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THE EFFECT OF INOCULATION TIME AND INOCULUM CONCENTRATION ON THE PRODUCTIVE RESPONSE OF *TETRASELMIS CHUII* (BUTCHER, 1958) MASS CULTURED IN F/2 AND 2-F MEDIA.

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Abstract - Diverse protocols have been used in the shrimp industry to produce microalgae. In this work, we evaluated the effect of inoculation time (0600 and 1200) and initial microalgae density (0.4 and 0.8 x10^5cells mL^{-1}) in static outdoor cultures of *Tetraselmis chuii*, using F/2 and 2-F media. A significant effect on cell density (> 6.7 x 10^5 cells mL^{-1}) was found after treatments with a high initial density and in the F/2 medium. The higher productive responses were observed in treatments inoculated at 0600. The better productive response was found in treatments inoculated at 0600 with 0.8 x 10^5 cells·mL^{-1} in the F/2 medium.

Key words: Cell density, green flagellate, mass cultures, outdoor cultures, microalgae production, production routine.

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INTRODUCTION

Shrimp aquaculture has had an explosive growth during the last 20 years, with positive economical and social impacts (Botacio 2006, FAO 2006, Gutiérrez-Venegas et al., 2006). Postlarvae production is one of the main aspects to consider for this success. The nutrition of postlarvae is based on using live feeds such as marine microalgae and Artemia nauplii (Merchie et al., 1997, Støttrup and McEvoy 2007). In particular, microalgae are used during the first larval stages of the penaeid shrimp and other fish species (Coutteau et al., 1997, Martínez-Córdova 1999).

The production systems of phytoplankton commonly used in aquaculture employ "multi-step" systems that initiate the microalgae culture with small inoculums in marine water enriched with nutrients, then make a complete harvest and scale the microalgae from one volume level to a higher one, *i.e.* the microalgae culture starts in 1 L flasks, followed by 20 L carboys and 200 L fiberglass columns (López-Elías et al., 2007). Most of these systems are placed outside to take advantage of natural light (López-Elías et al., 2005a). Such systems provide enough microalgae to cope with the laboratories' demand for production of shrimp larvae, zooplankton species or bivalve mollusks. Several microalgae species can be used for these purposes.

Planktonic microalgae, *Tetraselmis chuii* serve as live feed for shrimp larvae and postlarvae, as well as for bivalve mollusks (D´Souza and Loneragan 1999, Patiño-Suárez et al., 2004). Some commercial labo-

ratories worldwide have used *T. chuii* in combination with other diatom species, with successful results in terms of larvae growth and survival (D´Souza and Loneragan 1999).

Despite the extensive use of *T. chuii* in aquaculture, to date, there is not a clear protocol or methodology describing the optimal culture conditions for this species. For instance, commercial laboratories usually maintain their microalgae in enriched medium F/2 (Guillard and Ryther 1962) while others use 2-F medium. In addition, the inoculum density used to start a microalgae culture varies between laboratories and farms - some use inoculums with cellular concentrations as low as 0.04 x 10^6 cells mL^{-1}, whereas others use 0.60 x 10^6 cells mL^{-1} (Sáenz-Gaxiola 2000, Chavira-Ortega 2000, Cuevas-Rocha 2001). Another aspect that has not been clearly established is the optimal time at which a microalgae culture is initiated. Since most of outdoor cultures are carried out within 3-4 days, the inoculation time becomes an aspect of concern. Thus, many laboratories have their own "recipes" to produce microalgae for feeding shrimp larvae or bivalve mollusks.

Some authors have found that the above factors can influence the productive results of microalgae culture (Richmond 1986, López-Elías et al., 2005b). Therefore, it is important to establish a protocol based on scientific experimentation. The aim of this work was to study the effect of initial microalgae density and the inoculation time on the productive response of the microalgae *T. chuii,* cultured outside in static systems, using F/2 and 2-F media.

MATERIALS AND METHODS

T. chuii microalgae were obtained from the microalgae laboratory at the Department of Scientific and Technological Research (University of Sonora, Hermosillo, México).

A randomly factorial design with three replicates per treatment was performed. It consisted of an evaluation of the productive responses of the microalgae after exposure to three different factors. The factors evaluated were: inoculation time (0600 and 1200 h), initial microalgae density (0.4 and 0.8 x10^5cells·mL^{-1}) and culture medium (F/2 and 2-F). The cultures were maintained during spring at the experimental laboratory of Bahía Kino. The experimental units consisted of plastic tanks (250 L) that were kept outside. Constant aeration was provided by means of a blower (1 ½ HP), (Table 1).

Table 1. Characteristics of the different treatments.

Treatment	Inoculum density (x10^6cells mL^{-1})	Culture medium	Inoculation time
A	0.4	F/2	0600
B	0.4	F/2	1200
C	0.4	2-F	0600
D	0.4	2-F	1200
E	0.8	F/2	0600
F	0.8	F/2	1200
G	0.8	2-F	0600
H	0.8	2-F	1200

The eight treatments were labeled as A, B, C, D, E, F, G and H, and their characteristics are shown in Table 1. Treatment B was considered a conventional system, because its characteristics were similar to the methodologies used by some local commercial farms and/or laboratories. The culture medium (F/2 or 2-F) was added to the marine water at a rate of one mL of medium per liter of water.

Once the experiment began, physiochemical parameters were periodically monitored. The pH was measured every six hours with a pH meter (Hanna Instruments, MI, USA) and the temperature and light intensity were measured every two hours. Light intensity was monitored by means of a portable photometer (Fisher, NJ, USA).

Cellular counts were carried out every 12 h from the beginning (T0) to the end of the experiment (three days). Four samples were collected in each experimental unit, using assay tubes (5 mL) and were preserved with lugol. Thereafter, the number of cells

per volume unit was calculated using a Neubauer chamber (0.1 mm, Hausser Scientific, PA, USA) and an optical microscope (Carl Zeiss Axiostar plus, Germany).

The growth rate was calculated using the following equation:

$$m = \frac{(Log_2 B_n - Log_2 B_o)}{(t_n - t_o)},$$

with μ is the growth rate, B_0 is the microalgae density (cells·mL^{-1}) at the beginning, Bn the microalgae density at any time, and t_n-t_0 is the period of the culture since inoculation.

The generation time was calculated as follows:

$$gt = \left(\frac{1}{m}\right)(t_n - t_0),$$

with t_n-t_0 estimated as the number of experimental hours (Table 1).

A multivariate analysis of variance (MANOVA) was performed to evaluate the effect of the different factors on the productive response of the microalgae. A confidence level of 95% was considered. To make global comparisons of the eight treatments and the physiochemical parameters, a repeated-measures analysis of variance was done.

RESULTS

The temperature levels ranged from 24 to 32ºC, without significant differences between treatments. Light intensity was similar and varied from 76.6 μmoles·m^2·s at night to 1711 μmoles·m^2·s at day. The pH levels (8-10) were also similar for all treatments.

With regard to production parameters, similar tendencies were observed for all treatments (Fig. 1). An acclimation phase during the first 36 h was followed by an accelerated growth phase during the last 32-36 h. However, significant differences were registered between treatments. The final microalgae concentration was affected by the initial microalgae density ($F=9.2$; $p=0.00$) and the culture medium ($F=7.20$; $p=0.01$). Microalgae cultured in F/2 medium yielded a greater concentration at the end of the experiment when the initial density was high (0.8 x10^{5}cells mL^{-1}) compared to the microalgae cultured in 2-F. In contrast, the microalgae cultured in 2-F medium had a greater final concentration when their initial density was low (0.4 x10^{5}cells mL^{-1}; Table 2). An interaction between the inoculum density and the culture medium was observed.

Table 2. Production parameters of the microalgae *T. chuii* cultured under different protocols of inoculation density, culture media and inoculation times.

Treatment	Initial microalgae density (x10^{5}cells·mL^{-1})	Culture medium	Inoculation time	**Final microalgae density (x10^{5}cells mL^{-1})**	**Growth rate (divisions day^{-1})**	**Generation time (days)**
A	0.4	F/2	0600	6.06 ± 0.35 b	1.31 ± 0.03 d	20.3 ± 1.2 cd
B	0.4	F/2	1200	4.20 ± 0.40 a	1.13 ± 0.01 c	23.3 ± 1.4 bc
C	0.4	2-F	0600	6.14 ± 0.65 b	1.31 ± 0.05 d	20.8 ± 2.8 cd
D	0.4	2-F	1200	6.34 ± 0.58 bc	1.33 ± 0.09 d	18.8 ± 1.2 d
E	0.8	F/2	0600	7.48 ± 0.31 d	1.09 ± 0.03 c	22.9 ± 0.5 b
F	0.8	F/2	1200	6.82 ± 0.36 c	1.03 ± 0.03 b	23.7 ± 0.3 b
G	0.8	2-F	0600	5.95 ± 0.13 ab	0.91 ± 0.05 a	28.7 ± 1.8 a
H	0.8	2-F	1200	4.96 ± 1.11 a	0.84 ± 0.04 a	37.0 ± 7.1 a

*Different letters in the same column indicate significant differences ($p<0.05$).

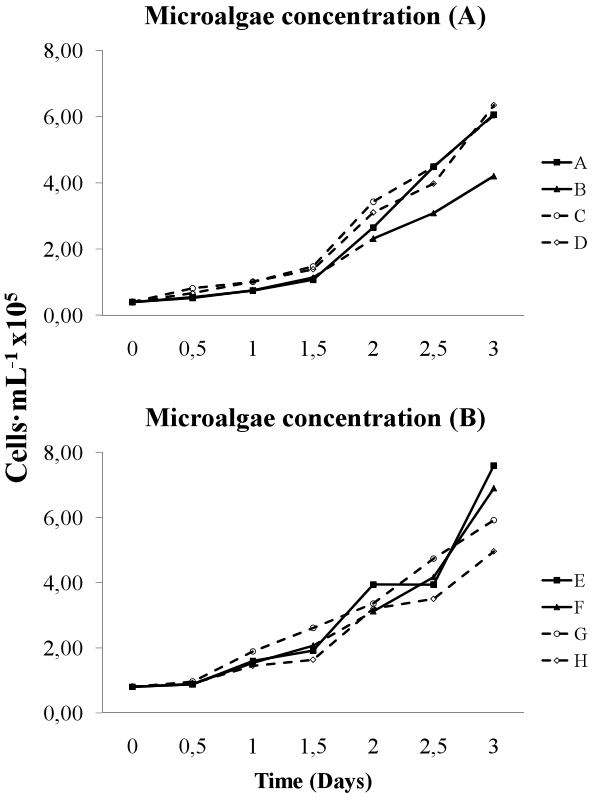

Figure 1. Cell concentrations of *Tetraselmis chuii*, cultured under different protocols. A – A, B, C and D, and B – E, F, G and H. Treatments in which the inoculation was with 0.4 x10^5cells·mL^{-1} are shown in graph A, whereas inoculation with 0.4 x10^5cells·mL^{-1} is shown in graph B.

Greater concentrations of microalgae were observed in treatments E and F (> 6.7 x 10^5 cells mL^{-1}) which had a high initial density and were cultured in F/2. These were followed by C and D (> 6.1 x 10^5 cells mL^{-1}) which had a low initial density and were cultured in 2-F. The lowest concentration was registered in the B treatment (4.2 x 10^5 cells mL^{-1}), which was considered as the traditional method. The inoculation time did not have any significant effect on the final concentration of microalgae (F= 0.94; p=0.33), but had an effect on other productive responses.

The growth rate was also affected by initial microalgae density (F=293; p=0.00), culture medium (F=6.72; p=0.03) and inoculation time (F=11.73; p=0.00). The greatest growth rates were achieved at lower initial density (~1.3 divisions day^{-1}). Interaction between the initial density and the culture medium was observed. Treatments employing high initial density exhibited higher growth rates (> 1.03 divisions day^{-1}) when cultured in F/2 medium (E and F), compared to those cultured in 2-F (G and H; < 0.91 divisions day^{-1}). Treatments inoculated at an early time (0600) had higher growth rates ($p<0.05$) compared to those inoculated at 1200.

The generation time was affected by the initial microalgae density (F=14.3; p=0.00), while the culture medium (F=4.2; p=0.06) and inoculation time (F=1.3; p=0.27) did not have a significant effect on this response. Lower generation times were achieved in treatments with lower initial density (18.8-23.3 h), compared to those with higher initial density (22.9-37.0 h).

DISCUSSION

The physiochemical parameters registered in this experiment were within the optimal range reported for planktonic microalgae (Nelson et al., 1992, López-Elías 2005b, Martínez-Córdova 1999). The treatment considered as the conventional method (B) showed a poor productive response, however, modifications of different methodological steps improved the response, in some cases for more than 35%, although, some interactions between factors were observed. For instance, the effect of the initial microalgae density depended on the culture medium and the inoculation time which had an effect in most treatments. Sáenz-Gaxiola (2000) found that higher inoculum densities of microalgae *T. suecica* were related to higher cellular concentrations. We observed higher cellular concentrations of microalgae when they were grown at a higher initial density and cultured in F/2 medium. However, this effect was not observed for microalgae cultured in 2-F medium. Combinations of three different factors could be the key for improving the productive response, as was suggested by the interactions found after the statistical analysis.

Despite the treatments with higher initial density (E and F) yielding greater final concentrations,

the productive response in terms of growth rate and generation time might be more attractive for some laboratories. The reason for this is as follows: by the beginning of the culture, the microalgae concentrations in treatments E, F, G and H were 100% higher than in the rest of the treatments, and by the end of the culture cycle, the concentrations were only 18% higher than in treatments A, C and D. Considering that raising the microalgae concentration from 0.4 to 0.8 x 10^5 cells·mL^{-1} takes around 20-24 h, the production cycle can be carried out in less time and with lower amounts of inoculum.

The higher growth rates and generation times observed in treatments with low inoculum densities could be attributed to an earlier reproduction of microalgae due to a greater volume or better nutrient availability per microalgae cell. Becerra-Dórame et al. (2010) found that the growth rate of *Dunaliella* sp. was similar or sometimes greater when the initial microalgae density was 40 x 10^3 cells mL^{-1} in comparison to the treatments inoculated with 80 x 10^3 cells mL^{-1}.

The greater yield observed in treatments at the earlier inoculation time (0600) could be attributed to the longer exposure to natural light during the first hours. In contrast, samples that were inoculated at 1200 h had less hours of light at the beginning of the culture. In spite of the fact that all treatments had the same period of light exposure, the early growth of microalgae seemed to be more affected by the initial exposure period. For instance, the traditional system (B) had a poor productive response, but when the inoculation time was 6 hours earlier (A), the response was much greater. It has been argued that planktonic microalgae enhance their growth when exposed to longer periods of light (Meseck et al., 2005).

The microalgae yield observed in treatments C and D were similar or better than those obtained in different commercial laboratories after three days of culture (López-Elías et al., 2003). Moreover, the growth rates were higher (~ 1.3 divisions·day^{-1}) than those found in indoor cultures (0.74 divisions day^{-1}; Meseck et al., 2005).

Despite treatments E and G allowing for higher concentrations of microalgae at the end of the experiment, considering the higher inoculum density it took more time and resources to achieve these higher yields. The traditional system did not have the expected response in yield terms, although a modification in the inoculation time improved the results. Also, better results can be achieved by using medium 2-F instead of F/2, in this particular case.

REFERENCES

Becerra-Dórame, M., López-Elías, J. A., Enríquez-Ocaña, F., Huerta-Aldaz, N., Voltolina, D., Osuna-López, I., and *G. Izaguirre-Fierro* (2010). The effect of initial cell and nutrient concentrations on the growth and biomass production of outdoor cultures of *Dunaliella* sp. *Annales Botanici Fennici*, **47**, 109-112.

Botacio, A. (2006). Reseña del cultivo de camarón en México. *Industria acuícola* **3**, 1- 30.

Chavira-Ortega, C. O. (2000). Evaluación del sistema de producción de microalgas de un Laboratorio comercial. Tesis de Licenciatura Facultad de Ciencias del Mar, Universidad Autónoma de Sinaloa, Mazatlán, Sinaloa 53 pp.

Coutteau, P., Geurden, I., Camara, M. R., Bergot, P., and *P. Sorgeloos* (1997). Review on the dietary effects of phospholipids in fish and crustacean larviculture. *Aquaculture* **155**, 149-164.

Cuevas-Rochas, F. (2001). Evaluación del área de microalgas de tres laboratorios comerciales de producción de larvas de camarón. Tesis de Maestría en Ciencias Universidad de Sonora. Hermosillo, Sonora 71 pp.

D'Souza, F.M.L. and *N.R. Loneragan* (1999). Effects of mono specific and mixed algae diets on survival, development and fatty acid composition of penaeus prawn (*Penaeus* spp.) larvae. *Marine Biology* **133**, 621-633.

FAO (2006). State of world aquaculture 2006. *FAO Fisheries Technical Paper* **500**, 1-134.

Guillard, R. R. L. and *R.L. Ryther* (1962). Studies of marine planktonicdiatoms. I *Cyclotella nanahustedt* and *Detonula confervalea* (cleve) gran. *Canadian Journal of Microbiology*. **8**, 229-239.

Gutiérrez Venegas, A., Correa, T., Muñoz, V., Santibáñez, A., Marcos, R., Cáceres, C. and *A.H. Buschmann* (2006). Farming of the giant kelp *Macrocystis pyrifera* in southern Chile for development of novel food products. *Journal of Applied Phycology*. **18**, 259–267

López-Elías J.A., Voltolina D., Chavira-Ortega C.O., Rodríguez B.B., Sáenz-Gaxiola L. M., Cordero-Esquivel B. and *M. Nieves* (2003). Mass production of microalgae in six commercial shrimp hatcheries of the Mexican northwest. *Aquacultural Engineering* **29**,155-164.

López-Elías J.A., Voltolina, D., Ávila-Mercado, I. S., Nieves, M. and *B. Cordero-Esquivel* (2005a). Growth, composition and biomass yields of *Chaetoceros muelleri* mass cultures with different routines and tank depths. *Revista de Investigaciones Marinas* **26**, 67-72.

López Elías, J. A., Voltolina, D., Enríquez Ocaña, F., and *G. Gallegos Simental* (2005b). Indoor and outdoor mass production of the diatom *Chaetoceros muelleri* in Mexican commercial hatchery. *Aquacultural Engineering* **33**, 181-191.

López-Elías, J. A., Huerta-Aldaz, N. and *F. Enríquez-Ocaña* (2007). El alimento vivo en el cultivo de camarón: investigaciones aplicadas al sector productivo. *EPISTEMUS* **2**, 15-20.

Martínez-Córdova, L.R. (1999). *Cultivo de Camarones Peneidos. Principios y Prácticas*. 283 pp. AGT Editorial, S.A., México.

Meseck, L.S.T., Jennifer, H.A. and *H.G. Wikfors* (2005). Photoperiod and light intensity effects on growth and utilization of nutrients by the aquaculture feed microalga, *Tetraselmis chuii* (PLY429). *Aquaculture* **246**, 393–404.

Merchie, G., Lavens, P. and *P. Sorgeloos* (1997). Optimization of dietary vitamin C in fish and crustacean larvae: A review. *Aquaculture* **155**, 165-181.

Nelson, R. J., Guarda, S., Cowell, L. E. and *P.B. Heffernan* (1992). Evaluation of microalgal clones for mass culture in a subtropical green house bivalve hatchery; growth rate and biochemical composition at 30 °C. *Aquaculture* **106**, 357-377.

Patiño-Suárez, V., Aldana Aranda, D. and *G. Zamora* (2004). Food ingestion and digestibility of five unicellular algae by 1-day-old *Strombus gigas* larvae. *Aquaculture Research* **35**, 1149-1152.

Richmond, A. (1986). *Handbook of microalgal mass culture*. CRC Press, Boca Raton, Florida, USA. 528 pp.

Sáenz-Gaxiola, L.M. (2000). Producción de microalgas del laboratorio de cultivo de camarón #2 de Maricultura del Pacífico, S. A. de C. V. Tesis de Licenciatura Facultad de Ciencias del Mar, Universidad Autónoma de Sinaloa, Mazatlán 49 pp.

Støttrup, J.G. and *L.A. McEvoy* (2007). *Live Feeds in Marine Aquaculture*. 318 pp. Blackwell, E.U.A.