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Efficiency of cattle manure increase in *Daphnia magna* diet: effects on fertility and sensitivity.
Eficiência do incremento de esterco bovino na dieta de *Daphnia magna*: efeitos sobre a fecundidade e sensibilidade

Gildete de Souza Bezerra[1*], Juliane Alessandra Cavaliere Soares[1], Aldo Felipe Fava[1], Vanilva Pereira de Oliveira[1], Pitágoras Augusto Piana[1], Gilmar Baumgartner[1], Nyamien Yahaut Sebastien[2]

[1*-] Programa de Pós-Graduação em Recursos Pesqueiros e Engenharia de Pesca, Departamento de Engenharias e Ciências Exatas, Universidade Estadual do Oeste do Paraná – UNIOESTE/TOLEDO-BRASIL, Rua da Faculdade, 645 - Jardim Santa Maria, Bloco C, CEP: 85903-000, Toledo, PR, Brasil.
*Corresponding author: gildetesb@gmail.com

[2-] Programa de Pós-Graduação em Ciências Ambientais, Departamento de Engenharias e Ciências Exatas, Universidade Estadual do Oeste do Paraná – UNIOESTE/TOLEDO-BRASIL, Rua da Faculdade, 645 - Jardim Santa Maria, Bloco C, Paraná, 85903-000 Toledo, Paraná, Brasil

Abstract

The objective was to evaluate if the cattle manure in diet alters sensitivity and reproduction. Three diets were evaluated: Diet A, *Scenedesmus acuminatus* ($1.5x10^7$ cells / ml / individual); diet B, cattle manure (6.5 g / L) and diet C, A and B combined. Being developed for 66 days, with n = 3 and 10 females in each. Newborns were counted every 2 days for fertility assessment. The sensivity was study through exposure of neonates to potassium dichromate. Diet C was the most efficient for fertility and all were within the sensitivity curve. Thus, it is possible to use cattle manure in the cultivation of Daphnia.
Keywords: Neonates. Reproduction. Feeding. Toxicity test.

Resumo

Objetivou-se avaliar se esterco bovino na dieta de Daphnia altera sensibilidade e reprodução. Três dietas foram avaliadas: Dieta A, *Scenedesmus acuminatus* ($1,5x10^7$ células / ml / indivíduo); dieta B, esterco bovino (6,5 g / L) e dieta C, A e B combinadas. Sendo desenvolvido por 66 dias, com n = 3 e 10 fêmeas em cada. Os neonatos foram contabilizados a cada 2 dias para avaliação da fertilidade. Já a sensibilidade foi estudada através da exposição de neonatos ao dicromato de potássio. A dieta C foi a mais eficiente para a fertilidade e todos os estavam dentro da curva de sensibilidade. Sendo assim, possível utilizar esterco em cultivo de Daphnia.
Palavras-chave: Neonatas. Reprodução. Alimentação. Teste de toxicidade.

Introduction

Commonly known as “water fleas” Daphnia magna is a zooplanktonic microcrustacean of the order *Cladocera*, that presents a short life cycle, asexual reproduction (parthenogenesis) and intake food by filtration (RODRIGUES; PPWLOWSKY, 2007; CASTRO et al., 2009).

Due to ease cultivation under controlled laboratory conditions, ease handling, and great sensitivity to environment variations and toxic agents, this specie is internationally known and have been broadly cultivated for toxicity tests (HEBERT, 1978; ALVES; SILVANO, 2006; BEATRICI, 2006; RODRIGUES; PPWLOWSKY, 2007; LOPES et al., 2009). As a subsidy for the development of this tests, as well as maintenance and farming of test organisms, standards and methodologies exist, such as USEPA (USEPA, 1993), ISO 6341 (ISO, 1996) e NBR 12713 (ABNT, 2004).

Usually, given its biology, it is recommended to feed *D. magna* with unicellular green algae, however, several studies have been suggesting the use of different diets, according to the characteristics of the culture water, and conditions and needs of each laboratory (ALVES; SILVANO, 2006; BEATRICI et al., 2006; PEREIRA, 2014). Among these diets, it is possible to note the dietary supplementation for rations to fish and crustaceans, oatmeal, soy flour or yeast, both with the aim of improve organisms biological development (PLATTE, 1993; BEATRICI, 2004; OCAMPO, 2010).

It should be noted that the nutritional quality of food supplied to crops, can to determine differences in its development and to cause positive and negative effects about longevity and fecundity and can even alter their sensitivity to toxic agents and environmental samples in toxicity tests (BAILEY et al., 2000; BURATINI; ARAGÃO, 2012). In this sense, among all the variables involved in cultivation for toxicity tests, the diet of the organisms has been a determining factor in their results (BEATRICI, 2006).

Thus, this study aimed to evaluate the efficiency of cattle manure increase, (resource very abundant, with important nutritional elements) in *D. magna* diet, checking if the supplementation has influence on cultivation conditions of test organisms, on fertility of adult females and on organism’s sensitivity. Specifically, we intended to evaluate the feasibility of making cattle manure increment in the diet an alternative for the farming of these test organisms for toxicity tests.

Material and methods

Daphnia magna cultivation for toxicological tests

The matrices of *D. magna* were obtained from cultivation tanks of large scale on Planktology laboratory, belonging to Institute of research in environmental aquaculture (InPAA) of the State University of Western Parana (Unioeste - Toledo/PR). The experiment was carried out at the limnology, ecotoxicology and biomonitoring laboratory (LEB) of this institute, where proceeded fertility trials, obtaining third generation neonates and sensitivity tests, all submitted to three diets

(A, B and C). Diet A consisted of suspension of *Scenedesmus acuminatus*, who was cultivated and administered on crops in concentrations of $1,5x10^7$cells.ml^{-1}.$individual^{-1}$.day^{-1}, following recommendations from NBR 12713 (ABNT 2004).

The diet B had as only source of food a cattle manure concentrate. Therefore, cattle manure *in nature* was collected on a farm close to InPAA, dried in a close circulation stove for 48 hours at 60 degrees, crushed with the aid of a processor, sieved in 0,3 mm mesh, and stored in polyethylene container. Each day, 13 g of this dry manure was dissolved in 500 ml of water, being the same homogenized and after resting for 24 hours, filtered through 0,5 mm sieve opening and administered daily in the amount of 3 ml.$individual^{-1}$. Diet C was made by joining diets A and B, in the quantities described above. It was performed dietary nutrient analyzes (table 1) in order to characterize the components present in each treatment.

Table 1- Total phosphorus concentrations (TP), nitrate (NO_3), nitrite (NO_2), ammonia nitrogen (NH_3), organic nitrogen (Norg) and total nitrogen (TN), in $mg.l^{-1}$, with the mean values and coefficients of variation (values in parentheses) observed in each diet.

Diet	TP	NO_3	NO_2	NH_3	N_{org}	TN
A	0,045 (1,0)	0,21 (98,5)	0,00 (41,3)	0,16 (40,0)	14,65 (4,41)	14,68 (5,5)
B	0,036 (0,0)	0,22 (14,3)	0,00 (66,4)	0,15 (26,0)	30,33 (10,1)	29,26 (10,5)
C	0,055 (3,0)	0,38 (26,4)	0,00 (2,5)	0,14 (48,5)	29,86 (4,33)	29,7 (3,96)

Fertility test

Ninety matrices from InPAA were evenly distributed in nine experimental units (2 liter glass containers), which were packaged inside BOD incubator with 16h photoperiod of light and temperature between 19 and 22 ^{0}C, where remained for 22 days. Three replicas of each treatment were allocated randomly to experimental units and the matching diets were administered daily at the concentrations described above. After observing the appearance of first neonates, we started the process of fertility measurement (total of neonate produced per female). This consisted of withdrawal and count the neonates produced every 2 days, with the aid of trays and Pasteur pipettes. According to recommendations of NBR 12713 (ABNT, 2004), cultivation waters were exchanged two times per week.

Third generation neonatal trial

In parallel to the fertility test, the test for obtaining third generation neonatal trial was conducted on three 2l beckers inside the BOD incubator. The beckers received 20 neonates from first fertility test, each coming from one of the diets treatments. These neonates were grown under

the same conditions as the fertility test until they gave rise to the second generation, which were also grown to generate the third generation, from which neonates of *D. magna* were obtained for the sensitivity test.

Sensitivity test

The sensitivity test was conducted to verify if the growing conditions of *D. magna* were favorable, as well as if the experimental diets are able to be provided to organisms in toxicity tests. The essay was static, without shift of water, during 24 hours. The substance used was potassium dichromate (K_2 Cr_2 O_7), in the following concentrations: 0; 0,55; 0,70; 0,85; 1,00; 1,15; 1,30; 1,45; 1,60 e 1,75 $mg.l^{-1}$ (ALVES; SILVANO, 2006). For each concentration we allocated 4 experimental units (glass tubes containing 15 ml of test solution) with 5 neonates with a lifetime between 2 and 24 hours each. The glass tubes containing the test solution and neonates were capped and environmentalized in a BOD incubator, with temperature between 19 and 22º C, photoperiod of 16 hours/light, where the organisms remained for 24 hours deprived of food. After this period, the immobile organisms were quantified to determine the effective concentrations average (CE_{50}). This procedure was repeated for third generation neonates of diets A, B and C.

Regarding the pH values of dilution water used in sensitivity tests (control and dilution of potassium dichromate), which were read before and after the tests, it was found that had variation between 7,70 and 7,72 in the diet A, between 7,56 and 7,74 in the diet B, and between 7,66 and 7,70 in the diet C. However, variation is within the allowed by NBR 12713 (ABNT, 2004).

Data analysis

To increase the statistical power of results, the 3 tests were repeated three times, obtaining 3 times the number of experimental units described above. In first test, *D. magna* fertility was estimated from the average number of neonates produced by female. Day^{-1} and the information obtained was submitted to analysis of one-way variance, follow by Tukey test at the significance level of 5%. In the test of sensitivity, the values of immovable individuals in the experimental units were used to obtain CE_{50} (mean effective concentration after 24h of test), and the results were evaluated using the Trimmed Sperman-Karber software (HAMILTON, 1986), with a confidence level of 95%.

The experimental sequence, since the experimental design until housekeepers' analyses are summarized in figure 1.

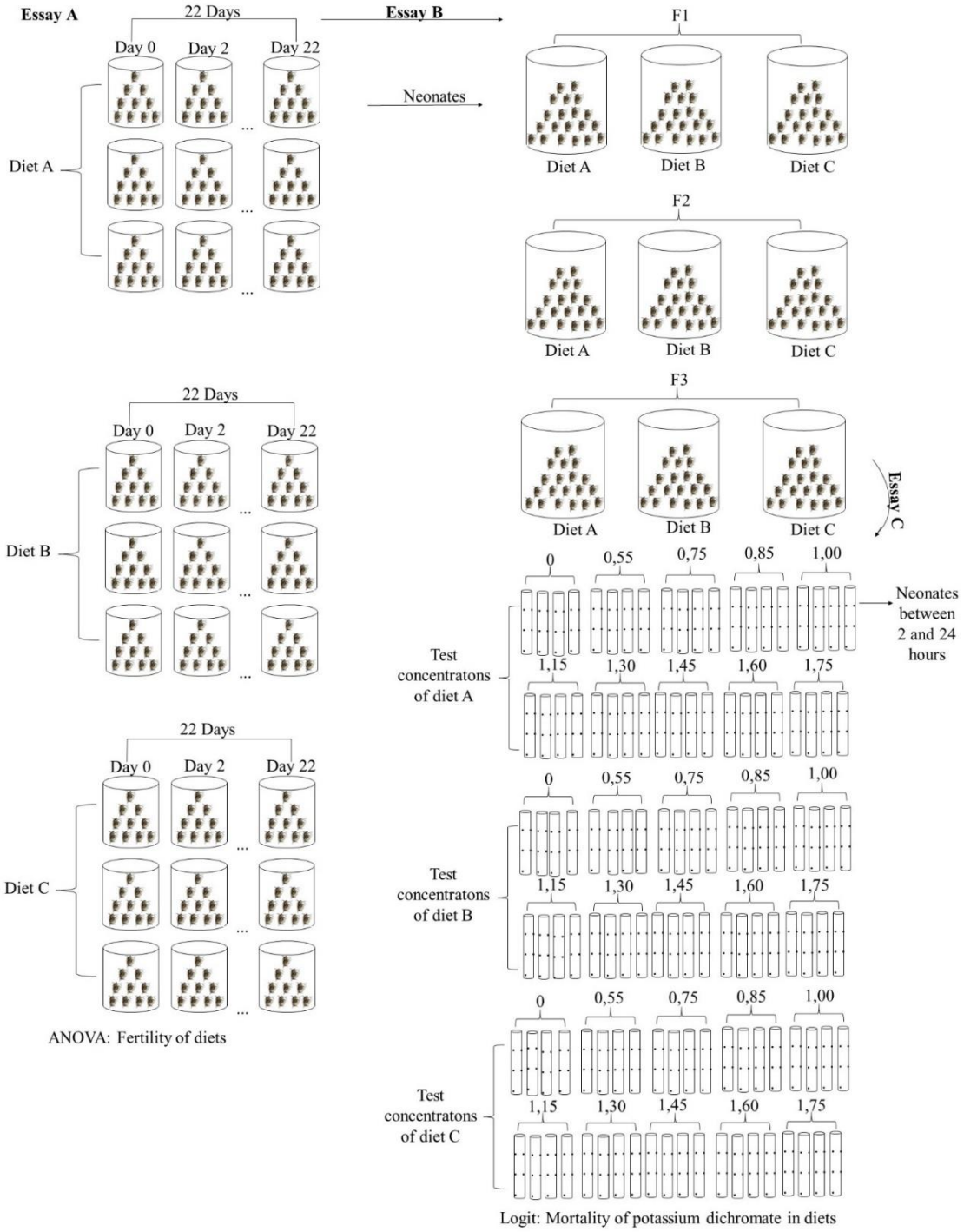

Figure 1 - Test Scheme developed to evaluate the fecundity and sensitivity of *Daphnia magna* feed with diets A (*Scenedesmus acuminatus* offered in concentration of $1{,}5x10^{7}$ cells.ml^{-1}.individual^{-1}.day^{-1}), B (cattle manure administered in an amount of 3 ml.individual^{-1}) and C (*S. acuminatus* and cattle manure in the same concentrations of diet A and B). The test A consisted of *D. magna* cultivation in glass containers, with cultivation medium in the digestive chamber during 22 days for each diet (A, B and C) in order to determine the fecundity of organisms submitted to each diet. The test B is the cultive of *D. magna* from F1 generation to F3, to be used in the sensitivity test. The test C is the execution of sensitivity test of neonates, with age between 1h and 24h, to obtain the CE_{50} of each diet.

Results

The *D. magna* submitted to cattle manure increment diets in algae suspension of *Scenedesmus acuminatus* (diet C) got the best answer regarding the fertility, with total production of 16.821 neonates, follow by suspension algae of *Scenedesmus acuminatus* (diet A) and of cattle manure concentrate (diet B), in which were verified productions of 11.020 and 3.323 neonates, respectively. Thus, average daily productions of *D. magna* adults were 2,82 neonates/female/day in Diet C, 2,04 neonates/female/day in Diet A, and 0,55 neonates/female/day in Diet B.

The ANOVA one-way showed significant differences between the three experimental diets ($F_{2,22}$=77, p<0,0001), being that the average number of neonates produced by female in diet C was significantly higher (p<0,05) than the average number of neonates produced per female in diet A, and both were significantly higher than average number of organisms produced by Diet B (Fig. 2).

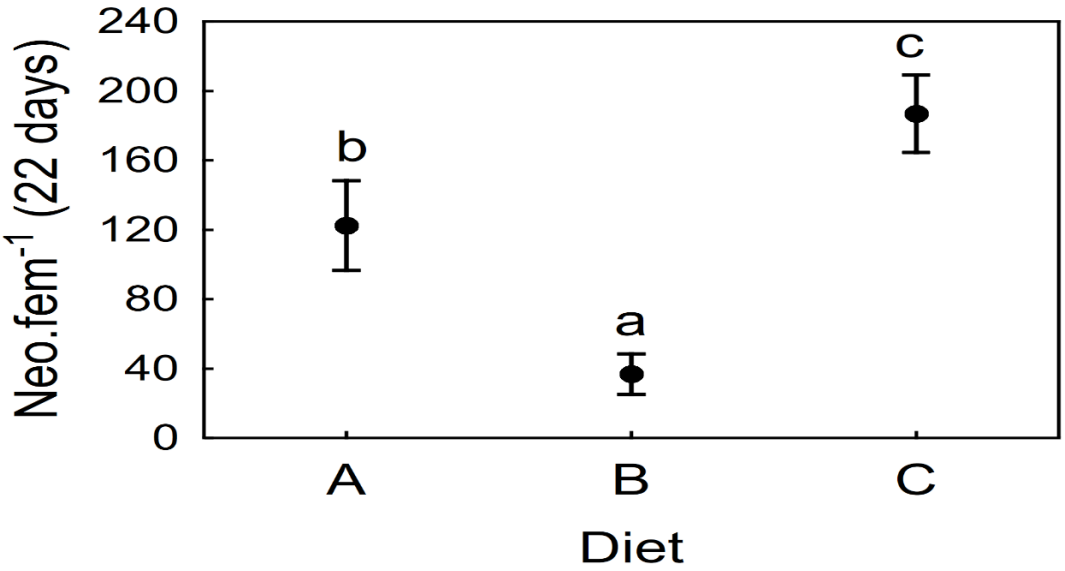

Figure 2 - Average and 95% of IC for fecundity (Neo.fem^{-1}) obtained by female submitted to diets A (*Scenedesmus acuminatus*), B (cattle manure administered in an amount of 3 ml.individual^{-1}) and C (*S. acuminatus* and cattle manure in the same concentrations of diets A and B). Small letters express statistical differences between the neonate production according to diet by Tukey test.

Sensitivity tests performed for each experiment repetition indicated that the CE_{50} of the organisms submitted to different diets differed statistically (p<0,05), being the organisms submitted to Diet B showed higher resistance to potassium dichromate, whereas the organisms submitted to diet C and A showed lower resistance to the same reference substance (Figure 3).

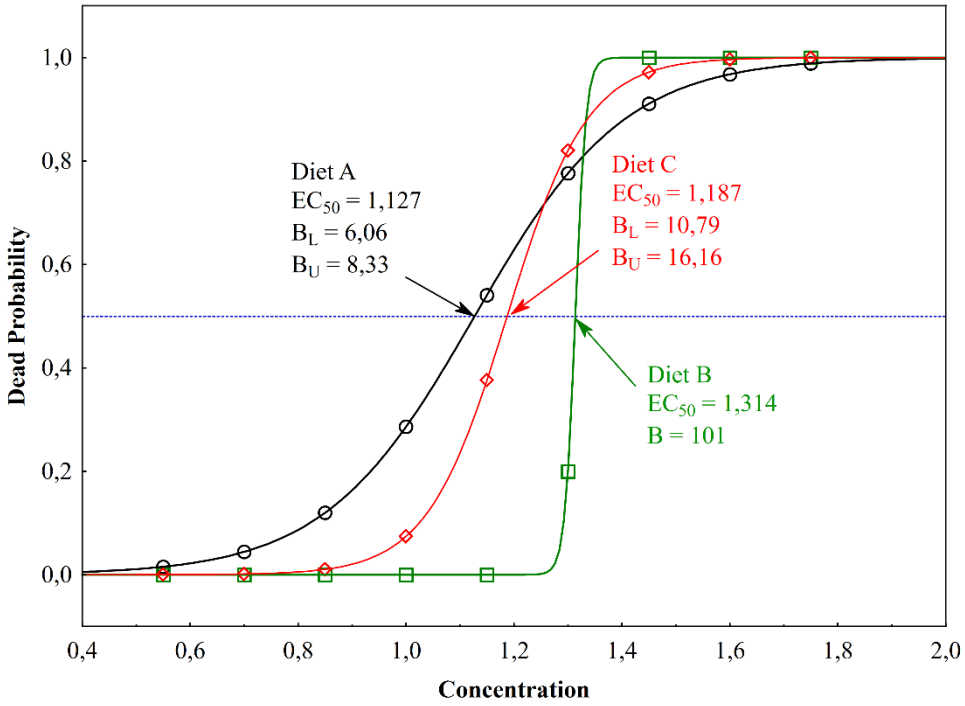

Figure 3 - Results of sensitivity tests performed during the experiments repetitions, with their respective CE_{50}-24h (effective average concentration) for each experimental diet.

Discussion

The experimental diets demonstrated to have a relevant interference in *D. magna* fecundity, that may be related to the quantity and quality of food, because it influences growth, reproduction and locomotor capacity of these organisms (PEREIRA, 2014). Diets formed by different compositions of micro crustaceans, that increased the diets previously composed of algae, also proved to be determinants in *D. magna* fertility (BEATRICE, 2006; CARABALLO et al., 2011; CHEBAN et al.,2018). In the same perspective, Lewis and Make (1981) submitted *D. magna* to diet composed by *Pseudokirchneriella subcapitata* (seaweed) increased with ration for trout and observed the increase of 70% in fertility rate in relation to isolated diets. Beatrici (2004), that also evaluated the increment of ration for trout in algae suspension of *Selenastrum capricornutum*, obtained similar results, since when the organisms were submitted to mixed diet, more neonates were obtained than isolated diets.

Similarly, Platte (1993) aiming to achieve a way to increase the productivity of *Ceriodaphnia dubia* cultive, got similar results when using artemia based food supplement as way of to increase the based algae diet of organisms. In this sense, Diet C, composed of cattle manure increase in algae suspension of *Scenedesmus acuminatus*, that isn't cited in the standards related to defined cultivation, perhaps due to absence of studies, proved to be an alternative diet, quite effective in these micro-crustaceans reproducing.

About diet A (*Scenedesmus acuminatus*), despite it was not as efficient about *D. magna* fecundity, the dates obtained are in expected in international norms as in USEPA (1993), that

stipulated a minimum of 40 neonates per adult female in period of 21 days, similarly to verified in this study. In relation to Diet B (cattle manure isolated), whose fertility rate was much lower, it was considered unsatisfactory, if it is using as refer internationally recognized standards (BEATRICI, 2006).

In this sense, Ocampo et al. (2010) reinforce the importance of using of supplemented food in dafinid cultivation, since complementation helps to increase production of neonates. On the other hand, the use of only one food may impair the organisms reproduction. Thus, it is worth mentioning the study of Beatrici (2004), that when evaluating *D. magna* and *D. similis* fertility and sensitivity in different diets found higher fertility in the supplemented diets.

Regarding the sensitivity of organisms, Winner et al. (1997) observed that the nutritional state directly influences the toxicity of chemical substances for organisms, mainly in the chronic essay in which the growth and reproduction were evaluated. In this study, although there are differences between resistances to concentrations, *D. magna* submitted to both diets were fit to be used in toxicity tests, since the values of CE_{50} for reference substance, potassium dichromate, were within the recommended range by the standard ISO 6341 (ISO, 1996), indicating, thus, that the diets did not affect their sensitivity, similarly to described by Alves and Silvano (2006).

About the values of pH measured from dilution waters used in sensitivity tests, it was verified that they were in agreement to NBR 12713 (ABNT, 2004), that is, they were between 7,00 and 8,00, no adjustments needed, and so no influence on the test results occurred. Regarding to nutritional analyses of the diets, both were within as expected when compared to Gomes and Silva (2004), that showed low nitrogen in manure. It is worth mentioning that this composition may vary depen ding on the animal's diet (SEVERINO et al., 2006). With respect to phosphorus, this also remained in the same standards, according to Gomes and Silva (2004).

Conclusion

From the results obtained in this study, we can concluded that the quality and quantity of food influenced directly the *D. magna* reproduction cultivated in laboratory. However, sensitivity of organisms do not changed due the diet.

The increase of cattle manure in algacea suspension of *S. acuminatus*, submitted to *D. magna* with Diet C, showed to be an efficient supplementation as regards to fecundity increase, when compared to isolated diets tested. Thereby, we concluded cattle manure is an efficient food and viable to maintain a good level of fertility, enough to produce the number of neonates required for ecotoxicological tests.

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