Digital Journal of
El Hombre y su Ambiente Department
ISSN: 2007-5782

Vol. 2 (9): 31-40. July to December 2015.

Technologies for indoor or outdoor microalgae culture. Practical proposals to operate in small-scale aquaculture.

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ABSTRACT

One of the main problems that freshwater ornamental fish producers have is low disponibility of live food demanded by many species during initial stations of development. Technologies for constant development of this type of food are beyond reach of most farmers due to high costs and complicated procedures involving. In the search of simple and economic techniques for microalgae production, with potential to use in small aquaculture farms, procedures for semi-continuous massive culture were develope, inoculating with surface water samples and using commercial fertilizers, in two ways: 1) indoor conditions, with continuous artificial lightning, and 2) outdoor conditions with natural lighting and photoperiod. For indoor conditions, a metallic photo bioreactor that supports pendant polyethylene bags of 10 L capacity was design, with continuous lighting of fluorescent lamps, constant aeration and room temperature; for indoor conditions, plastic carboys of 20 L disposed in a metal foil shelf were use, continuous aeration, sunlight and ambient temperature. Both modes of production generated green microalgae cultures with predominance (>90% of cell mL^{-1} in both cases) of complex *Desmodesmus* (R. Chodat) S. S. An, T. Friedl & E. Hegewald, 1999 - *Scenedesmus* Meyen 1999, proving being efficient in the type of produced population and biomass. The estimated development times of the optimum population density for harvesting, maintenance conditions and the functional culture duration, facilitate its implementation in small and medium farms to establish a constant live feed production. It is consider viable for technology transfer.

Key words: Live food, indoor/outdoor cultures, freshwater microalgaes, technologic transfer.

RESUMEN

Uno de los principales problemas que afrontan los productores de peces de ornato dulceacuícolas, es la escasa disponibilidad del alimento vivo demandado por muchas especies durante las fases iniciales de desarrollo. Las tecnologías para la producción constante de este tipo de alimento no están al alcance de la mayoría de los productores debido a sus altos costos y lo complicado de los procedimientos que involucran. En la búsqueda de técnicas sencillas y económicas para la producción de microalgas, con potencial de ser efectuadas en pequeñas granjas acuícolas, se desarrollaron procedimientos para cultivo masivo de forma semi-continua, inoculando con muestras de cuerpos de agua superficiales y usando fertilizantes comerciales, en dos formas: 1) en condiciones de interior, con iluminación artificial continua, y 2) en condiciones de exterior, con iluminación y fotoperiodo naturales. Para el primer caso se diseñó un foto-biorreactor metálico que soportaba bolsas de polietileno colgantes de 10 L de capacidad, iluminación continua con lámparas fluorescentes, aireación constante y temperatura ambiente; en el segundo, se emplearon garrafones de plástico de 20 L dispuestos en un anaquel de lámina metálica, aireación las 24 h, luz solar y temperatura ambiente. Ambos modos de producción generaron cultivos de microalgas verdes con predominancia (> 90% de cél mL^{-1} en cada caso) del complejo de géneros hermanos *Desmodesmus* (R. Chodat) S. S. An, T. Friedl & E. Hegewald, 1999 - *Scenedesmus* Meyen 1999, demostrando ser eficientes en cuanto al tipo de poblaciones y biomasa producidas. Los tiempos estimados de desarrollo de la densidad poblacional óptima para la cosecha, las condiciones del mantenimiento y la duración funcional del cultivo, facilitan su puesta en práctica en granjas pequeñas y medianas para establecer

Receive: October 30nd 2015 Accepted: November 25th 2015 Published: December 15th 2015

una producción constante de alimento vivo; se considera viable su transferencia tecnológica.

Palabras clave: Alimento vivo, cultivos interior/exterior, microalgas dulceacuícolas, transferencia tecnológica.

INTRODUCTION

One of the main worldwide problems that freshwater ornamental fish producers have and specially in Mexico, where most people works in small-scale (Ramírez *et al.*2010), is low disponibility of life food required by many species in brooding stage (Polanco 1999; Dhert *et al.* 2001; Lim *et al.* 2003), where there is a high mortality rate. Culture of species used as food is the base of development and diversification of aquaculture production (Polanco 1999).

Continuous productions techniques and life food production are beyond within reach of most farmers, due to high costs and complications to implement monoclonal algal cultures to sustain zooplankton production. Also considering construction cost or installations conditions for aseptic conditions for adequate microalgae monocultures growth, acquisition cost of a pure strain and maintenance, cost of reagents and complicated culture media elaboration used in laboratories, coupled with high time demand and required qualified labor.

At biological level, massive algae production is conditioned by diverse factors: temperature, pH, salts concentration, nutriment disponibility, type and intensity of light, culture density and presence of consumer organisms (Malgas 2013). Additionally, at technological level, success of microalgae production depends, between other factors, in development of profitable cultivation systems, which is a gradual process (Borowitzka 1999). Light was the mean component in microalgal growth, because nutrients and in some cases carbon dioxide, can incorporate to medium in excess to avoid as limiting factor, meanwhile light must be supplied during all culture time, because irradiance is not accumulative (Molina-Grima et al. 1996; Borowitzka 1999; Janssen 2002). Therefore, a basic principle in photo bioreactors is to take into account light quality and quantity, available for each cell (Borowitzka 1999). Necessary light energy for production is one of the most important costs in indoor systems (Borowitzka 1999), so, sunlight as energy source is preferable use (Janssen 2002).

Searching a simple and economic technique for freshwater microalgae production that can use in small aquaculture farms, it were implemented and adjusted both indoor conditions with artificial illumination and outdoor with natural irradiance techniques in semi continuous way (Leavens and Sergeloos 1996). Taking samples from surface water bodies and using common and economic fertilizers from plant market; it was searched: 1) evaluate efficiency of microalgae production process regarding quantity and biomass generated, observe simultaneous effect of fertilizers selection and culture conditions on inoculated photosynthetic organisms mixture, and 2) prove efficiency of two commercial different fertilizers in three different concentrations.

MATERIAL AND METHODS

Production systems

Two culture systems were use:

Indoor (with conditions of constant light and aeration and room temperature): A photo bioreactor of 2 x 2 m was design. Consisting in 1.2 x 0.45 x 2.0 m metallic structure with eight fluorescent cool white light lamps of 40 watts, placed back and lateral walls. With air supply system made with PVC-polyvinylchloride- tubes, connecting to external pump (Fig.1). On this metallic structure, low-density polyethylene bags were hanging of 5-10 L capacity, using removable metallic rods (Helm and Bourne 2006).

Outdoor (subjected to weathering): 10 to 20 L food grade plastic beakers capacity were used, arranged in a standard sheet shelf of 2.1 x 0.85 x 0.3 m, with 24 hours aeration proportionated by five

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watts aquarium pump, at sunlight and room temperature (Fig. 2).

Culture media

For culture media it was used chemically sterilized tap water, adding a solution of commercial sodium hypochlorite (NaClO at 5 %) at the rate of 1 mL 10 L^{-1} of water, during 24 hours and neutralizing one hour before its use with sodium thiosulfate ($Na_2S_2O_3$) in proportion of 50 mg per 1 mL^{-1} NaClO used (Torrentera and Tacon 1989).

Fertilization

Two commercial fertilizers were use: Triple 17 of agricultural use (T-17) and a fertilizer without brand for hydroponic cultures, identified as "Ferticiencias" (FCh) or being prepared and sale in Science Faculty of UNAM. Both fertilizers were prove in three concentrations: 0.15, 0.10 and 0.05 mg L^{-1}, with three replies of each one, in both systems.

Inoculum and spread

The inoculum was took from a surface water body located in lake area of Xochimilco, which was observed with an optic microscope Leica DM750 with digital camera ICC50HD, to assure that it contain at least one species of unicellular or synovial green algae. If it was detected cyanophytes presence, linked to blue-green or brown colorations, that water sample was discard for experimentation.

Tracing

Temperature and pH were record daily with a multi parametric Hanna HI 98129, a sample was observe in the optic microscope to prove composition and effect of conditions selection of culture; counts

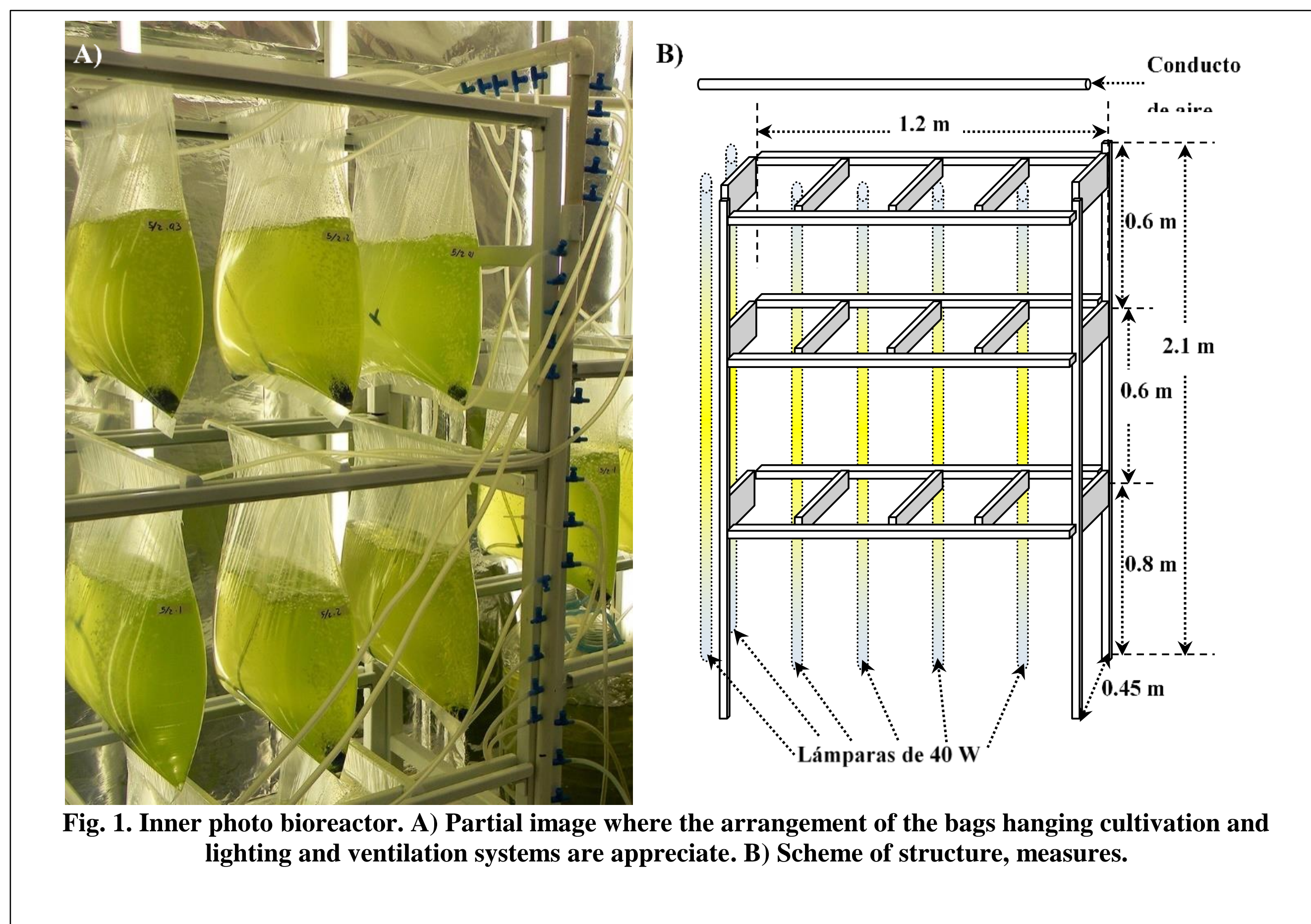

Fig. 1. Inner photo bioreactor. A) Partial image where the arrangement of the bags hanging cultivation and lighting and ventilation systems are appreciate. B) Scheme of structure, measures.

Receive: October 30nd 2015 Accepted: November 25th 2015 Published: December 15th 2015

Digital Journal of
El Hombre y su Ambiente Department
ISSN: 2007-5782

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were made in Neubauer chamber to evaluate population growth; changes in coloration and/or composition in media were registered. At harvest time, an analysis of nitrites, nitrates and ammonia made by colorimetry with Nutrafin Test of Hagen equipment.

Harvest time and culture useful lifetime

Adequate time for harvest was estimate according to density of cell mL^{-1} and, in indirect way, according to coloration and culture bulk density. Useful lifetime was determined proceeding to harvest-re-inoculum cycle, that consist in harvest 50% of volume in each container, replenish it with conditioned water and fertilizing it; this cycle was repeated until there was a coloration and/or texture change in media, indicative of a succession of organisms. This change was confirm by optical microscopy observation.

RESULTS

In Table 1 it is shown average results of the three replicas for both proposed systems. Throughout the work period, indoor and outdoor conditions, variable values were temperature of 20-25ºC and 10.2-25ºC respectively; pH 8.1-9.8 and 8.1-10 respectively. Independently to production system, culture behavior showed a similar pattern in fertilizers efficiency and concentrations for biomass generation (Fig. 3), as in exerted selection on algae mixture.

Biomass production was directly proportional to fertilizer concentration, being able to appreciate more production in cell mL^{-1} in concentration of 0.15 g L^{-1} , a lower production in 0.05 g L^{-1} concentration and a medium production in 0.1 g L^{-1} in all cases; this behavior showed the same pattern between fertilizers and between indoor/outdoor conditions.

Nitrites, nitrates and ammonia concentrations were slightly elevated for cultures of T-17 only in 0.15 g L^{-1} concentration, being minimum in the rest

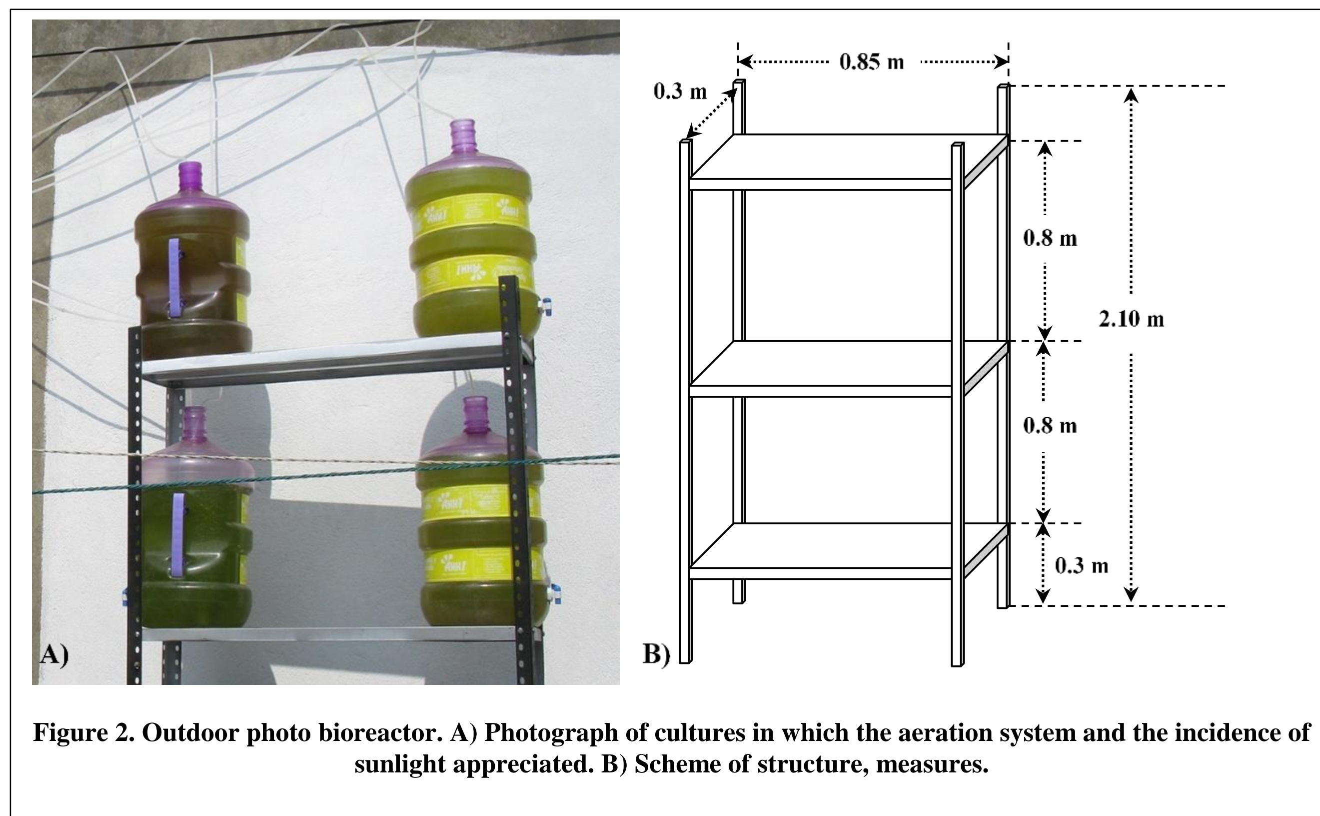

Figure 2. Outdoor photo bioreactor. A) Photograph of cultures in which the aeration system and the incidence of sunlight appreciated. B) Scheme of structure, measures.

Receive: October 30nd 2015 Accepted: November 25th 2015 Published: December 15th 2015

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of cases. Adequate population densities for harvest were reached (between 3.1 and 7.5 x 10^6 cel mL^{-1}, that not necessarily are maximum densities) between 72 and 96 hours after beginning of culture (Fig. 3), reaching again appropriate density every 72 hours during the cycle harvest-re-inoculum and semi-continuous maintenance.

Time duration of cultures in a same container was efficient during 10 days (initial inoculum and two or three harvests after re-inoculum with culture). After those days it was necessary to spread again- in indoor cultures, in new bags, throwing away used ones, and in outdoor cultures, in clean and disinfected carboys with a sodium hypochlorite solution-, previously filtrating with a net of 34 µm to avoid formation of suspended aggregates or films adhered to walls. In all generated microalgal cultures, in first place were unicellular chlorophytes or cenobial populations. After 24 hours, the culture mediums changes to five or six types of chlorococcales chlorophyta *sensu lato (s.l.)* populations, with higher abundance of one or two genus. After fourth day, one of these is more abundant until it conforms up to 90 to 95% of total. Predominant genus obtained in this experiment were complex sibling genres *Desmodesmus - Scenedesmus*.

Both in indoor and outdoor conditions, cultures development times with proved fertilizers and dosages were similar (Table 1), having similar responses in growth and scope of maximum density, also in genres predominance composition and/or

Table 1. Main results (mean values and intervals) with three concentrations of the two fertilizers used in growing experimental systems.

Culture system	Temp (°C)	pH	Fertilizer	Doses (g L^{-1})	Maximum mean density (cells mL^{-1})	Viability days culture	Crop nutriments concentration (mg L^{-1})		
							NO_2	NO_3	NH_4
Indoor	20 – 25	8.1 – 9.8	T-17	0.05	3.1 x 10^6	91	0.3	5	0.6
				0.10	5.6 x 10^6	91	0	5	0
				0.15	7.9 x 10^6	91	0	0	0
			FCh	0.05	3.1 x 10^6	85	0	5	0
				0.10	5.4 x 10^6	85	0	0	0
				0.15	8.0 x 10^6	85	0	0	0
Outdoor	10.2 - 25	8.1 – 10.0	T-17	0.05	3.2 x 10^6	91	0.3	5	0.6
				0.10	4.9 x 10^6	91	0	5	0
				0.15	7.4 x 10^6	91	0	0	0
			FCh	0.05	3.3 x 10^6	85	0	5	0
				0.10	5.9 x 10^6	85	0	0	0
				0.15	7.5 x 10^6	85	0	0	0

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chlorococcales chlorophyta *s.l.* species, in initial and established populations during culture.

Cultures lifetime considered in function with coloration change of these, an indicator that microalgae composition had changed, varied between 85 and 91 days, allowing 28 to 30 harvests respectively, depending in initial inoculum. It was verify by observation in optical microscope when coloration changes in cultures from bright green to brown, or blue, dark yellow, among others, it implied a change in predominant populations.

DISCUSSION

In the design and implementation of productive systems, it were consider fundamental requirements for an adequate microalgae photosynthetic production, disponibility of nutrients, continuous mixture and elimination. Nutriment input was through fertilization. Permanent mixture, necessary for nutriment homogenization and avoid cell sedimentations was made through continuous aeration, also allowed to maintain cells in constant movement (Torrentera and Tacon 1989), contributing to avoid cells self-shadowing effects by accumulated density in media and photo inhibition caused by excessive permanency of individual cells in an excessively lit area (Molina-Grima *et al*. 1996). Also it facilitated escape of excessive O_2 produced, which accumulation has an inhibitory effect on photosynthesis (Janssen 2002), while it is favoring incorporation of atmospheric CO_2 into culture media, avoiding to become a limiting factor (Borowitzka 1996). Light energy for photosynthesis came from two sources: artificial light through fluorescent light in indoor system and sunlight in outdoor system.

Processes efficiency for microalgae production:

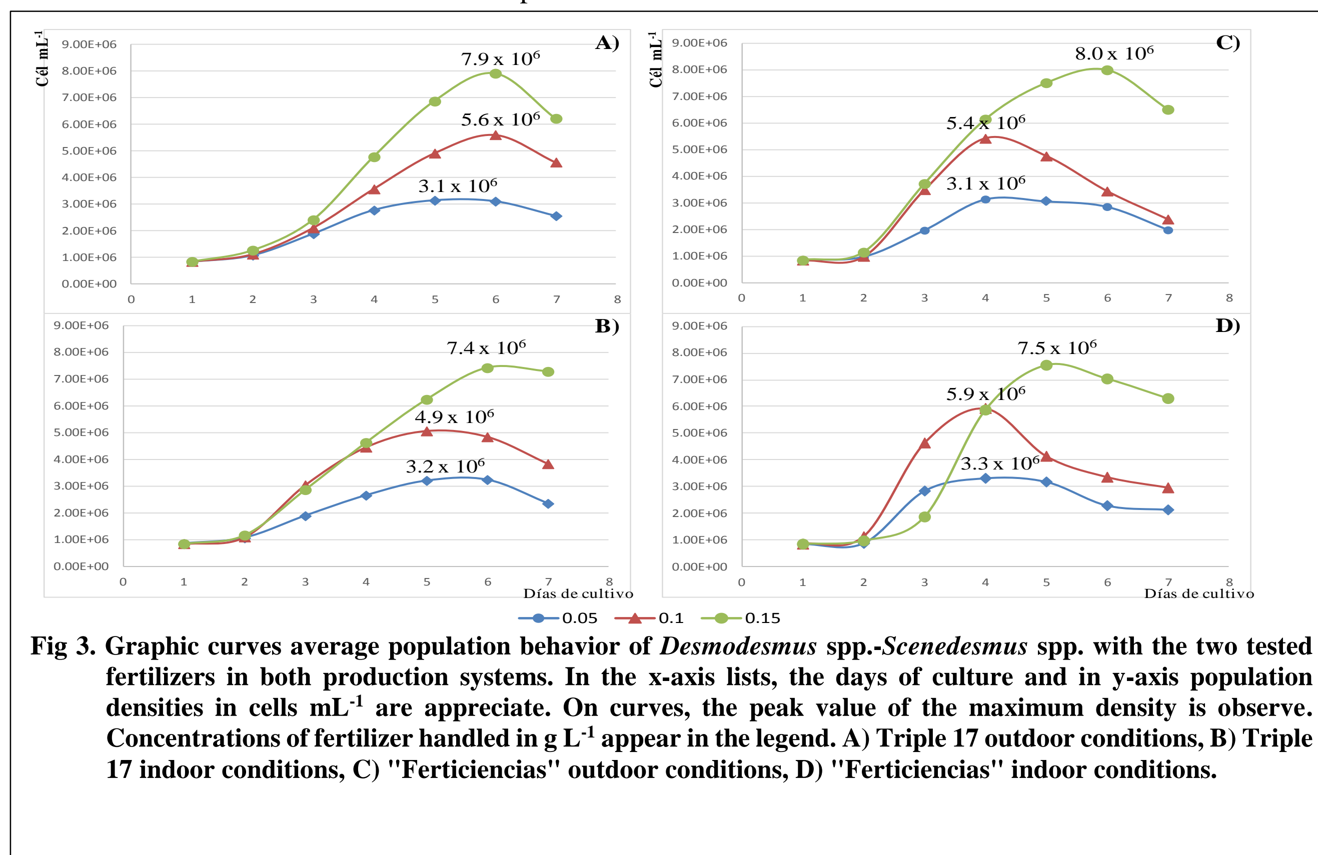

Fig 3. Graphic curves average population behavior of *Desmodesmus* spp.-*Scenedesmus* spp. with the two tested fertilizers in both production systems. In the x-axis lists, the days of culture and in y-axis population densities in cells mL^{-1} are appreciate. On curves, the peak value of the maximum density is observe. Concentrations of fertilizer handled in g L^{-1} appear in the legend. A) Triple 17 outdoor conditions, B) Triple 17 indoor conditions, C) "Ferticiencias" outdoor conditions, D) "Ferticiencias" indoor conditions.

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Biomass generation.

According to cultures density counts (cell mL^{-1}) indoor culture results are not different to those ones in outdoor cultures. Nevertheless indoor experiments who had artificial light 24 hours day, while outdoor experiments were under natural light and photoperiod conditions (shorter light exposure times) it is probably related to quality of available luminous energy. Chlorophyll *a* (Cl-*a*) and chlorophyll *b* (Cl-*b*) presented in this experiment, showed two absorption peaks, red (663-430 nm) and violet region (645 to 435 nm) respectively (Lee 2008). For all, shorter exposition time to light, sunlight was apparently more favorable than artificial for photosynthetic production, due to range of available wavelengths.

Cold white light lamps cover the daylight wave spectrum and showed peaks at 586, 558, 440 and 406 nm, showing a weak emission in rest of spectrum (Philips 2015). These wavelengths does not match with absorption peaks of chlorophylls *a* and *b*, which leads to a less efficient capture of available light energy and therefore a lower photosynthetic production, however constant illumination was provide in this experiment.

Kommareddy and Anderson (2003) evaluated six radiant efficiency energy sources (cold fluorescent, incandescent, halogen and LEDs) to growth microalgae, using a bioreactor equipment. They found that intensity and percentage of light, with respect total light emitted favorable for photosynthesis (to blue-green: 400-500 nm; to chlorophyte: 600-700 nm) was 45.65% for cold fluorescent light; 4.28% for high LED source; and 93.38% for medium LED light (643 nm).

The source light was settle at 2.4 m distance to culture mediums. Light source showed the photosynthetic efficiency resulting in biomass production in the experiments. This information corroborate the finding results in this study.

It is also important to mention that light intensity, photoperiod and illuminations and darkness hour's period affected cell division and their growth (Torrentera and Tacon 1989). Photoperiod that combined light/darkness phases is adequate for microalgae growth, but intense and/or constant illumination, like only factor which stimulate photosynthesis, can produce a metabolic stress factor for many other microalgae species. It is assume that use of prolonged illumination in microalgae cultures produce fast cell growth. Nevertheless, it also must be consider that a photoperiod with light and darkness lapses similar to solar photoperiod, maintains normal and healthy growth of microalgae populations (Torrentera and Tacon 1989), because it allows metabolic recover or rest in organisms. When intensity or exposure time of light increase in microalgae culture mediums, their cells showed auto-protection mechanisms like photophobia behavior of microalgae's cells with

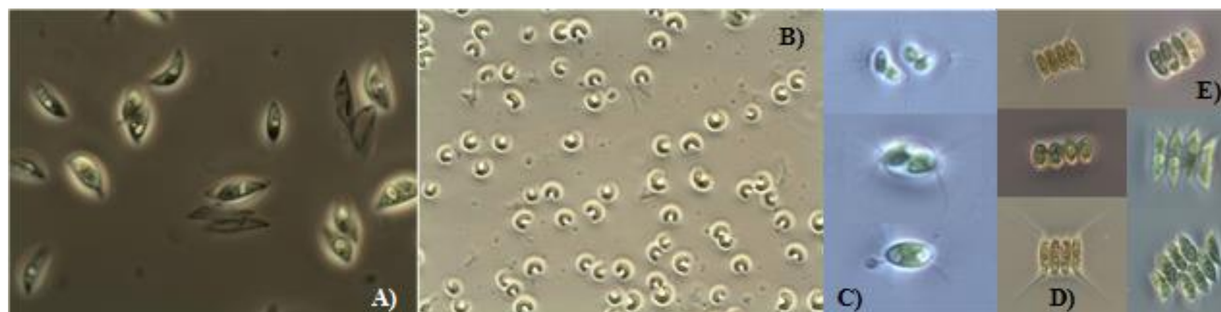

Fig. 4. Images of some cultures generated with production systems described. A) *Monoraphidium* sp., B) *Kirchneriella* sp., C) *Lagerheimia ciliata*, D) *Desmodesmus* spp. E) *Scenedesmus* spp.

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movements to less illuminated regions or production increase in accessory pigments (Lee 2008).

When such cells mechanisms are not enough to decrease metabolic stress, as it occurs in photo bioreactor system, it presents a photo inhibition or photo synthetic activity suspension, being able to occur a cell damage (Molina-Grima *et al.* 1996) although it is not irreversible, it results in a population growth decrease and therefore, a lower microalgae culture productivity. Regarding to indoor cultures, photo inhibition caused by continuous illumination could have added to effect of non-optimal efficiency of used type of light, resulting in a non-differentiated population growth with respect to outdoor cultures.

The above, was congruent with culture coloration behavior, which were different between indoor and outdoor systems. Outdoor cultures had a bright green aspect during four to five days before passing to green-yellow tones that indicate the senescence of population, while indoor cultures had a pale green coloration and only maintained for three to four days. These colorations can be associated to health condition of cultures in different culture production environments, if considered, as in terrestrial plants, which intense bright green is an indicator of wellness in this type of photosynthetic organisms, the outdoor cultures under solar photoperiod and probably without stress by light duration and/or intensity, would be healthier.

Effect of selected type of fertilizer and of culture conditions

In all produced cultures, unicellular or cenobial green algae populations were developed which mixed composition presented predominance (>90%) of one or two genres and/or species. The produced populations in this experiment belong to sibling genres *Desmodesmus*-*Scenedesmus*. Two species from the last genus: *S. obliquus* and *S. quadricauda* were report by Becker (2013) as common used in agriculture as food for freshwater zooplankton, marine rotifers and *Artemia.* About *Desmodesmus* sp. there was no information about it.

Others studies using same or different surface water microalgae inoculum with same or different fertilizers, showed after four days and throughout their useful lifetime, similar result with this study. With predominance (>90% of cell mL^{-1}) of chlorococcales s. l. clorophyta with the taxa: *Franceia* sp. Lemmermann, 1898, *Golenkinia radiata* Chodat, 1894, *Kirchneriella* sp. Schmidle, 1893, *Lagerheimia ciliata* (Lagerheim) Chodat 1895 and *Monoraphidium* sp. Komárková-Legnerová, 1969 (Fig. 4), reaching densities between 4 x 10^6 cell mL^{-1} up to 9.7 x 10^6 cell mL^{-1}. The microalgae cultures were efficiently prove as food to produce freshwater rotifers and ciliates, also extracted from natural waters.

Proved procedures and culture conditions, helped the growth of adequate algae as food for zooplankton organism production, with advantage of low or non-remanence of toxic substances for aquatic animals. Considering that general behavior described for detailed experiment in this study has repeated in other mentioned experimental blocks, it is observe a group of repeatable and replicable procedures with similar results. It support the possibility of being implement in other contexts with favorable results.

Fertilizers and its concentrations efficiency

The two used fertilizers and its respectively concentrations gave convincing results by generating a microalgae production with appropriate densities to use as food; difference in this aspect between the two types of implemented systems is not very notorious and it is explained in function of irradiance quality. Regarding to freshwater microalgae biomass production produced in this study, Andrade *et al.* (2009) obtained *Scenedesmus* sp. densities of 8.05 ± 0.55 x 10^6 cell mL^{-1} and 7.39 ± 0.18 x 10^6 cell mL^{-1} in fertilized cultures with fishery wastewater and Nitrofoska Foliar (5 mL L^{-1}), respectively, made in natural illumination and photoperiod conditions. Toyub *et al.* (2008) reported highest densities in 10th culture day in *Scenedesmus obliquus* cultures (9.7, 8.32, 6.52 and 5.12 x 10^6 cell mL^{-1}) using candy factory waste as fertilized medium in 2.5, 2.0 and 1.5

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% respectively, and 13.63 x 10^6 cell mL^{-1} in Bold Basal medium as control culture.

Eustance *et al.* (2013) reported obtained cell densities with *Scenedesmus* sp. of 2 x 10^6 cell mL^{-1} (4th day) using ammonium and 11.5 x 10^6 cell mL^{-1} (8th day), with nitrate and urea as nitrogen source, cultivating in a closed tubular photo bioreactor with 5% of CO_2. Microalgae densities obtained in this study, match in upper limit reported by Andrade *et al.* (2009) and Toyub *et al.* (2008). Nonetheless, the sophisticated culture system used by Eustance *et al.* (2013), matches with obtained by them in media fertilized with ammonia, while media fertilized with nitrate and urea are way above generate in this experiment.

At harvest moment, with respect to nitrogenous remnant concentrations compounds: nitrites (NO_2), nitrates (NO_3) and ammonia (NH_4), Camargo and Alonso (2007) reported 50% at 96 h lethal concentration (CL50-96 h) of nitrogenous compounds for relatively sensitive aquatic animals: 0.01 mg L^{-1} nitrites for salmonid *Oncorhynchus mykiss*. Upper 10.9 mg L^{-1} for mollusk *Planorbella trivolvis*. For nitrates, 17 mg L^{-1} for anuran *Seudacris triseriata*; 269.5 mg L^{-1} for caddis *Hydropsyche exocellata*. For ammonia, 0.08 mg L^{-1} to salmonid *Oncorhynchus gorbuscha*; 0.65 mg L^{-1} concentration for amphipod *Echinogammarus toletanus* and in rotifers and cladocerans nonspecific data was found. However, if it is take as reference what studies reported for other animals in order to minimize losses by unsuccessful trials, only use of 0.15 g L^{-1} concentration of T-17 is present as toxicity risk, while rest of trails does not present any one.

As to culture lifetime, it showed more longevity cultures generated with T-17 than the ones growth with “Ferticiencias”; this may be due to difference in initial concentrations of nutriments between the two fertilizers that, close to harvest time, it is evident higher concentration of remaining nitrogenous compounds from first dose with respect second when use 0.15 g L^{-1} concentration. In T-17 experiments, microalgae succession could have been slower because those concentrations of nutriments were fertilizer was enough toxic to prevent heterotrophic organisms as protozoa like ciliates and amoebas, and small size opportunistic rotifers. Succession in this experiment was due to cyanophytes proliferation, meanwhile in microalgae cultures generated with “Ferticiencias”, succession and consequent end of useful life of crop was due to excessive proliferation of small opportunistic heterotrophs and cyanophytes.

According to Chaumont (1993), compare productivity between different massive culture systems of microalgae is difficult due to factors as different geographic locations, culture strategies (*e.g.* in batches, semi continuous and continuous), different species of algae, and others, affect in a imponderable way the biomass production. Nevertheless, the two proved systems in this work, no matter its fundamental differences in illumination type and photoperiod duration, and inherent variables to its development condition in indoor/ outdoor, they showed very similar results and, therefore, similar and adequate efficiencies as production systems susceptible of being transfer for its use for producers, in its actual condition.

CONCLUSIONS

Low cost procedures for freshwater microalgae production in indoor and outdoor conditions described in this study are easy to implement and operate. Population densities in cultures, obtained in relatively short times, and absence of toxicity at harvest time, prove the viability of systems and procedures used as primary life food. Used fertilizers, along with culture conditions, helped the growth of chlorococcales *s. l.* chlorophytes with predominance of one or two genres and/or species, appropriate as food for zooplankton organisms production. Estimated time for optimal development of population density for harvest, maintenance conditions in semi-continuous way and functional duration culture, facilitate their implementation in small or medium farms, allowing to take the necessary provisions to establish a culture escalation that maintain a continuous production of live food, which in turn allows to program larvae production and rearing.

Receive: October 30nd 2015 Accepted: November 25th 2015 Published: December 15th 2015

It is desirable to tune the technologies considering and evaluating in posterior experiments the role of photo inhibition, light source emission spectrum (proving the effect with use of special fluorescent lamps for gardens, terrariums and planted aquariums, or of LEDs lamps) and temperature. Nevertheless, in the present state of development of procedures, technology transfer can be done.

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Receive: October 30nd 2015 Accepted: November 25th 2015 Published: December 15th 2015