Bull. Environ. Contam. Toxicol. (1990) 44:59–66

Environmental Contamination and Toxicology

Use of *Selenastrum capricornutum* and Microfeast™ as Food for *Daphnia pulex*

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Daphnia magna and Daphnia pulex have been traditionally used as toxicity test organisms because: (1) they are members of an important phyla of aquatic organisms; (2) they are an important food source for many aquatic species; (3) their sensitivity to chemicals is similar or greater than other aquatic organisms. In addition, daphnids have a short life cycle and require limited laboratory space for culturing. Daphnia magna has been frequently used due to its larger size and hardiness. Recently D. pulex has become increasingly popular as a test organism due to its apparently greater sensitivity (Winner and Farrell 1976; Buikema et al. 1980) and more cosmopolitan distribution (Edmondson 1959) when contrasted with D. magna . The latter attribute makes this species especially suitable for site-specific toxicity investigations (USEPA, 1983).

Little attention has been devoted to conditions necessary for culturing D. pulex in the laboratory or to the quality and quantity of food required for maximum neonate production. Acute toxicity tests require large numbers of juvenile daphnids, < 24 hours old. Neonate production is also used as an endpoint in the 21 day chronic test to determine the effect of chemicals on reproductive capacity. Biesinger and Christensen (1972) have observed that sensitivity of daphnids to toxicants differs depending on diet and availability of food. Therefore, there is a need to standardize Daphnia culture techniques in order to ensure production of constantly healthy neonates for toxicity testing purposes.

Different investigators have utilized a variety of algal diets for daphnia: Ankistrodesmus sp. (Daniels and Allen 1981; Keating and Dagbusan 1984), Chlamydomonas reiinhardti (Marshall 1962; Starkweather 1976); Scenedesmus sp.

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(Horton et al. 1979), Selenastrum capricornutum (Goulden et al 1982). Feeding concentrations for D. pulex have ranged from 3 x 10^3 algal cells ml^{-1} to as high as 1 x 10^6 algal cells ml^{-1}. Some investigators have advocated algal vitamin enrichment (Goulden et al. 1982). Non algal diets, such as trout chow or yeast, have been used due to their ease in preparation. However, these diets have shown extreme variability in their successfulness and in general are thought to be inferior to algal diets (Winner 1976; Buikema et al. 1980).

As part of a program to develop a protocol to maximize D. pulex neonate production. We have (1) examined the effect of stored (4°C) vs. fresh S. capricornutum on neonate production, the former diet was tested in order to take advantage of an algal batch culture system; (2) determined a feeding level which maximizes neonate production; and (3) examined the effect of a yeast extract (Microfeast™) addition to the optimum S. capricornutum feeding concentration. Results of these activities are discussed in this manuscript.

MATERIALS AND METHODS

Fresh and stored S. capricornutum were prepared in the following manner. A fresh source of Selenastrum capricornutum was obtained daily from a 2-L culture flask containing 1400 mls of medium (Miller et al. 1978). The flasks were placed on stir plates in an environmental growth chamber maintained at constant temperature (24°C) and light intensity (cool white fluorescent 105 microeinsteins m^{-2} sec^{-1}). One flask was inoculated each day. Upon reaching maximum growth, an aliquot was used to inoculate the new flask. The volume of the inoculum was adjusted to yield an initial algal concentration of 7 x 10^4 cells ml^{-1} in the new flask. The culture was then harvested in a sufficient volume of moderately hard reconstituted water (APHA 1980) to give an absorbance of 0.14 at 660 nm. This then served as the freshly prepared food source. Selenastrum was also grown in a 5 gallon pyrex batch culture system. The culture chamber was filled with ten liters of double strength medium, placed on a stir plate in an incubator maintained at constant temperature (24°C) and light intensity (cool white fluorescent 72 microeinsteins m^{-2} sec^{-1}). The system was aerated. One hundred ml of inoculum were used to initiate the culture resulting in an initial algal concentration of 3 x 10^4 cells ml^{-1}. Growth dynamics were monitored spectrophotometrically. Algal cells were harvested with a continuous centrifuge while still in the exponential growth phase. The resulting pellet was resuspended in reconstituted water and placed in 250 ml polyethylene jars which were then stored in the refrigerator for two weeks prior to the onset of the experiment.

Algal density was quantified by deriving regression equations for algal concentration (cells ml^{-1}) and dry weight (mg l^{-1}) as a function of absorbance at 660 nm using a Bausch & Lomb Spec-20.

Algal concentrations and dry weights were determined for a concentrated sample. Cell counts were enumerated using a hemacytometer (APHA 1980) while dry weight was determined by passing six 100 ml aliquots through six 0.45 um glass fiber filters (Schleicher and Schuell) which were then dried at 105°C for 24 hr and subsequently weighed. Serial dilutions of this sample were then used to model the above two parameters spectrophotometrically.

At the start of an experiment, and then as needed, one of the refrigerated algal concentrates was diluted with reconstituted water. Six 600 ml beakers each containing 500 mls of reconstituted water (APHA 1980) and eight < 24 hour-old neonates were used for each replicate of the two treatments, fresh versus stored food. Culture water was replaced at approximately one week intervals and the experiment was conducted under ambient room temperatures and light conditions. Each replicate beaker received a daily addition of 3×10^7 cells 500 $ml^{-1}d^{-1}$ or a resultant algal feeding concentration of 6×10^4 cells $ml^{-1}d^{-1}$ (0.42 mg dry weight d^{-1}). After daphnids attained reproductive maturity, neonate production in each replicate was determined by first removing adults and then pouring the contents of the beaker through a 250 micron mesh screen to trap neonates. The adults were returned to the culture water. The neonates remaining on the screen were counted under a dissecting microscope. Whenever a replicate was reduced to less than eight organisms the neonate counts were discarded in statistical analyses.

Three feeding level experiments were conducted to determine at what S. capricornutum concentration D. pulex neonate production was maximized. Stored food was used exclusively in all the experiments. The first (I) and second (II) experiments utilized the preceding experimental design. In the third experiment (III) culture media volume and feeding concentration were reduced in a replicate if mortality occurred. In this way the remaining organisms were maintained at a constant organism volume ratio and were thereby fed a constant algal concentration equivalent to the original value employed. In experiments II and III, algal cells were not centrifuged and resuspended in culture water but were refrigerated directly in the algal medium in which they were cultured. Ten rather than eight < 24 hr old neonates were used in experiment III. Feeding levels for each experiment are shown in Table 1.

The effect of a defined organic supplement (Microfeast™, Provesta Corporation, Bartleville, OK 74004) on neonate production was examined utilizing the optimum S. capricornutum concentration. Microfeast™ is produced as a byproduct of an industrial biochemical process and contains yeast, vegetable protein, marine and vegetable oils, lecithen, cholesterol, amino acids, vitamin C, B complex, A, D, E and K. Experimental design was the same as for feeding level experiment III. An aqueous solution of extract was prepared in reconstituted water and aged uncovered under ambient laboratory conditions for three days. This solution was then fed to yield a constant concentration of 5 $\mu g\ ml^{-1}$ with the optimum algal concentration.

Use of a CAHN model C-31 microbalance allowed determination of adult D. pulex weights for feeding experiments II, III and the Microfeast™ experiment. Adult daphnid dry weights were determined by placing organisms on glass petri plates and removing excess water. The organisms were dried for 24 hr at 60°C, after which they were allowed to cool in a desiccator before being weighed.

RESULTS AND DISCUSSION

Growth curves based on both concentration and dry weight for S. capricornutum were calculated. The regression models developed for algal quantification are as follows:

$$\lambda = 660 \quad r = 0.999$$

$$\text{Conc (cells ml}^{-1}) = 2.335 \times 10^{7} \text{ (ABS)} - 1.42 \times 105$$

$$\text{Dry Weight (mg 1}^{-1}) = 314.4 \text{ (ABS)} - 1.9 \quad r = 0.999$$

Mean neonate production per brood for fresh versus stored food treatments based on five replicates over the 21 day test period were not normally distributed. In view of non-normally distributed data, the nonparametric Wilcoxon test was applied. Within treatment variability between individual replicates of both diets was not significantly different (stored p = 0.9620; fresh p = 0.8253). Mean number of neonates per brood for fresh ($\bar{X} = 3.5 \pm 2.6$) versus stored ($\bar{X} = 3.1 \pm 2.1$) food treatments was not significantly different (p = 0.5417). These data suggest that culturing large volumes of algae and then refrigerating the algae for up to five weeks until needed affords a viable alternative to the more laborious task of preparing a fresh algal diet daily. This approach simply requires that a stock S. capricornutum culture be maintained for inoculation purposes.

The second set of experiments were conducted to determine an appropriate feeding level of stored food for

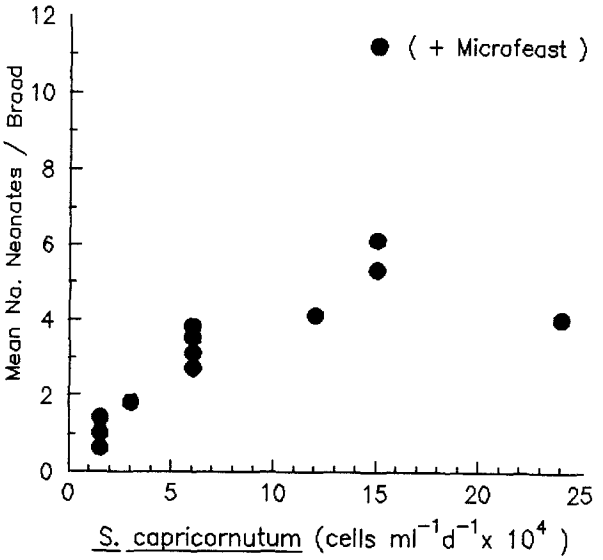

Figure 1. Mean D. pulex neonate production per brood at various S. capricornutum feeding concentrations.

Table 1. Neonate production and adult weight as a function of increasing algal concentration

S. capricornutum[1] Feeding concentration (cells ml^{-1}day^{-1})	Feeding Study	Mean No. Neonates/ Brood	SD	Mean Adult wt.(mg)	SD
A 1.5 x 10^4	I	0.6	0.6		
B 1.5 x 10^4	II	1.4	0.8	0.0257	0.0047
B 1.5 x 10^4	III	1.0	0.6	0.0300	0.0038
C 3.0 x 10^4	III	1.8	0.1	0.0367	0.0058
D 6.0 x 10^4	I	3.5	2.6		
D 6.0 x 10^4	II	3.8	1.4	0.0368	0.0062
D 6.0 x 10^4	III	2.7	1.0	0.0513	0.0090
E 1.2 x 10^5	III	4.1	1.5	0.0655	0.0125
E 1.5 x 10^5	I	5.3	4.5		
E 1.5 x 10^5	II	6.1	2.1	0.0480	0.0120
D 2.4 x 10^5	III	4.0	1.7	0.0814	0.0303
F 1.5 x 10^5 + micro	III	11.2	4.4	0.1430	0.0323

1 Neonate production in concentrations with the same letter were note significantly different.

optimizing production. Statistical comparisons were based on four replicates. Neonate production between replicates of the same treatment within each experiment were not significantly different ($\alpha = 0.05$), except at 1.5×10^4 cells $ml^{-1}d^{-1}$ where the neonate production in Experiment I was significantly different from the neonate production in Experiments II and III ($p < 0.001$). At the completion of feeding Experiment I we observed greater than 20% mortality in the highest two feeding treatments (6.0×10^4 and 1.5×10^5 cells $ml^{-1}d^{-1}$). The trend towards greater mortality at the elevated feeding levels may have been a consequence of adverse changes in culture water quality due to algal metabolism or accumulation of metabolic waste products produced by the animals. Consequently, in the succeeding experiments culture water was changed three times a week.

During the course of these experiments several treatment levels were repeated with comparable neonate production (Table I, Figure 1). Mean number of neonates per brood increased as a function of increasing algal concentration until at a concentration of 2.4×10^5 cells $ml^{-1}d^{-1}$ a decrease occured (Table I and Figure 1).

Grouped feeding treatments for all three experiments did not meet normality assumptions; consequently, the non-parametric Kruskal Wallis procedure was applied to these data. The analysis showed that neonate production was significantly affected by feeding level ($p < 0.0001$). Duncans multiple range test applied to the ranked means revealed that mean neonate production per brood for the fourth and fifth food concentrations, 1.2×10^5 and 1.5×10^5 cells $ml^{-1}d^{-1}$ were the optimum feeding levels (Table 1). For comparison, Craddock (1976) using an experimental procedure similar to that used in this study investigated the effect of temperature on D. pulex neonate production. At a temperature of 20°C mean neonate production per brood was observed to be 3.1 and 3.7 using a mixture of Chorella and Chlamydomonas, however, algal feeding concentrations were not reported.

At the conclusion of feeding experiments II and III adult weights were obtained. The weight distribution did not meet normality assumptions, therefore the Kruskal Wallis test was used to evaluate treatment effects on adult weight. There was a significant effect of algal concentration on adult weight ($p < 0.001$). Adults fed an algal concentration of 2.4×10^5 cells $ml^{-1}d^{-1}$ gained the most weight (Table I). Thus, the reduction in neonate production observed at this highest algal concentration was not reflected in a concommitant weight loss. These findings suggest that above a feeding concentration of about 1.5×10^5 cells $ml^{-1}d^{-1}$ additional energy assumilated by each female is channeled into growth at the expense

of increased fecundity.

Supplementing cultures fed at the algal concentration shown to maximize neonate preduction (1.5×10^5 cells $ml^{-1}d^{-1}$) with 5 μg $ml^{-1}d^{-1}$ of Microfeast™ resulted in a significant increase in fecundity ($p < 0.001$) and adult weight ([$p < 0.001$] [Table 1]). These results clearly demonstrate the benefits of a Microfeast™ supplemented algal diet. Improved culture health probably resulted due to the supply of essential trace organics and/or the promoted growth of bacterial populations upon which the daphnids could feed.

In this study storing algae for up to five weeks had no effect on D. pulex neonate production. Mean neonate production when daphnids were fed S. capricornutum stored for five weeks was not significantly different from freshly prepared algae. Batch culturing algae will reduce the number of hours spent in daphnia food preparation.

Mean neonate production per brood and adult daphnia weight increased with increasing algal concentration. The optimal algal feeding concentrations for D. pulex were 1.2×10^5 and 1.5×10^5 cells $ml^{-1}d^{-1}$. A decline in neonate production was noted above these concentrations.

The addition of a yeast extract (Microfeast™) at 5 μg $ml^{-1}d^{-1}$ to the optimum algal concentration increased both adult weight and neonate production. Use of this extract as a supplement with S. capricornutum can reduce culture size to obtain required neonates for testing and therefore reduce culture maintenance.

Daphnia pulex cultures designed for toxicological applications should ensure large numbers of consistently healthy neonates yet require minimal amounts of effort expended in routine culture maintenance. The findings presented in this paper can serve to aid researchers in achieving this goal.

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Received June 3, 1989; Accecpted June 20, 1989.