of development, shrimp larvae are fed microalgae as only food item or as a complement to their zooplankton-based diet. For this reason, all commercial shrimp hatcheries need to grow phytoplankton in mass cultures (Alfonso, 1993; Duerr *et al.*, 1998) and the species used for this purpose in the majority of the hatcheries of NW Mexico is the diatom *Chaetoceros muelleri,* that is grown in all cases with the multi-step procedure.

This consists of batch cultures of progressively increasing volumes, that in most hatcheries are kept indoors in a temperature-controlled environment and with continuous artificial lighting until volumes of 200-400 L. These serve as starters

for the mass cultures of the final step, which are grown outdoors with a natural light-dark photoperiod, either without any protection or protected by a semitransparent roof or by shade cloths.

In a formal survey of 14 hatcheries between 1999 and 2000, confirmed later with *in situ* visits and informal interviews with the operators of 25 of the 51 hatcheries operating in 2003 (Alvarado, 2004), we found that the volumes of the outdoor tanks ranged between 0.8 and 20 m^3, with depths from 0.6 to 1.0-1.5 m. Most had concrete or opaque fiberglass walls, but some were semitransparent high density polyethylene (López-Elías, 2002; Voltolina and López-Elías, 2002).

In a previous study we found that the mean yields of commercial scale cultures varied widely and that there were differences between hatcheries, but it was not possible to separate the effect of the local seasonal and meteorological condition, from that caused by differences in production routines and type of tanks (López-Elías *et al.*, 2003).

In this study we compared the growth rates and the mean organic biomass yields and composition of the mass microalgae cultures of two nearby hatcheries of the Mexican NW, that were chosen because the meteorological conditions were the same, but they used different routines of production and the tanks for outdoor cultures had different depths.

MATERIALS AND METHODS

The observations were carried out at 1-1.5 months intervals during two consecutive production cycles at the hatcheries Camarón Dorado (CD) and Acualarvas (AL). These are located in the Huatabampo Municipality in the southern part of the State of Sonora (CD: 26º 43' 30.72" N, 109º 41' 57.81" W; AL: 26º 41' 57.81" N; 109º 37' 16.68" W), within less than 5 km from each other. Because of differences in seasonal demand, the first production cycle of both hatcheries lasted from late February-early March to June, 2000 and the second from March to August 2001.

The microalga used was the same (*Chaetoceros muelleri*, strain CCMP 1316, also known as CHGRA) and the multi-step routine was based on cultures of the same volume for the first four steps (0.25, 1, 15 and 300 L), but all were maintained indoors in CD whereas the last two were grown outdoors in AL. In CD, two 300 L cultures were used to inoculate one concrete outdoor tank (0.83 x 3.0 x 1.2 m, with water depth of 1 m and volume of 2.5 m^3). In AL, the outdoor tanks were 2.0 x 3.3 x 0.6 m, the water depth was 0.5 m and the volume was 3.3 m^3. During the first half of the 2000 cycle these were inoculated with one 300 L culture, and with two cultures for the rest of that cycle and during the following one.

In both hatcheries the growth medium was modified f/2 prepared with different fertilizers, with an approximate cost of 0.10-0.15 US $/$m^3$ (CD: Nutrilake© enriched with industrial-grade monobasic sodium phosphate; AL: urea, industrial-grade monobasic sodium phosphate and pentahydrate sodium silicate), but the concentrations of the nutrients available, including vitamins and trace minerals, were the same. Earlier laboratory-scale experiments with *C. muelleri* showed that these media gave the same growth and the same biomass yields.

Apart from the location of the 300 L cultures, that were grown in both cases in high transparency (90% light transmission) fiberglass cylinders with a diameter of 0.5 m and 1.5 m high, other differences were the duration of the last two steps. In AL the 300 L cultures were used after two days, whereas in CD they lasted three days. The outdoor mass cultures were harvested after 48 hours in both hatcheries during the first year, but in the second cycle some lasted only one day in AL and some were harvested after three days in CD.

During each visit, samples of 3-4 cylinders and 2-3 outdoor tanks started on the same day in both hatcheries were obtained at 24 h intervals from the day of inoculation to that of harvest. These were used to evaluate the cell concentration of each culture with a 0.1-mm deep hemacytometer and the resulting values served to calculate the growth rates after $\log_2$ transformation (Fogg and Thake, 1987).

Additional samples were obtained each day from two-three outdoor tanks when they were being used for larval feeding. These were concentrated in triplicate Whatman GF-C glass fiber filters of known dry weight, washed with 4-5 mL of a 3% solution of ammonium formate to eliminate sea salt, dried to constant weight in an oven at 60 ºC and ashed in a muffle furnace at 500 ºC to obtain the inorganic weight. This was used to calculate by difference the organic content of the biomass.

Samples taken from the same cultures and concentrated, in triplicate for each type of analysis, were used to evaluate their protein, carbohydrate

and lipid content using the methods described in Lowry *et al.* (1959), Dubois *et al.* (1955) and Pande *et al.* (1963).

The tests of normality and equal variances gave negative results in all cases. Therefore, the mean values of organic biomass yields and composition calculated for both cycles in each laboratory were compared using two way nonparametric analysis of variance tests with $\alpha = 0.05$ (Wilson, 1956), followed by Dunn's multiple comparison tests (Zar, 1996).

RESULTS

The 300 L indoor cultures of CD were maintained with continuous lighting (7.9 ± 1.5 mol/m^2/d) and near constant temperature (21 ± 1ºC). Outdoors, the water temperatures and the light levels were similar and ranged from 17ºC to 34ºC (mean 29 ± 3ºC) and from 68 to 80 mol/m^2/d (73.9 ± 19.9 mol/m^2/d).

The 300 L cultures grown outdoors at AL gave final cell yields between 2.5 and 2.7 times higher than those maintained indoors at CD. In part, this was due to the difference in initial mean cell concentrations, that were higher in the case of AL, but the main reason was the difference in growth rates.

These were twice as high outdoors than indoors and the mean values from inoculation to harvest were 1.39 divisions/d in AL and 0.74 divisions/d in CD. Comparing the mean cell densities determined at daily intervals and the respective growth rates, the indoor cultures of CD appeared to be light-limited from the day of inoculation, whereas light started to be limiting in the outdoor cultures of AL only during the second day of growth (Table 1A).

In addition to the advantages of a faster growth and of higher cell numbers in the outdoor cylinders of AL, growth was better also in the shallow outdoor tanks of this hatchery. These yielded an average of $1.4 \cdot 10^6$ cells/mL in two days, and were harvested after only one day when the initial cell concentration was higher, as a response of better growth in the outdoor cylinders during July and August 2001.

In contrast, the deep tanks of CD yielded consistently cell concentrations lower than $1 \cdot 10^6$ cells/mL, and to attain these levels of production the cultures lasted between two days throughout most of both cycles, and three days during the colder months (Table 1B).

Possibly because of the lower percentage of cells recently divided in the cultures of AL harvested after the normal two day routine, their organic biomass yields were significantly higher than those harvested after only one day. There were no significant differences in production between the cultures harvested after two days or three days in CD, and both were lower than those of AL.

There were also significant differences in composition: independently from the routine, the mean protein yields at AL were similar and close to 29.5 g/m^3, but the yields of lipids and carbohydrates obtained after one day were 50% lower than those obtained after two days. In CD, the protein yields after two days were 17.6 g/m^3, that is significantly lower than in AL. After three days the yields increased to 26.4 g/m^3, but there were no significant differences with the routine of two days of this hatchery and with both those of AL.

The yields of carbohydrates were similar with the two routines of CD and with that of one day of AL. There was no difference between the lipid yields of the cultures harvested after two days in both hatcheries and these were significantly higher than those obtained with the other routines (Table 2).

DISCUSSION

Laboratory experiments showed that the combination of low temperature and low light level used for the indoor cylinders at CD is growth-limiting for *C. muelleri*. Temperatures of between 20 and 25ºC and 7.8 mol/m^2/d of photon flux gave significantly lower yields than at 30ºC ($0.8\text{-}0.9 \cdot 10^6$ cells/mL, compared with $1.3 \cdot 10^6$ cells/mL). With 30.2 mol/m^2/d the yields were 1.1 ± 0.2, 1.5 ± 0.1 and 2.0 ± $0.2 \cdot 10^6$ cells/mL at 20, 25 and 30ºC, that were significantly better than the respective values found with 7.8 mol/m^2/d (Peraza-Contreras, 2002).

This explains the consistently slow growth of the indoor cylinders, as well as the high variability of the outdoor ones of AL, that were grown with high light levels but within a wide range of water temperatures.

In the case of the outdoor tanks, the water temperatures, as well as the surface light were the same, and light penetration should have been initially better in the tanks of CD because of the low concentration of the inocula. However, light

Table 1. Mean cell concentrations (N, in 10^6 cells/mL) and growth rates (μ, in divisions/d) in the 300 L cylinders (A) and 2.5-3.3 m^3 tanks (B) used for mass cultures of *Chaetoceros muelleri* in the hatcheries Acualarvas and Camarón Dorado. Standard deviations in parenthesis. $\bar{\mu}$: mean growth rates in divisions/d, from inoculation to harvest.

	Acualarvas (n=26)		Camarón Dorado (n=25)	
	A			
Day	N (x10^6 cell/mL)	μ	N (x10^6 cell/mL)	μ
0	0.36 ± 0.19	-	0.21 ± 0.07	-
1	1.34 ± 0.40	2.01 ± 0.45	0.38 ± 0.10	0.98 ± 0.39
2	2.04 ± 0.46	0.65 ± 0.54	0.56 ± 0.17	0.54 ± 0.19
3	-	-	0.77 ± 0.21	0.46 ± 0.25
$\bar{\mu}$	-	1.39 ± 0.36	-	0.74 ± 0.24

	B							
	(n=6)		(n=12)		(n=16)		(n=6)	
Day	N	μ	N	μ	N	μ	N	μ
0	0.51 ± 0.21	-	0.44 ± 0.27	-	0.22 ± 0.13	-	0.17 ± 0.07	-
1	1.84 ± 0.64	1.89 ± 0.32	1.08 ± 0.57	1.36 ± 0.52	0.58 ± 0.26	1.32 ± 0.45	0.27 ± 0.09	0.70 ± 0.48
2	-	-	1.38 ± 0.55	0.41 ± 0.28	0.88 ± 0.33	0.54 ± 0.65	0.48 ± 0.22	0.81 ± 0.24
3	-	-	-	-	-	-	0.72 ± 0.33	0.59 ± 0.27
$\bar{\mu}$	-	1.89 ± 0.32	-	0.88 ± 0.30	-	0.94 ± 0.35	-	0.70 ± 0.19

Table 2. Mean yields of ash-free biomass (AFW) and proteins, carbohydrates and lipids (in g/m^3) of the outdoor mass cultures of *Chaetoceros muelleri* at the hatcheries Acualarvas (AL) and Camarón Dorado (CD), harvested 1, 2 and 3 days after inoculation. Different letters indicate significant differences between values in the same column (two way nonparametric analysis of variance and Dunn's multiple comparison tests. $\alpha = 0.05$). a≤ab≤b and a<b.

Hatchery	Days	n	AFW g/m^3	Proteins g/m^3	Carbohydrates g/m^3	Lipids g/m^3
AL	1	19	39.98 ± 10.77b	29.54 ± 7.30b	5.40 ± 2.45a	5.06 ± 1.76a
	2	65	48.65 ± 16.32c	29.47 ± 12.85b	10.15 ± 6.38b	10.13 ± 5.25b
CD	2	65	32.84 ± 6.95a	17.60 ± 3.78a	5.57 ± 1.96a	9.66 ± 3.57b
	3	14	33.62 ± 6.57a	26.36 ± 6.57ab	4.85 ± 1.31a	2.40 ± 0.61a

availability decreases exponentially with depth and therefore the percentage of cells present at any time in the thin surface layer where light levels are not limiting, is lower in a deep tank.

Fast-growing cells such as those harvested after one day in AL have a low individual weight and a high percentage of proteins, because their content of storage products is low (Nieves *et al.*, 2005), that explains the low biomass yields and the 70% protein content, in comparison to the 12.5-13.5% of lipids and carbohydrates, that coincide with the values found by López-Elías *et al.* (2005) in the shallow outdoor tanks harvested at the peak of the growth season of *C. muelleri* in a different Sonora hatchery.

More mature cultures, such as those harvested in both hatcheries after two days are also rich in storage products, with a consequent lower percentage of protein (53 to 60%) and between 17% and 20% of carbohydrates. In this case, the lipid content was lower in AL (20%) than in CD (29%), possibly because of an incipient senescence of the light-limited cultures of CD (Fogg and Thake, 1987).

Finally, the cultures maintained for three days at CD under continuous light-limiting conditions did not yield higher cell concentrations or a significantly higher organic biomass than those grown only during two days, and the high protein content of those cultures (78% of the organic yield) was the consequence of the high numbers of protozoa and of bacteria, that were observed as free cells, or adhering to the microalgae or in clumps. These are considered poor sources of lipids and carbohydrates, but are protein-rich (Tacon, 1990).

CONCLUSIONS

The production of the microalgae section of most Mexican hatcheries is limited by the excessive depth of the tanks used for mass cultures and it is also inefficient and unnecessarily expensive, because the intermediate inocula are usually maintained under conditions that may be limiting for growth. In this study, the growth of the indoor 300 L cultures was slow because of low light and temperature levels, whereas, as a consequence of higher light and mean temperature, the mean growth rate was twice as high outdoors than indoors.

The cell and organic biomass yields obtained in the shallow tanks were significantly higher than those of the 1-m deep tanks, because of the difference in light availability within the water column. In the summer months, with higher temperatures, the growth rate of the cultures in the shallow containers was high (1.9 divisions/d) and this, combined with the higher yields of the previous step, allowed to harvest the cultures after only one day.

However, these cultures had a high proportion of cells recently divided. These have a low individual weight because of their low organic content, that is especially poor in storage products. As a consequence, their protein content was higher than that of the cultures harvested after two days, but they had a low content of lipids and carbohydrates.

The production of the deeper containers was significantly lower. These were harvested after two or after three days, but the yields in organic biomass were similar and in addition after three days bacteria and protozoa contamination was high, with a consequent increase of the protein yields and similar or lower contents of lipids and carbohydrates.

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