MAINTENANCE OF *DAPHNIA CARINATA* CULTURES FOR USE IN TOXICITY TESTING

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ABSTRACT
A growing concern for use of local species for toxicity testing leads to the need to create toxicity data banks for these species. In order to have healthy animals to conduct the tests, procedures for maintenance of stock cultures of these species should be developed. The most widespread daphnid in Australia, *Daphnia carinata,* is considered to be one of the most suitable for toxicity testing of contaminants entering Australian freshwaters. However very little data are available on the culture requirements of the species. In this study different types of food were tested: *Chlorella vulgaris* cultured in two different media - Keating and Tamiya, *Chlorella pyrenoidosa* cultured in the same two media, and a suspension of trout pellets. Intrinsic rates of natural increase of individual cultures of *D. carinata* were determined from "life tables". The best food from among those tested were *C. pyrenoidosa* cultured in either Keating or Tamiya medium. Two different procedures of individual cultures are proposed for the maintenance of *D. carinata* for use in toxicity testing, based on tests conducted using different culture volumes.

Key words: *Daphnia carinata,* culture requirements, toxicity testing.

INTRODUCTION
Cladocerans of *Daphnia* species have been widely used for many years as test organisms for toxicity testing under laboratory conditions. Different guidelines for testing of chemicals are used in different countries (US EPA, OECD Guidelines, National standards etc.), and *Daphnia magna* and *Daphnia pulex* are among the most frequently used species in the northern hemisphere. A considerable amount of toxicity data is available on these species together with requirements for their cultures (Lee *et al.* 1985; Elendt 1990a, 1990b; Elendt and Bias 1990). In Australia and South-East Asia the most abundant and widely distributed daphnid is *Daphnia carinata* (Benzie 1988). A growing concern for use of local species in ecotoxicology creates an agenda for establishing toxicity data for such species. In Australia *Ceriodaphnia dubia* is commonly used for toxicity testing (Rose *et al.* in press; Warne and Schifko 1999; NSW EPA 1999, 2003a). However very little data are available not only on toxicity testing, but also on the culture and requirements of the common Australian cladoceran *D. carinata* (NSW EPA 2003b). In our study we tested several experimental procedures in order to choose the most suitable among them (in terms of medium, volume requirements and food type) for culturing *D. carinata* for use in toxicity testing.

METHODS
Our experiments consisted of three parts:
1. Growth rates of algae in two culture media
2. Effects of different diets on daphnid survival and reproduction
3. Effects of culture volume on daphnid survival and reproduction

Experiment 1. Growth rates of algae in two culture media
Two species of freshwater green unicellular alga *Chlorella vulgaris* and *Chlorella pyrenoidosa* were cultured in order to determine their growth rates. Two different media were used for culturing each of the species: (a) Keating MS medium with low concentrations of nutrients and pH=8.0 (Keating 1985) and (b) Tamiya medium with high concentrations of nutrients and pH=5.0 (cited in Vasser 1989). Algae were cultured axenically in cotton-stoppered 250-mL conical flasks on a light table (luminosity 3000±10 lux, continuous (no photoperiod), temperature 25±1°C). Cultures were aerated continuously using aquarium air pumps. There were four replicates of each culture (16 total). Cell density was counted every six hours for 60 hours, using a haemocytometer. On each occasion four subsamples of 0.01 cm^3 were taken from each replicate and all algal cells in the grid were counted.

Growth rates of the cultures (μ) were calculated as

$$\mu = (\ln N_t - \ln N_{t-1})/\ t$$

where N_t is algal cell density at time t,
N_{t-1} is algal cell density at time of previous observation,
t is time between the observations.

Algae from the exponential phase of growth were then used as food for daphnids (along with trout pellets) in a feeding trial.

Experiment 2. Effects of different diets on daphnid survival and reproduction
Female neonates of *D. carinata* (< 24 hours of age) were placed individually into 28-mL McCartney bottles with 25 mL of medium in each. The medium was carbon-filtered tap water with 0.5 g/L of scientific grade sea salt "Coralife" (Coralife Scientific Grade Marine salt, Energy Savers Unlimited, Inc, Carson, CA USA) (nitrates and phosphates free). The temperature of the media was 25±1°C, pH = 7.0±0.1, luminosity was maintained at 500 lux at daytime, photoperiod 15 h day/ 9 h night. Twenty animals were tested for each type of food. The experiment was conducted for 21 days. Medium was replaced daily, daphnids were fed with algae or trout pellets once a day. Concentration of algae was 2×10^5 cells/mL when algae were added to the culture. The solution of trout pellets was prepared as follows (on recommendation from ARI, see acknowledgments): 20 g of trout pellets were ground and suspended in 150 mL of distilled water, then filtered three times using a fine strainer (pore size 140 μm). The suspension was then stored in the refrigerator for up to three weeks and used for the feeding trials. Daphnids were fed daily with 10 ÏL of the suspension. Survival

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and fecundity of females were recorded and the intrinsic rate of natural increase was computed using the Lotka formula (Lotka 1913):

$$l_x m_x e^{-rx} = 1$$

where l_x is the proportion of individuals surviving to age x, m_x is the age specific fecundity (number of females produced per surviving female at age x), x is days.

The type of food that produced the best results in terms of intrinsic rate of natural increase was then used as food in the test of different culture volumes.

Experiment 3. Effects of culture volume on daphnid survival and reproduction

Individual culture of *D. carinata* was proposed as an alternative to the use of cohorts for toxicity testing recommended by OECD (1996). In the chronic (21-day) toxicity testing, according to the OECD guidelines, the volume of medium provided per female should not be less than 40 mL (for *D. magna*), and it is recommended that the medium be replaced every other day (for a cohort of 10 animals). However, the issue of preference of individual culture over the use of cohorts of daphnids in toxicity testing has been widely discussed (Sims and van Dijk 1996). According to these researchers there is no difference in statistical power between the OECD-recommended procedure and testing using individual culture of daphnids. However, biological information obtained from individual culture is greater than can be obtained from cohort culture, since individual daphnids could not be identified in the cohort. Therefore two types of individual culture were investigated:

- Individual culture of *D. carinata* in 25 mL of medium with daily feeding and daily replacement of medium.
- Individual culture of *D. carinata* in 75 mL of medium with daily feeding and replacement of medium every alternate day.

Conditions and end-points were as in Experiment 2. The aim of these experiments was to check if both of these conditions satisfy the requirements for long-term toxicity testing using *Daphnia*, ie. not more than 20% mortality and not less than 25 offspring per female 14 days of age (OECD 1996).

Statistics

Data derived from the experiments (algal growth rate, body length of females, time to the first brood, number of offspring per female) were analysed in paired comparisons using analysis of variance. The mean value of intrinsic rate of natural increase and its error were determined using a jackknife approach as described by Taberner *et al.* (1993).

RESULTS AND DISCUSSION

Experiment 1. Growth rates of algae in two culture media

The highest growth rate was recorded for *C. pyrenoidosa* cultured in Keating medium - 0.129 h^{-1}. *C. pyrenoidosa* also had a higher growth rate in Tamiya medium than *C. vulgaris* - 0.102 h^{-1} (Table 1).

The higher growth rates obtained in Keating medium are the results of the following effect. *C. pyrenoidosa* stock culture was maintained in Tamiya medium and then taken for the experiment and placed in the two different media. Though the cell size of *C. pyrenoidosa* is reported to be 3-6 µm (Bellinger 1992), we observed cells up to 20 µm in diameter in Tamiya medium. It seemed that cells were not dividing, but accumulating biomass. When placed in Keating medium these cells multiplied by producing up to 8 new small (3-6 µm) cells at a time. This indicates that cell density is not always an accurate measure of growth. We noted that though growth rates of *C. vulgaris* were also higher when cultured in Keating medium than in Tamiya medium, the enlarged cells were not observed.

Experiment 2. Effects of different diets on daphnid survival and reproduction

Survival and fecundity were higher for daphnids fed with *C. pyrenoidosa*, than those fed with *C. vulgaris* or trout pellets (Table 2, Figure 1). In daphnids fed on *C. pyrenoidosa* (cultured in Tamiya medium) total mortality was lower than 20% as recommended by the OECD (1996), while for those fed with the same algae cultured in Keating medium, mortality was only 10%. After 21 days, survival of daphnids fed with *C. vulgaris* cultured in Keating medium was 35% with production of very few offspring. There was 100% mortality in those fed with *C. vulgaris* grown in Tamiya medium by the 13th day of the experiment, and they failed to reproduce. This could be a result of the antibiotic chlorellin, produced by *C. vulgaris* (Pratt and Fong 1940; Pratt *et al.* 1945). This antibiotic is reported to adversely affect the feeding rate of daphnids (Ryther 1954). As we can see from Figure 1, feeding on *C. vulgaris* affects to a greater degree the juvenile stages of daphnids, compare to animals fed with *C. pyrenoidosa*, reducing their survival. The feeding trial with trout pellets produced some offspring, but all daphnids had died by the 18th day of the experiment. This confirms the work of previous researchers who have recommended that daphnids be fed with live algae rather than solely on trout pellets (Cowgill 1989; Sergy 1990), because trout pellets lack essential nutrients.

Tong et al. (1996) suggest that instead of the intrinsic rate of natural increase (r) calculated over a 21-day experiment, one can use 14-day values. They argue that the first three broods mostly contribute to this value. However, in our experiments, 14-day and 21-day values differ in some cases (*C. vulgaris* in Keating medium and trout pellets suspension, Table 2), and this leads to underestimation of r-values in 14-day computations. These errors can lead to crucial mistakes in modelling.

The time to the first brood is supposed to be the most significant contributing factor in r-value, however, it is not the only one determining it. Though the time to the first brood is significantly different for *C. pyrenoidosa* cases (columns 2 and 3 of Table 2), their r-values for both 14 and 21 days are not. Significantly different total number of offspring per female nullified the differences in the time to the first brood. As we showed, the length of the test is also important for r-value. When the length is increased from 14 to 21 days, this changes the r-value markedly in some cases - from negative (meaning that population is eliminated) to positive (indicating some growth) (Table 2, two last columns).

Though the r-value is highest with *C. pyrenoidosa* (Keating)-fed animals, the mean number of offspring produced per female was highest with *C. pyrenoidosa* (Tamiya)-fed animals (69 compared to 42 in the case of those fed *C. pyrenoidosa* (Keating)). It should be noted that the number of offspring was chosen as an endpoint (not the biomass produced by a female), because a certain number of offspring over 14 days is a requirement for long-term toxicity

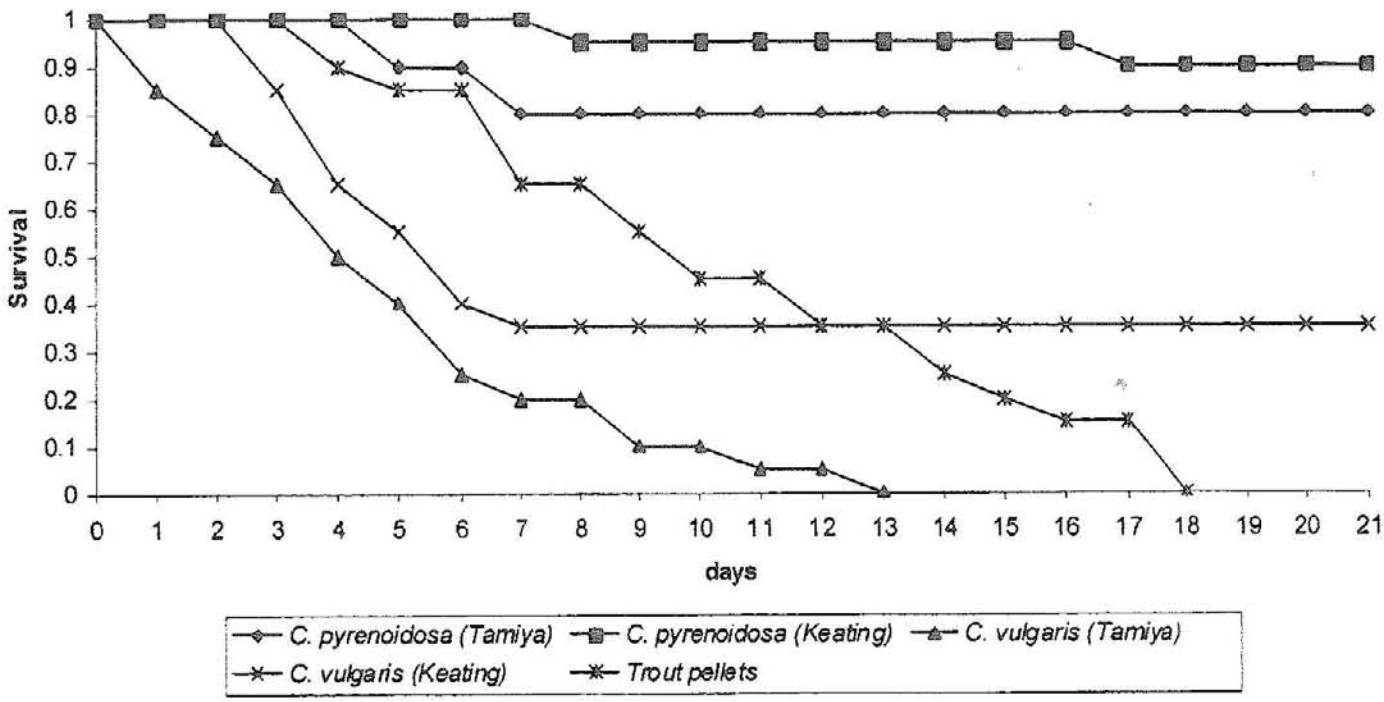

Figure 1. Survival of D. carinata fed with different types of food (experiment 2)

testing (OECD 1996). Based on these results, *C. pyrenoidosa* cultured in Tamiya medium was used as the food for daphnids in later experiments. In addition, the recommended pH of the culture medium for *C. pyrenoidosa* is 5.0, therefore Tamiya medium is the preferred medium for culturing *C. pyrenoidosa* (Myers 1947).

Experiment 3. Effects of culture volume on daphnid survival and reproduction

Standard 21-day observations showed that there were no significant ($P>0.05$) differences between all calculated values (Table 3) for both experimental procedures.

However, in the 75-mL beakers mortality was higher (10%) than in the 25-mL vials (0%). Our observation showed that the antennae of daphnids in the beakers were clogged with algae when they died. The test vessels could not be aerated (daphnids do not tolerate vigorous agitation), and algae settled on the bottom (after 24 h, algal concentrations reduced from $2x10^5$ to $2x10^4$ cells/mL). On the days when the media were not replaced, new algae had to be mixed with those that had settled. This could cause overfeeding (despite attempts to minimise this) and consequent clogging of daphnids' antennae, leading to reduction of the feeding rate.

Though 25 mL per female is less than the volume of medium recommended by OECD (1996), with daily replacement of medium it is possible to achieve virtually the same (if not better) results than with the conditions recommended by the OECD (1996). Moreover, 40 mL is recommended for *D. magna*, which is larger than *D. carinata* and thus would produce more metabolites. It is possible, as demonstrated in this experiment, that the volume of medium used for individual culture be reduced. Also in toxicity tests with rapidly degrading chemicals it is preferable that the medium be replaced every day in order to maintain a constant concentration of the pollutant. In addition small vials are easier to handle, and they require less algae, water and chemicals, making toxicity testing less expensive. There are, however, some negative issues potentially associated with the use of smaller volumes, which should be considered when conducting a toxicity test. Changes in the surface-to-volume ratio of containers can change the rate of adsorption of chemicals on the walls of a container, thus affecting the amount of toxicant in the medium. Replacing the medium every day can help alleviate this problem. Also the absorption rate depends on the chemical, and this should be taken into account while conducting a toxicity test.

CONCLUSIONS

This study has developed some improved conditions for the culture and maintenance of *D. carinata* for use in toxicity testing. It was shown that *C. pyrenoidosa* cultured in either Tamiya or Keating medium can be used successfully as food for *D. carinata*, while trout pellets are not recommended for prolonged use as the only food for *D. carinata*. The volume of culture medium can be reduced from the 40 mL per female recommended by the OECD (1996) to 25 mL per female for *D. carinata* without compromising the quality of daphnid culture (in terms of survival and reproduction), in order to conduct toxicity testing.

This paper also demonstrated that individual culture provides a significant number of different endpoints that cannot be measured in cohorts. We also recommend that when using *D. carinata* in individual culture, the number of animals be reduced from the OECD-recommended 40 (4 cohorts of 10 animals) to 20 or 15.

Table 1. *Comparison of growth rates (μ, h^{-1}) of* C. vulgaris *and* C. pyrenoidosa *cultured in different growth media, Mean±SE, (n=4).*

C. pyrenoidosa (in Keating medium)	*C. pyrenoidosa* (in Tamiya medium)	*C. vulgaris* (in Keating medium)	*C. vulgaris* (in Tamiya medium)
0.129±0.023[a]	0.102±0.027[a]	0.075±0.006[b]	0.068±0.003[b]

Superscripts *a* and *b* indicate significant ($p<0.05$) differences between values.

Table 2. *Comparison of end points of individual culture of* D. carinata *fed with different types of food, Mean±SE (n=20).*

Endpoint	**Food provided**				
	C. pyrenoidosa (in Tamiya medium)	***C. pyrenoidosa*** (in Keating medium)	***C. vulgaris*** (in Tamiya medium)	***C. vulgaris*** (in Keating medium)	**Trout pellets suspension**
Time to the 1st brood (days)	8.1±0.2[a]	6.6±0.2[b]	--	11.7±1.6[c]	11.5±1.1[c]
Total number of offspring per female	69±4[a]	42±4[b]	_	7±1[c]	5±1[c]
Intrinsic rate of natural increase after 21 days (day^{-1})	0.279±0.012[a]	0.287±0.012[a]	NA	0.047±0.025[b]	0.024±0.038[b]
Intrinsic rate of natural increase after 14 days (day^{-1})	0.260±0.013[a]	0.274±0.013[a]	NA	-0.045±0.042[b]	-0.001±0.055[b]

Superscripts a, b and c indicate significant ($p<0.05$) differences between values for each endpoint on any given row.

Table 3. *Comparison of end points for D. carinata cultured in different volumes of medium, Mean±SE, (n=20). There were no significant differences between treatments.*

End points	**Volume of medium**	
	25 mL	**75 mL**
Time to the 1st brood, days	8.2±0.2	7.8±0.3
Total number of offspring per female	60±4	56±3
Body length of females after 21 day, mm	3.72±0.04	3.61±0.07
Intrinsic rate of natural increase (over 21 days), day^{-1}	0.300±0.007	0.298±0.007
Intrinsic rate of natural increase (over 14 days), day^{-1}	0.283±0.009	0.282±0.007

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