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Population density comparison of *Daphnia pulex* (Linnaeus, 1758) fed with bacteria obtained from Biofloc system

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Abstract
The use of heterotrophic bacteria as only food source in cladocerans diet to make massive production cultures has been little studied. So, this experimental study used *Daphnia pulex* cultivated in triplicate in 20 L plastic beakers at 19°, 21° and 25 °C temperature, with continuous light and aeration (24 hours), during 60 days. The bacteria source was obtained from screened liquid (20 µm) grown in Biofloc tilapias system. Every third day a sample of 500 mL was taken from each beaker and all organisms were counted. The highest density was found at 19 °C experimental test but the organisms died on third day. The maximum density was obtained at 21°±2 °C was of 24,414±244 org, whereas, lower density was found at 25°±2 °C with 19 259±252 org. The ANOVA analysis showed significant (P<0.05) differences between experimental treatments. Obtained reproduction rates values were r = 0.095-0.10; Ro = 17-21 org.female-1 and Tc = 29-30 days. The total density barely exceeded 2 org.mL-1. However, heterotrophic bacteria can be use as food to maintain low density rotifer culture or in mixed diets with microalgae.

Keywords: *Daphnia pulex*, cladocerans, bacteria, culture, temperature, reproduction rates

1. Introduction
The zooplanktonic species like ciliates, insects, crustaceans and *Artemia* are used as live food in aquaculture industry for their facility to cultured and their capacity to change their nutritional value [1]. Cladocerans are found inside this crustacean group, which are called "water flies" because of their little size and characteristic movement in water [2].
Many species of this freshwater Daphniidae family are cosmopolite, they have a short life cycle. Depending temperature conditions culture, their longevity ranges between 13 to 60 days [3]. The water quality, nutritional value and food quantity applied in their culture system have influenced in their reproduction rate and reproductive frequency of cladocerans, determined population growth [4]. *Daphnia pulex* species have been used in different studies because they can tolerate diverse environmental conditions with high organic matter concentration, grow better in 27-28 °C range conditions and survive in extreme environmental changes of temperature and oxygen. *Daphnia pulex* can grow in oxygen saturation conditions and can survive below concentrations of 2 mg. L-1 [1] and reach measures of 4 mm in body size [3].
The highest production of *Daphnia* sp. is consequence of their resistance to environmental changes, also for their high reproductive rates and because they are not selective filter feeders of microscopic microalgae, bacteria, fungi, ciliates like *Paramecium* sp. and detritus, if their particle size is <60 µm. This characteristic facilitates the use of different diets to make massive cultures production [5].
Cladocerans showed different responses and sensibility to diverse diets, low content of nutrients in microalgae or in their consumed particles [6]. This condition affect directly organism's development. A technique that has not been employed to feed cladocerans directly is the provision of heterotrophic bacteria produced in Biofloc system, which can use as vitamins and minerals sources, especially phosphorus [7].
Therefore, the goal of present study was to consider heterotrophic bacteria source like food, produced in tilapia Biofloc system, to produce massive cladocerans culture (*Daphnia pulex*) in 20 L plastic beakers in laboratory, considering their use as only food source when microalgae culture fall or they do not have optimal cell concentration.

3. Material and methods

3.1 Organisms supply

Daphnia pulex strain was obtained from cladocerans ceparium of Live Food Production Laboratory from Universidad Autónoma Metropolitana, Xochimilco unit.

3.2 Experimental design

Twenty-liter plastic beakers, filled with 15 L of freshwater were used to made culture experiments. Light (40 w, white tube) and aeration were constant during 60 experimental days (Fig.1). They were tested three experimental temperatures: 19°, 21° and 25 °C for triplicate. The organisms were fed with heterotrophic bacteria produced in tilapia Biofloc system. Every third day, organisms were sampled and counted to determined population density. This was made during 60 culture days.

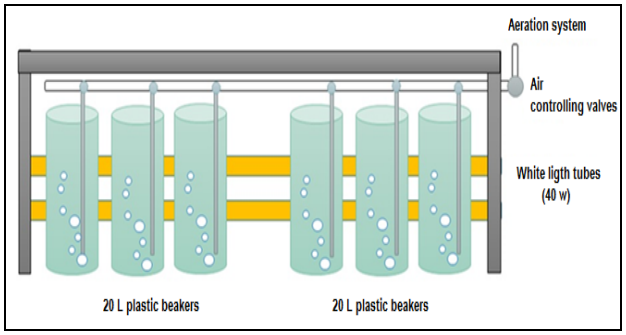

Fig 1: Experimental design of *Daphnia pulex* at three experimental temperatures (19°, 21° and 25 °C) fed with heterotrophic bacteria produced in tilapia Biofloc system.

3.3 Bacteria production (Biofloc)

Biofloc system was installed three weeks before initiated *Daphnia pulex* experiments, to obtain heterotrophic bacteria. Four 200 L plastic beakers were fill with 160 L of freshwater, with vigorous aeration and temperature of 25 °C. Juvenile stage tilapia (35) were introduced and fed with extruded pellet (45% protein) and enriched with molasses as carbohydrates source.

3.4 Cladoceran feeding

From each Biofloc beaker, every week were extracted 5 L of culture medium and sieve through a 20 µm mesh to obtain bacteria. For each experimental cladoceran beaker, 2 L of this bacteria medium was added.

3.5 Sampling

Every third day (during 60 days), from each experimental beaker, 500 mL was taken and sieve through 20 µm mesh. The organisms were concentrated in 50 mL and three subsamples were taken of 1 mL, fixed with Lugol solution (5%) and counted in Stereoscopic Microscope Leica EZ4HD. Data were extrapolated to 20 L culture media.

3.6 Data Processing

Population density values were introduced in Excel 2010 base data to obtain mean values (±S.D.) and tendency growth curves.

Density values were introduced in Life Table Program (Excel 2010) to obtain reproductive parameters:

Reproduction rate: $Ro = \sum l_x * m_x$

Where:

$\sum$= summatory

lx = survival proportion from each phase

mx = produced organisms from each survival organism from each phase

Growth intrinsic rate: $r = \log_e R_o / T_c$

Where:

$\log_e R_o$ = reproduction rate natural logarithm

T_c = Cohort generational time

Cohort generational time: $Tc = \sum x * lx * mx / Ro$

Where:

$\sum$= summatory

lx = survival from each phase

mx = produced organisms from each phase

Ro = Reproduction rate

3.7 Statistical analysis

With population density mean values obtained from each experimental temperature, significant differences ($P<0.05$) were determined by ANOVA analysis using Systat 13.0 statistical program and multiple mean values comparison (Tukey's test) was made.

4. Results

Table 1 show mean values of population density every third culture day. The experimental cultures at 21°±2 °C temperature conditions obtained highest density with 24 414±244 org. 20L-1, whereas at 25°±2 °C showed the lowest density with 19 259±252 org.20L-1. The ANOVA analysis showed, at final of experiment, significant differences ($P<0.001$) between experimental temperatures.

Table 1: Mean values (±S.D.) of population density of *D. pulex* cultured at three experimental temperatures.

Sampling	Experimental culture temperatures		
	19 °C	21 °C	25 °C
0	1 067±13	1 067±14	1 067±14
3	1 440±9	1 221±15	963±18
6	1 460±11	2 441±11	1 926±18
9	1 482±15	3 662±17	2 889±27
12	1 563±14	4 883±19	3 852±15
15	1 771±71	6 104±64	4 815±85
18	3 270±37	7 324±34	5 778±57
21	6 200±62	8 545±85	6 741±74
24	10 477±147	9 766±79	7 704±70
27	11 634±116	10 986±190	8 667±67
30	12 403240	12 207±270	9 630±63
33	13 381±133	13 428±128	10 593±150
36	14 591±154	14 648±164	11 556±155
39	16 058±158	15 869±186	12 518±152
42	17 807±178	17 090±190	13 481±148
45	19 224±291	18 311±138	14 444±442
48	19 491±194	19 531±135	15 407±147
51	19 863±186	20 752±205	16 370±176
54	20 113±113	21 973±219	17 333±173
57	21 640±264	23 193±293	18 296±281
60	22 596±259	24 414±244	19 259±252

Fig 2: show tendency population growth curves of *Daphnia pulex* produced at three experimental temperatures.

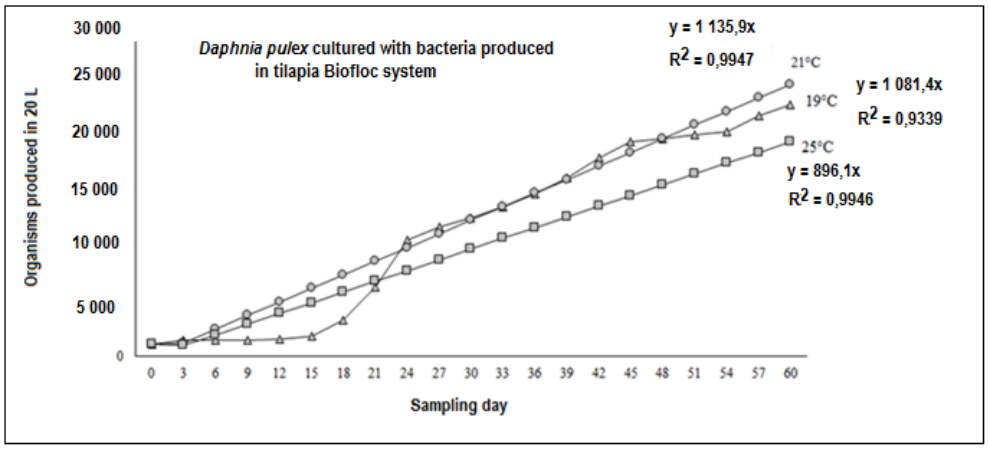

Fig 2: Population density tendency growth curves of *Daphnia pulex* produced in laboratory at three experimental temperatures and fed with bacteria produced in tilapia Biofloc system.

Table 2 show Life Table values. Organisms produced per female (Ro) changed according temperature condition, obtaining for 19 °C experimental test 20 organisms; for 21 °C, 22 organisms and for 25 °C only 17 organisms. The reproductive variables (Tc and r) did not show differences.

Table 2: Production values of *Daphnia pulex* produced in laboratory at three experimental temperatures.

Culture temperature	Reproduction rate ∑lx*mx Ro	Cohort reproduction time ∑x*lx*mx/Ro Tc	Instantaneous growth rate logeRo/Tc r
19 °C	20,182	30,023	0,100
21 °C	21,881	29,803	0,104
25 °C	17,147	30,000	0,095

5. Discussion

For a massive cladocerans culture, is not only important to consider food type and their concentration, but also their nutritional composition. In *Daphnia* sp. culture it is important to maintain constant carbon (C) and phosphorus (P) levels [8], since in diets with deficiency of phosphorous, *Daphnia* organisms must increase their carbon excretion and oxygen consumption, causing morphological changes in cladocerans populations. In massive cladocerans cultures it is important to increase in diet the polyunsaturated fatty acids (PUFA) of w3 chain concentration, which help organisms to get better growth and egg reproduction. This condition can be increased with mixed microalgae diets and enriched with bacteria, because C: P ratio maintained constant in culture medium. Is important to consider not only use green microalgae in cladocerans culture diet, but also diatom microalgae which was rich in essential fatty acids (EFA) [8].

Bacteria present in cladocerans culture medium or applied externally like complement diet [9] can be use as food, because as well as providing EFA, provide essential amino acids and vitamins. These authors mentioned that it has not been able to determine the activity that bacteria have in cellular digestion in cladocerans group, because microalgae digestion can observe in presence or absence of bacteria. Likewise, it is not observed a general pattern about abundance and retention time in digestive tract of daphnias, but only observed and increase or decrease of their survival along digestive tract. Bacteria which are resident in digestive tract of cladocerans, have ability to adhere and avoid their expulsion. So, it could be interesting to take samples of *Daphnia pulex* own digestive tract, make *in vitro* culture, determine their probiotic characteristic, and applied, not as only food diet in cladocerans culture, but as complement diet or enriched diet with microalgae source.

To make a successful cladocerans culture is important to considered allelopathic interaction between organisms, caused by food insufficiency, reducing feeding rates and decreasing population growth and reproduction [10]. This conditions can be present when there is an excess in food concentration, either with microalgae, bacteria, or mixed diets. These authors also mentioned that it needs to take care of culture population density, because it can diminish population reproduction due to physical contact between organisms, this phenomenon only was observed in *Simocephalus vetulus* specie. High densities in cladocerans culture can provoke that invested energy in motherhood changes life cycle strategies in next cladocerans generations, producing bigger females that growth and mature quickly. When cultured densities were 1 org. mL-1, first generation produce 16 neonates and for second generation 19; when densities were 20 to 40 org.mL-1 produce 2,5 to 4,9 neonates in first generation and for second generation only produce 2,1 to 3,1 neonates [10].

Another important variable was temperature. Daphnias showed adaptive plasticity at different oxygen concentrations (O_2) and temperature wide ranges values [11]. This regulation is expressed for their own genetic condition, in which interfere certain type of proteins to maintain cellular function in this altered environment. That's why important diet source, not only as easily digestion, but with high protein value must be apply. The temperature affect principally biochemical reaction in organism's physiology, which modified their fecundity and reproduction, but also can affect daphnias heart beats, respiration process and muscular activity. For this reason, cultures at different temperatures, the organisms need to take an acclimation period to withstand changes. This period was considered in this study. In *Daphnia* sp. genus, which is an eurythermal organism, exposed to dramatic temperature changes (2-30 °C), need to accumulate in their body high unsaturated fatty acids (HUFA), principally 18:3w3 and 18:2w6, as EPA precursor and arachidonic acid (ARA),

obtained for diet lipids, which allow controlled temperature changes, used microalgae or bacteria as food source. This can be observed clearly when cladocerans culture was made at <15 °C temperature [11]. There are studies that EPA and ARA presence in diet were not only important to cladocerans culture, but also sterols. Essential amino acids are important too, because not only were used to maintain and welfare of cladocerans [12], but also deficiency of Arginine: Histidine or Lysine: Threonine induce ephippia formation in *Daphnia pulex* populations [12].

With respect to growth rates in *Daphnia* sp. populations, it is mentioned that not all cladocerans species or populations respond equally to different environmental conditions and food sources, especially in Life Table values [13]. These authors founded better r values (0,236), Ro (27 org.female-1) and Tc (24 days), than our research, but it must be considered that *Daphnia* sp. is low stress susceptibility to growth, mortality and fecundity reduction levels, which impact in different way, not only to different cladocerans species, but also between population from different geographical localities. Authors who make a reproduction rate compilation of different species of cladocerans [14], founded values of r = 0, 08-0,51; Ro = 3-41 org.female-1 and Tc = 8-10 days. In genus *Daphnia* sp. cultures fed with *Scenedesmus acutus* [15], founded values of r = 0,156 when microalgae concentration was 0,2 mg C.L-1 and 0,370 with 1 mg C.L-1 concentration. These authors mentioned that in low food concentration, females produce big size eggs with a lot of yolk to resist food low concentration and began diminished organism production. The apply to cladocerans culture a poor nutritional diet cause that daphnias modified their reproduction rates, interchanging growth, and reproduction type at population level. This was observed apparently when in cladocerans culture microalgae diet was suppressed.

Studies made with four microalgae as unique diet (*Chlamydomonas reinhardtii, Scenedesmus acutus, Synedra tenuissima* [diatom] *Cryptomonas pyrenoidifera* [cryptophyta] in comparison with Cyanobacteria (*Microcystis aeruginosa*) [16], in 20°C temperature cultures, founded values of r = 0,39-0,50; 0,42; 0,44; 0,46 and 0,16 respectively. This values were similar with cyanobacteria that founded in this study with r = 0.10 and low value with respect microalgae diets. When microalgae mixed in a diet [17], like *Chlamydomonas reinhardtii* and *Ankistrodesmus falcatus* with 500 x 10^3 cel. mL-1 concentration, obtained values of r = 0,28-0,31, this assert with our study that bacteria diet was useful as complement diet and not for unique diet in this cladoceran, because r values are below with respect to microalgae diet. Reproduction rate values above this study with *Daphnia pulex* were obtained with *Ankistrodesmus* sp. (r =0, 91-0, 260) [18]. In cladocerans cultures with microalgae like *Chlorella* sp. and *Scenedesmus* sp., mixed with *Microsystis aeruginosa* (cyanobacteria) at different concentrations (0, 25, 0, 50, 0,75 and 100%) [19], founded values of r = 0,003-0, 21. Although an increase of reproduction rate with increasing cyanobacteria concentration is observed, r values are low with respect microalgae diets [16].

Although bacteria cannot be considered as high nutritional diet for zooplankton, but it can be ingested and settle in their digestive tract [20]. When bacteria were defecated, and get out to culture medium, they are exposed to environmental conditions and only few can be favored to reproduce in culture medium and used again as food to zooplankton. It was observed that bacteria community inside digestive tract of cladocerans and copepods were different and depends only on food source and bacteria community culture medium composition: So it is interesting to make experiments inoculating probiotic bacteria or their own digestive tract bacteria and allow zooplankton to survive, growth or reproduce appropriately.

Bacteria presence in culture medium for their ingestion in cladocerans must be sourced by decomposition of death organisms or their exoskeleton covers [21]. These organic matter sources were important to bacteria culture present in cladocerans culture medium, because it can be a nutrient regeneration source. Bacteria present in culture medium can provide a quarter part of carbon flux (27, 1 ± 25, 4%) [22], this bear out, that bacteria source as zooplankton diet only, was a complement diet. Bacteria can reincorporate 50% of free carbon release by phytoplankton. Cladocerans and protozoa organisms can be use as bacterial bio capsule, which other freshwater or marine organisms can be fed with. Transfer carbon efficiency between producers and consumers depends body size differences between these two groups. Also, cannot forget genetic component between cladocerans species or their different populations [23], which allow more efficiency organism's response to selective pressure for space, food, and temperature differences.

Bacteria plays an important role in zooplankton food, but can observed in this study, only allowed that *Daphnia pulex* can maintained in low growth range, which implies a successful research because in microalgae low production or absence of it. *Daphnia pulex* can maintained in low growth rates until obtained new microalgae culture or began a culture medium with high production rates.

6. Conclusion

Heterotrophic bacteria obtained in tilapia Biofloc system can be use as maintaining food source in microalgae absences or low production. These heterotrophic bacteria can be used as complement diet as mixed diet with one, two or three microalgae.

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