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Dietary supplementation of cattle manure in S*cenedesmus acuminatus* algae suspension in *daphnia magna* farming

Suplementação dietética de esterco bovino em suspensão algácea de *Scenedesmus acuminatus*, no cultivo de *daphnia magna*

Suplementación dietética de estiércol bovino en suspensión de algas de *Scenedesmus acuminatus*, en el cultivo de *daphnia magna*

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Abstract
The use of live food has been recommended in the larval stage of several species of fish, providing better survival and growth results when compared to those obtained with artificial diets. This study aims to evaluate the biomass and protein content of *Daphnia magna* submitted to three diets: Diet A (*Scenedesmus acuminatus*) at a concentration of $1.5x10^7$ cells/mL per individual, diet B (cattle manure) at 6.5g/L, and diet C at the same diets and concentration as in diets A and B for a period of 21 days. The experiment was repeated three times, totaling 63 days of study with a completely randomized design, with three replicates in each treatment. The experimental unit consisted of polyethylene containers with 20L, containing 10 *D. magna* neonates. Every two days, temperature, pH, dissolved oxygen and saturation were measured. At the end of the experimental period, nutrient quantification of the culture water and diets (phosphorus, nitrate, ammoniacal nitrogen, total nitrogen, and organic nitrogen), determination of the final biomass and protein content of the individuals were performed. Diet C provided a higher mean biomass value, followed by diet A and B, with values of 344.12 g, 157.71 g and 81.93 g, respectively. The *D. magna* fed with diet B had a higher protein content, with 2.56%, followed by those fed diets C and A, with 2.17 and 1.32%, respectively. At the end of the experiment, it was found that the organisms fed diet C had a larger reproduction rate when compared to the other diets.
Keywords: Biomass; Crude protein; Microcrustacean.

Resumo
O uso de alimento vivo é recomendado na fase de larvicultura de várias espécies de peixes, proporcionando melhor resultado de sobrevivência e crescimento, quando comparado ao obtido na dieta artificial. O presente estudo objetivou avaliar a biomassa e teor proteico de *Daphnia magna* submetida a três diferentes dietas. Dieta A (*Scenedesmus acuminatus*) na concentração de 1,5x107 célula/mL por individuo, dieta B (esterco bovino) na concentração de 6,5g/L e dieta C com a mesma concentração das dietas A e B na mesma, durante um período de 21 dias. O experimento foi repetido três vezes, totalizando 63 dias de estudo com delineamento experimental inteiramente casualizado, com três réplicas por tratamento, sendo a unidade experimental constituída por recipientes de polietileno com capacidade para 20L contendo 10 neonatas de *D. magna*. A cada dois dias, temperatura, pH, oxigênio dissolvido e saturação foram mensurados. Ao final do experimento, foram realizadas analises para quantificação de nutrientes da água de cultivo e das dietas (fósforo, nitrato, nitrito, nitrogênio amoniacal, nitrogênio total e nitrogênio orgânico), determinação da biomassa final e teor de proteico dos indivíduos. A dieta C proporcionou maior valor médio de biomassa, seguida da dieta A e B, com valores de 344,12 g, 157,71 g e 81,93 g, respectivamente. As *D. magna* alimentadas com a dieta B

apresentaram maior teor proteico, 2,56%, seguidas das alimentadas com as dietas C e A, com 2,17 e 1,32%, respectivamente. Ao final do experimento contatou-se que os organismos alimentados com a dieta C obtiveram reprodução maior, quando comparados as demais.
Palavras-chave: Biomassa; Proteína bruta; Microcrustáceo.

Resumen
Se recomienda el uso de alimento vivo en la fase de larvicultura de varias especies de peces, proporcionando mejores resultados de supervivencia y crecimiento, en comparación con el obtenido en la dieta artificial. El presente estudio tuvo como objetivo evaluar el contenido de biomasa y proteína de *Daphnia magna* sometida a tres dietas diferentes. Dieta A (*Scenedesmus acuminatus*) a una concentración de $1.5x10^7$ células / mL por individuo, dieta B (estiércol de ganado) a una concentración de 6.5g / L y dieta C con la misma concentración de dietas A y B, por un período 21 días. El experimento se repitió tres veces, totalizando 63 días de estudio con un diseño completamente aleatorizado, con tres réplicas por tratamiento, estando constituida la unidad experimental por recipientes de polietileno con capacidad de 20L conteniendo 10 neonatos de *D. magna*. Cada dos días se midieron la temperatura, el pH, el oxígeno disuelto y la saturación. Al final del experimento se realizaron análisis para cuantificar nutrientes en el agua para cultivo y dietas (fósforo, nitrato, nitrito, nitrógeno amoniacal, nitrógeno total y nitrógeno orgánico), determinación de la biomasa final y contenido proteico de los individuos. La dieta C proporcionó el valor medio de biomasa más alto, seguida de la dieta A y B, con valores de 344,12, 157,71 y 81,93 g, respectivamente. *D. magna* alimentada con la dieta B tuvo el mayor contenido de proteínas, 2.56%, seguida por las alimentadas con las dietas C y A, con 2.17 y 1.32%, respectivamente. Al final del experimento, se encontró que los organismos alimentados con la dieta C tenían una mayor reproducción en comparación con los demás.
Palabras clave: Biomasa; Proteína cruda; Microcrustáceo.

1. Introduction

The use of live feed has been recommended in the larval stage of several fish species, because it provides better survival and growth results when compared to those obtained with the use of artificial diets (Montchowui, *et al*., 2012; Akbary, *et al*., 2010; Soares, *et al*., 2000). These results have been attributed to the optimal consumption of this food, induced by visual and chemical stimuli, presence of enzymes that contribute to digestion and greater digestibility when compared to inert diets (Beux & Zaniboni Filho, 2006). As the majority of post-larvae fish do not have a fully developed digestive and enzymatic system (Dabrowski, 1984; Zavala-Camin, 1996), the use of live food becomes essential at the beginning of exogenous feeding, in order to ensure good rates survival and growth (Beux & Zaniboni Filho, 2006).

Zooplankton constitutes a group with an enormous diversity of organisms, with different sizes, shapes, lifecycles and functions, being able to respond promptly to environmental changes (Crispim & Watanabe, 2000). The species belonging to this group are primary or secondary consumers, herbivorous or omnivorous. Some present sexual reproduction, while others, asexual reproduction. These individuals gain prominence in the food chain of aquatic ecosystems, as they represent the link between producers (phytoplankton) and consumers (Brandão, 2009).

The genus *Daphnia* comprises animals that live in the water column, feeding by microalgae filtration and constituting a relevant food source for the fish community (Castro, *et al*., 2009), particularly because they are organisms of easy cultivation and handling in experimental procedures of laboratory (Pereira, 2008).

A number of factors directly influence zooplankton populations, whose influence ranges from population growth to survival. Among the abiotic factors, photoperiod, temperature, pH, nutrients and others (Gyllström & Hansson, 2004) can be mentioned, whereas among the biotic factors, predation pressure, availability and quality of food can be mentioned (Rocha, *et al*., 1990).

Variables such as temperature and water hardness directly influence the cultures of these organisms. Nevertheless, among all variables, the diet (quantity and food quality) to which the organisms are submitted has been shown to be a determinant factor in their reproduction, growth, and locomotive capacity (Kawabata & Urabe, 1998; Tolardo, 2014).

The importance of the quality and quantity of the food supplied can be evaluated by the number of fingerling or the biomass produced in the farms, as the diet can directly influence their reproductive capacity (Vijverberg, 1989). In their study, (Tolardo, 2014) submitted the *Daphnia* to two treatments (very low food) and found that organisms that received high amounts of food presented the best reproductive performance, allocating part of the energy between growth and reproduction. According to (Beatrici, 2004), the nutritional conditions of the organisms can have effects on the future generations, as when they are submitted to poor diets, the *Daphnia* present poor reproductive performance and low concentrations of arginine, histidine, cystine, and methionine.

With a view to the farming of *D. magna* fed with an alternative food of low commercial and nutritional value, this study aims to evaluate the biomass production and protein content of *D. magna* submitted to three different diets.

2. Methodology

The experiment was carried out at the Limnology, Ecotoxicology and Biomonitoring Laboratory (LEB) of the Environmental Aquaculture Research Institute (InPAA) at the State University of Western Paraná, Toledo campus, using *D. magna* provided by the InPAA Planktology laboratory.

In order to reach the objectives, *D. magna* neonates were submitted to three types of diets: diet A, in which the organisms received the microalga *Scenedesmus acuminatus*, cultivated and administered in the concentration of 1.5x107 cells/mL per individual, as single source of food, in accordance to NBR 12713 (ABNT, 2004); diet B, which consisted of cattle manure as a single source, feed in the concentration of 3 mL per organism; and diet C, which was prepared from a combination of diets A and B, in the same proportion, 1.5x107 cells/mL of seaweed plus 3 mL of manure concentrate preparation, thereby avoiding possible modifications in the food concentration variable.

Two growth curves of the microalgae were implemented to determine the exact concentration given to each organism, as the cell density offered for each organism should be the same throughout the experiment. To do this, microalga samples were collected for 22 days. The count was performed every two days in the Newbauer chamber under light microscopy, with 40x magnification. Parallel to the count, the samples were photographed to allow monitoring of the growth of the culture, facilitating the choice of the container from which the daily sample was collected to verify the cell density offered in the diet (Sarrafzadeh *et al*, 2015). In the second growth curve, in addition to counting and photos, the cell density of the microalga was evaluated by turbidity to ensure the amount of microalga delivered was always the same.

The feed composed of cattle manure was collected from a farm near the InPAA in the fresh state. The manure was placed in a closed circulation oven for 48 hours at 60°C in a glass vessel. After drying, the manure samples were crushed with the aid of a processor and then sieved and stored in a capped polyethylene container. To prepare the manure concentrate (7.5 g/L), a mixture of 13 g of manure diluted in 500 ml of water was used, being then allowed to stand for 24 hours and filtered through a sieve with a 0.5 mm mesh. Finally, the concentrate was stored in an Erlenmeyer flask and left at room temperature until use based on the methodology described by Pinto *et al*., (2016).

The design used was completely randomized (Banzatto & Kronka, 2006), consisting of the use of a total of 90 neonates of *D. magna* which were distributed in groups of 30 individuals per diet, which were submitted to a 24 hours acclimation period. They were subsequently divided into three replicates containing 10 individuals placed in polyethylene containers with a capacity for 20 L, duly labeled with farming start date, number of neonates and diet, on a random basis.

Biomass was obtained by means of collections made from the first reproduction of the organisms, which always occurred from the second week of the growing period, thus starting the total biomass estimation. To do so, the samples were randomly collected using a 200 ml beaker in three different regions in the farming media of each diet. At each treatment repetition, the *Daphnia* were transported to a 2 L container and counted for subsequent weighing, after which, the organisms were returned to the culture. The experiment had a duration of 21 days but was repeated three times, with the aim of increasing the sample N, totaling 63 days of experimentation.

Farming maintenance was performed twice weekly for removal of carapace and accumulated dead or weakened microalgae and *Daphnia*. Each day, for 1 hour, there was circulation of the culture water was carried out by means of biological filters coupled to each treatment, and every two days, the monitoring of the physical parameters (temperature, oxygen, pH, and dissolved oxygen) of the water for each diet was carried out.

Analyses were carried out in order to obtain the crude protein content obtained by the determination of the Kjeldahl Nitrogen by the semimicro process, as well as chemical analysis to determine the concentrations of nitrate (Mackereth, *et al*., 1978), nitrite (Strickland & Parson, 1972), total phosphorus (Adams, 1990), ammoniacal nitrogen (Koroleff, *et al*., 1976), organic nitrogen (Adams, 1990) and total nitrogen (total nitrate, nitrite, ammonia nitrogen, and organic nitrogen) of the water composition of the three diets. The analyses were carried out in the Applied Limnology Laboratory (GERPEL, UNIOESTE, Toledo, PR, Brazil).

A one-way analysis of variance (ANOVA) (Scheiner & Gurevitch, 2001) was used to analyze the data relating to biomass, protein content and water nutrients, and the differences were analyzed by the Tukey test at 5% significance using the Statistica 7.0 program.

3. Results

Daphnia submitted to diet C obtained the best total biomass response, followed by diet A and B, with total yields of approximately 1kg, 500 g, and 300 g of *D. magna*, respectively. These differences were considered significant ($p < 0.05$). Thus, it is possible to assume that each adult *Daphnia* produced the equivalent of 5.25g of *D. magna* individuals in diet A, 2.73 g in diet B, and 11.47 g in diet C during the experimental period. Regarding the mean and standard deviation of the biomass of the diets, it followed the same behavior as the total biomass, in which diet C had the highest value while diet b had the lowest value, 157.71±18.34 and 81.93±1.12 respectively (Table 1).

Table 1 - Values of total biomass, mean and standard deviation based on the diets offered to Daphnia magna, with diet A: Scenedesmus acuminatus at a concentration of 1.5x107 cells/mL per individual, diet B: bovine manure at a concentration of 3mL per organism and diet C: Scenedesmus acuminatus plus bovine manure concentrate in proportions of 1.5x107 cells/mL and 3mL, respectively.

Diet	Total biomass (g)	Mean	Standard Deviation
A	473.13^{b}	157.71^{b}	18.34
B	245.79^{c}	81.93^{c}	1.12
C	1032.37^{a}	344.12^{a}	8.84

Means followed by different letters differ from each other by the Tukey test at 5% probability. Source: Authors.

The p-value found in the Tukey test was lower than 0.05, showing statistically significant differences between the diets. This showed that diet C, based on *S. acuminatus* seaweed and cattle manure, obtained a greater efficiency in weight gain in relation to diets based on *S. acuminatus* (A) and cattle manure (B) alone.

The crude protein content of the *Daphnia* fed with different diets differed significantly between them ($p <0.05$). Diet B presented the highest protein content with 2.56%, followed by diet C, with 2.17%, and diet A, with 1.32%.

The analysis of the nutrients of the diets indicated that the diet C presented the highest concentration of total phosphorus (0.12 mg/L), followed by the diets A (0.08 mg/L) and B (0.04 mg/L). Regarding nitrite, diets A and C presented the same values (0.01 mg/L) – in this case, a value higher than that of diet B (0.00 mg/L). For nitrate, diet C presented a significantly higher value (0.11 mg/L) in relation to the other diets. As for the nitrogenous forms, diets A, B and C presented different values of ammoniacal nitrogen concentration, while diet C presented the highest values of ammoniacal nitrogen (0.010 mg/L), organic (6.71 mg/L),) and total nitrogen (6.93 mg/L), as shown in Table 2. Both values differed significantly from each other, with p <0.05 (Table 2).

Table 2 – Chemical composition of experimental diets with only *Scenedesmus acuminatus* at a concentration of 1.5x107 cells/mL per individual (diet A), bovine manure at a concentration of 3mL per organism (diet B) and the combination of *Scenedesmus acuminatus* plus bovine manure concentrate in the proportions of 1.5x10^7 cells/ml and 3mL (diet C).

Nutrients (mg/L)						
Diet	**Total Phosphorus**	**Nitrate**	**Nitrite**	**Ammoniacal Nitrogen**	**Organic Nitrogen**	**Total Nitrogen**
A	0.08^b	0.06^b	0.01^a	0.06^b	3.13^b	3.26^b
B	0.04^c	0.05^c	0.00^a	0.04^c	3.58^b	3.67^b
C	0.12^a	0.11^a	0.01^a	0.10^a	6.71^a	6.93^a

Means followed by different letters differ from each other by the Tukey test at 5% probability. Source: Authors.

Regarding the chemical analyses of the culture water, all nutrients analyzed maintained a standard regarding the significance level, in which diet C always presented the highest values for all water quality variables evaluated (0.07 mg/L of total phosphorus, 0.60 mg/L of nitrate, 0.15 mg/L of nitrite, 0.25 mg/L of ammoniacal nitrogen, 15.71 mg/L of organic nitrogen and 16.71 mg/L of total nitrogen), followed by diet A (0.04 mg/L of total phosphorus, 0.33 mg/L of nitrate, 0.08 mg/L of nitrite, 0.16 mg/L of ammoniacal nitrogen, 10.13 mg/L of organic nitrogen and 10 .70 mg/L of total nitrogen), while diet B obtained the lowest values (0.03 mg/L of total phosphorus, 0.20 mg/L of nitrate, 0.05 mg/L of nitrite, 0.07 mg/L of ammoniacal nitrogen, 5.58 mg/L of organic nitrogen and 5 .90 mg/L of total nitrogen) (Table 3).

Table 3 – Chemical composition of the cultivation water of experimental diets composed of *Scenedesmus acuminatus* at a concentration of 1.5x10^7 cells/mL per individual (diet A), cattle manure at a concentration of 3 mL per organism (diet B) and the combination of *Scenedesmus acuminatus* plus concentrated bovine manure in the proportions of 1.5x10^7 cells/mL and 3 mL (diet C).

Nutrients (mg/L)						
Diet	**Total Phosphorus**	**Nitrate**	**Nitrite**	**Ammoniacal Nitrogen**	**Organic Nitrogen**	**Total Nitrogen**
A	0.04^b	0.33^b	0.08^b	0.16^b	10.13^b	10.70^b
B	0.03^c	0.20^c	0.05^c	0.07^c	5.58^c	5.90^c
C	0.07^a	0.60^a	0.15^a	0.25^a	15.71^a	16.71^a

Means followed by different letters differ from each other by the Tukey test at 5% probability. Source: Authors.

The physical parameters of the culture water did not show major variations among the diets during the experiment. The temperature had a mean value of 17.11°C and a standard deviation of 2.47, whereas the pH obtained a mean value of 7.46 and a standard deviation of 0.26. In turn, the dissolved oxygen obtained a mean value of 4.30 mg/l and saturation of S% = 42.54%, with a standard deviation of 1.29 and 6.89, respectively.

4. Discussion

The diets influenced the reproduction of *D. magna*. According to (Tolardo, 2014), the reproduction factor is directly related to the quantity and quality of the food offered to the organism. Furthermore, this factor influences growth and locomotor capacity.

Still in the context between food and influence in the development of the organism, it is possible to observe that there was a relationship between reproduction and composition of diets according to several authors (Ocampo, *et al*., 2010; Beatrice, 2004; and Beatrice, *et al*., 2006), mainly in the overlapping of diets composed of algae and food supplements such as artemia or trout feed. (Lewis & Maki, 1981) exposed *D. magna* to a diet of *Pseudokirchneriella subcapitata* with a feed based on trout ration, observing that reproduction had an increase of 70% when compared to the individuals fed only the feed or *P. subcapitata*.

The diet C presented the highest biomass in relation to the others, possibly due to the components of the diet being a combination of algae – in this case *S. acuminatus* – and manure. This combination is not recommended by the standards (ABNT, 2004; USEPA, 2002), as they recommend that the food offered to the *Daphnia* be composed of alga plus an alternative food supplement. Notwithstanding, the results demonstrate the feasibility of introducing supplemented feeds into diets for *D. magna*, proving their efficacy. Similarly, (Platte, 1993), with the aim of achieving a way to increase the productivity of cultivars of *Ceriodaphnia dubia*, obtained similar results when using an artemia-based food complement as a means to increase the algae-based diet of the organisms.

The data of this present study show that the biomass presented the best result with a complementary food and that it can be acquired for a low value, being accessible to small producers with low purchasing power, who wish to feed fish in the larval phase with live food. (Corrêa, *et al*., 2010) cultivated artemia with plankton in treatment 1, named natural food, and maniva juice in treatment 2, named inert food, obtaining 0.030 kg / 0.068 m³ with 212.500 nauplii/l for T1 and 0.243 kg / 0.086 m³ with 175.000 nauplii/l in T2, showing the efficiency of the alternative food in relation to the biomass production for microcrustaceans.

Other authors as (Vinatea, 1995) produced biomass of *Artemia sp.* fed with inorganic fertilizers, soybean meal, soybean flour, fish meal, and a mixed diet using shrimp meal. The results showed that the biomass production was higher with the alternative food, including inorganic fertilizer with 7 g/40 m³ for 250 nauplii/l and 113.33 g for soybean meal, while other diets obtained lower values, showing an alternative for production of artemia with food of easy access and low commercial value.

Diet A did not show the best result for the production of biomass. Nevertheless, it is in accordance with the data obtained by other authors when analyzing the biomass of artemia, such as (Vivar, 1979) and (Burga, 2002), who used diets with microalga *Isochrysis galbana* and obtained 33.57 g artemia for diet with microalga and 70.30 g for mixed diet.

As for diet B, biomass was lower when compared to other diets, with 2.13 g per female per day, which was considered unsatisfactory, based on (Ocampo, *et al*., 2010), who affirms the importance of using food complemented in a *Daphnia* culture, as the complementation assists in providing a greater number of neonates. On the other hand, the use of only one food compromises the reproduction of organisms.

The data obtained in this present work corroborate those of (Beatrici, 2004), who evaluated the fertility of *D. similis* and *D. magna* submitted to three diets – *Selenastrum capricornutum* algae, trout, and algae supplemented with trout feed – noting that the *Daphnia* submitted to diet with seaweed and ration obtained a significantly higher number of neonates in relation to the other diets. (Sanches, 2015) evaluated the growth of *D. magna* in different storage densities and found that the increase in density decreases the production of neonates. The same was not observed in this study, as it maintained its density within the rates suggested by the NBR 12713 standard (ABNT, 2004), in which up to 25 organisms per liter should be farmed.

Based on the data of this study, it can be said that the introduction of alternative foods in the supplementation of the diet for *D. magna* was satisfactory in relation to biomass production, even when compared to crustaceans of other species. These results showed the feasibility of using alternative food sources or sources within the reach of the producer for maintenance of the culture. In this context, the results obtained by this study represent an important step for the intensive production of *D. magna* of good nutritional quality to be offered to fish larvae in farming.

The protein content found in the studied organisms can be considered satisfactory, as it is a microcrustacean with significant crude protein values. (Rocha, *et al*., 1990), when analyzing the crude protein of shrimp fillet, obtained values of 14.75%, whereas (Sriket, *et al*., 2007), when analyzing the muscle portion of shrimp cephalothorax, obtained 18% crude protein (CP).

It is possible to observe that in laboratorial cultures, many factors contribute to the limitation of the reproduction of organisms, including competition, predation and adverse chemical and physical conditions. These can be controlled by allowing them to develop rapidly and reach their population growth potential (Fonseca & Rocha, 2004). Nevertheless, in this study, the physical factors were not controlled, only monitored.

Daphnia are organisms that present variations in temperature and pH stipulated by standards such as (ABNT, 2004) and (USEPA, 2002), in which the temperature must be 19-22ºC and the pH, 7.0-8.0. Notwithstanding, only the pH was within these values, while the temperature obtained a lower variation for both diets. In turn, the non-variation of these values may not have influenced biomass production during the experiment. The oxygen also did not present great variations between the cultures. The same occurred for dissolved oxygen. (Assano, *et al.*, 2011) obtained average values of 30.91 mg/L for oxygen saturation in Nile tilapia cultures. In this study, in turn, it was 42.54 mg/L, being acceptable in the farming of organisms such as *D. magna*.

In relation to nutrients, (Nour, *et al*., 2014) studied total nitrogen and found concentrations of 0.64-0.69 mg/L in formaldehyde biological treatment in *Daphnia* bioassays. In this study, lower values were available in all diets, mainly in diet C, with values of 6.93 mg/L.

Similarly, oxidative nitrogen, nitrate, nitrite and phosphorus forms were compared with the study by (Marchello, 2013) in microalgae and zooplankton farming. The author a verified concentration of 1.0 mg/L for nitrate, 0.07 mg/L for nitrite and 4.8 mg/L for phosphorus. In this study, different results were found for nitrate, nitrite and phosphorus, which presented lower values.

In relation to ammoniacal nitrogen (considered a reduced nitrogen form), under the same conditions, (Herawati, *et al*., 2015) found a median of 0.108 mg/L. In this study, the values reached a concentration of 0.010 mg/L in diet C.

In relation to the physical-chemical composition of the diets, both are within the expected values when compared to those found by (Gomes & Silva, 2004), who analyzed the physical-chemical composition of cattle manure and verified that the nitrogen content was low. It should be noted that its composition can vary according to the diet of the animal (Severino, *et al*., 2008). In relation to phosphorus, the same patterns as those of (Gomes & Silva, 2004) were maintained. The same happened with diet C, the junction of food did not interfere as to its composition.

5. Conclusion

Based on the results obtained, it can be concluded that the supplemented diet presented the best results regarding the production of *D. magna* biomass, when grown in the laboratory under uncontrolled environmental conditions. On the other hand, diet B presented the best result for crude protein. Nevertheless, it is advisable to use the organisms grown in the C diet, as it enables a significant number of individuals with high protein content, providing a great availability of live food.

The authors suggest that future studies be carried out aiming to improving the replacement of alternative foods and even cattle manure in higher concentrations in diets for *Daphnia*, in order to verify the possible differences that were not addressed in this study.

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