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Growth Differences in Cultured Populations of *Brachionus plicatilis* Müller Caused by Heavy Metal Stress as Function of Microalgal Diet

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Bioassays with few rotifer species are commonly used in marine ecotoxicology (Snell & Janssen, 1995). These organisms play a key role in the coastal marine ecosystem (Wallace & Snell, 1991). In addition, rotifers show fast turnover rates, contributing to the nutrient recycling of aquatic habitats. By having a high assimilation efficiency, they convert most of food into biomass, making it available to higher trophic levels (Starkweather, 1987). *B. plicatilis* is a very common rotifer species used in ecotoxicology because of the easy culturing, and the large amount of biological information of this taxon (Pozuelo & Lubián, 1993). There is a wide variety of bioassays developed on these organisms, using population growth rate (Snell & Moffat, 1992), ingestion rate (Ferrando *et al.*, 1993), survivorship (Del Valls *et al.*, 1996), motility (Charoy *et al.*, 1995) or the enzymatic activity (Moffat & Snell, 1994) as endpoints. It is well known that a microalgal diet for rotifers of this species is more stable than others (such a baker's yeast diet) (Hirayama, 1987), but there is a wide list of microalgal species that can be used for feeding rotifers.

In this work differences between growth curves of populations of *B. plicatilis* fed with combinations of three different microalgae (*D. salina*, *N. gaditana* and *I.* aff. *galbana*), have been studied when populations are stressed by sub-lethal concentrations of two heavy metals (Cd or Cu). Toxicity of these heavy metals for rotifers (Snell & Persoone, 1989) and microalgae (Moreno-Garrido, 1997) has been demonstrated. On the other hand, biomagnification processes can be expected, because microalgae seem to be good bioaccumulators for heavy metals (Moreno-Garrido *et al.*, 1998).

MATERIALS AND METHODS

Rotifers were routinely cultured in natural sea water collected at shore in Figueira da Foz -Portugal- during rising tide, filtered by 0.45 µm mesh (Sartorius), and sterilised by autoclaving. For these cultures, Guillard's f/2 medium was used (Guillard & Ryther, 1962). During experiments, a modification of f/2 medium was used, lacking EDTA and trace metals. It is well known that EDTA decreases the toxicity of heavy metals (Moreno-Garrido *et al.*, 1997).

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Artificial sea water was used for both microalgal routine culturing and toxicity experiments. This medium was prepared following the formulation used by Moreno-Garrido (1997). Stocks of artificial sea water were sterilised by autoclaving and stored at 4 °C. Water more than five days old was automatically discarded in order to avoid potential differences caused by aged media (Snell & Persoone, 1989). All cultures and experiments were performed at 20±1°C, under continuous white light.

All marine microalgae cultured at IAV (Instituto do Ambiente e Vida) have been obtained from the Marine Microalgal Culture Collection of ICMAN (Instituto de Ciencias Marinas de Andalucia, CSIC) (Lubián & Yúfera, 1989). Species used were *Nannochloropsis gaditana* (Eustigmatophyceae), clone B-3; *Dunaliella salina* (Chlorophyceae) and *Isochrysis* aff. *galbana* (Chrysophyceae), clone T-Iso. Microalgal cellular densities were calculated by counting in a Neubauer chamber, under optical microscopy. A minimum of three counts were made for each sample. When adjustment of cellular densities were needed, adequate volumes of microalgal cultures were gently centrifuged (1700g, 40 minutes), in order to avoid cellular damages, and pellets were suspended in adequate volumes of fresh medium. Rotifer strain (S1) of *Brachionus plicatilis* was obtained from ICMAN. These organisms were routinely cultured in 100 ml capacity glass flasks, and fed with living cells of the marine microalga *Isochrysis* aff. *galbana*. Subitaneous (asexual) eggs were obtained from adult rotifers by shaking and pipetting. All of these eggs hatch during the 24 hours after collection.

Cadmium and copper were added as mono-hydrated chloride and penta-hydrated sulphide, respectively, both obtained from Merck (reagent grade). All glassware was cleaned before use with nitric acid and rinsed many times with ultra-pure water, before sterilisation by autoclaving. Handling of organisms was performed under a laminar flow chamber. All additional precautions were taken for avoiding external contamination. Levels of dissolved metals were checked using Atomic Absorption Spectrophotometry (flame technique), model Perkin Elmer 3110. Samples were digested with nitric acid, diluting to a half (90 °C, one hour), filtered and then measured. Detection limits of AAS apparatus are 0.032 mg L^{-1} for Cu and 0.016 mg L^{-1} for Cd.

Experiments were performed in sterile plastic plates of 48 wells (Costar Corporation) with 1 mL capacity each well. These plates were used once and discarded. Previous experiments determined that initial concentrations of 0.7×10^6 cells mL^{-1} for the microalga *I.* aff. *galbana* were optimal for culturing rotifers in the experimental conditions. Cellular concentrations of *N. gaditana* and *D. salina* were calculated and adjusted for obtaining the same microalgal biovolume as that of *I.* aff. *galbana*. Combinations of three parts of the final volume for each well were mixed for this three microalgae (D for *D. salina;* B for *N. gaditana* and T for *I.* aff. *galbana*), resulting in ten combinations (DDD; BBB; TTT; DDT; DDB; DTT; DBB; BBT; BTT and DBT). Original microalgal biovolume was constant in all them ($35 \times 10^6 \pm 0,3 \times 10^6 \mu m^3 mL^{-1}$). Cellular biovolume data were obtained from Lubián & Yúfera (1989). Eight replicates were examined for each

one of six series (blank; 1 mg L^{-1} Cd and 0,1 mg L^{-1} Cu; with or without rotifers). Initial metal levels in media were chosen because these concentrations are near the $EC_{50}\%_b72h$ (OECD, 1984) for *N. gaditana* and *I.* aff. *galbana* (Moreno-Garrido, 1997). In three of the six series, one neonate rotifer, hatched from asexual eggs no more than 24 hours before and not fed till that moment, were placed in each well. Other three series, lacking rotifers, were used in order to know competition capacity of each algae when not grazed. Final microalgal concentrations were determined for all wells. Number of rotifers in each well was checked daily during two weeks. ANOVA was applied for distinguishing significant differences between treatments.

RESULTS AND DISCUSSION

Results of experiment for determining the best cellular density of the marine microalga *I.* aff. *galbana* to be fed to cultures of *B. plicatilis* in the experimental conditions are shown in Figure 1. As can be noted, an initial cellular density of $0.7x10^6$ cells mL^{-1} *I.* aff. *galbana* seems to be optimal at our experimental conditions. Population growth (i.e. number of rotifers) in each 1 mL well, after two weeks, is shown in Figure 2. In our experimental conditions *D. salina* is the least suitable food source for rotifers: all cultures containing one, two or three parts of *D. salina* did not allow rotifers to grow. So, these population curves were excluded from Figure 2. Microalgal densities were counted at the end of the experiment in all wells, in order to know which species was dominant in each situation (Table 1). 1 mg L^{-1} Cd (but not 0.1 mg L^{-1}) Cu significantly reduced the final microalgal concentration of non-mixed cultures of *D. salina.* Presence of rotifers in the initial conditions did not imply variations in wells with non-mixed *D. salina.* By contrast, for non-mixed cultures of *N. gaditana,* Cd and, at higher levels, Cu, at the doses used, strongly reduced the final number of cells. Grazing of rotifers on microalgae in toxicant-free wells implied a decrease of about 30% of final cellular density in this series. This decrease is enough for generating near 30 rotifers per well, after two weeks. Rotifers, on the other hand, greatly grazed on *I.* aff. *galbana*. These rotifer populations reach a maximum number of animals, nearly 120 animals per well. As the microalgal population was drastically reduced, a decline in the rotifer growth curve would be expected. Cultures BBT and BTT did not show significant differences when compared with TTT. That means that food is not the limitant growth factor, at the experimental conditions, for cultures that contained *I.* aff. *galbana,* but not *D. salina.* Cultures BBB in blank experiments reached, after two weeks, densities nearly four times lower than cultures with *I.* aff. *galbana*. These series did not allow to growth rotifers when 1 mgL^{-1} Cd or 0.1 mgL^{-1} Cu was added. Rotifers cultured with TTT, with 1 Cd mgL^{-1} or 0.1 mgL^{-1} Cu, reach similar numbers of animals after two weeks (nearly 40 animals per well). Composition and caloric values of these microalgal species have been reported (Table 2, Lubián & Yúfera, 1989). Differences in nutrition must be due to digestibility of the cells or to the fine composition of cells, because there are not significant differences in total energy per well in the different combinations of microalgae (Table 3). There is some data about the good results obtained by feeding *B. plicatilis* with a combination

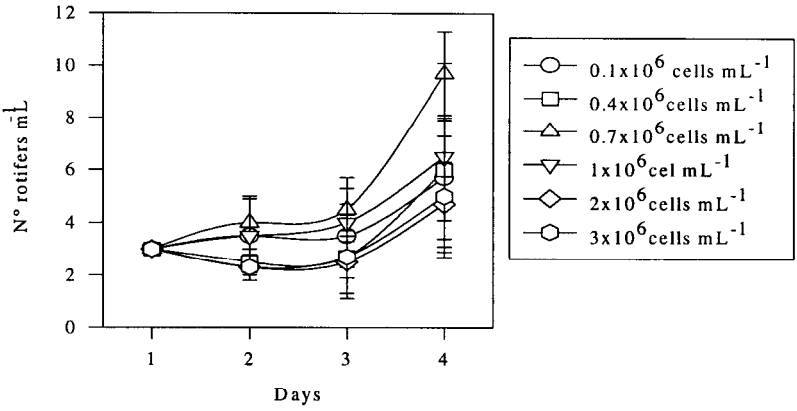

Figure 1. Determination of the best microalgal concentration for feeding *B. plicatilis.* Six initial cellular concentrations of *I.* aff. *galbana* were tested. Error bars mean standard deviation between replicates.

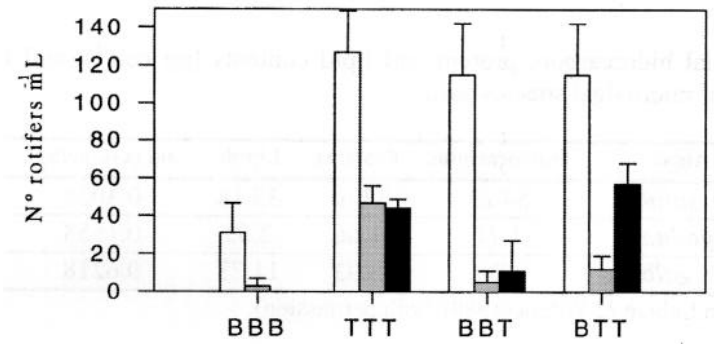

Figure 2. Number of rotifers per well after two weeks of exposition to different metal levels in media. White, control cultures. Light grey, 0.1 mgL^{-1} Cu. Dark grey, 1 mg L^{-1} Cd. Error bars mean standar deviation between replicates.

Table 1. Final microalgal concentrations ($x10^6$cells mL^{-1}) in each well. Standard deviation between replicates is indicated.

Algae	Rot-,Blank	Rot+,Blank	Rot-, Cd	Rot+, Cd	Rot-, Cu	Rot+, Cu
DDD	D 6.3±0.8	D 3.9±0.4	D 4±0.7	D 4.5±0.4	D 6±0.6	D 6±0.6
BBB	B 44±3	B 30±2	B 7.6±0.8	B 5.5±0.9	B 2.6±0.5	B 3±0.6
TTT	T 17±2	T 0.1±0.2	T 2±0.3	T 0.7±0.2	T 6±0.7	T 5.5±0.5
DDT	D 5±0.6	D 3±0.7	D 3±0.4	D 3.6±0.4	D 4±0.5	D 5±0.5
	T 9±0.1	T 10±1	T 1±0.1	T 1±0.4	T 1±0.4	T 3±0.2
DDB	D 5±0.6	D 4±1	D 4±1	D 5±0.5	D 5±0.6	D 6±0.8
	B 7±0.4	B 7±1	B 2±0.5	B 3±0.2	B 8±1.5	B 4±1
DTT	D 3±0.6	D 4.5±0.1	D 6.5±0.5	D 3±0.2	D 4.6±0.2	D 5±0.4
	T 9±1	T 9±0.7	T 1.1±0.3	T 1.3±0.4	T 2±0.2	T 3±0.6
DBB	D 7±0.5	D 4±0.2	D 2.5±0.9	D 4.6±0.2	D 4.7±1.4	D 4.6±0.5
	B 10±0.5	B 16±2	B 3.5±0.2	B 4.1±0.6	B 5±0.7	B 6.7±1
BBT	B 18±1	B 0.1±0.1	B 3±0.4	B 0.8±0.2	B 8±0.5	B 7±0.5
	T 13±3	T 0.4±0.3	T 0.4±0.1	T 0.2±0.2	T 0.7±0.2	T 0.4±0.1
BTT	B 15±0.7	B 0.3±0.3	B 3±0.7	B 0.6±0.1	B 17±3	B 0.3±0.2
	T 15±0.5	T 0.1±0.1	T 1±0.5	T 0.6±0.2	T 9±1	T 0.3±0.2
DBT	D 4±1	D 5±0.7	D 1.6±0.4	D 2±2	D 6±0.9	D 6±0.4
	B 8±0.6	B 10±0.5	B 2±0.7	B 2±0.3	B 4±0.4	B 5±0.6
	T 9±2.6	T 8±1	T 0.4±0.3	T 0.5±0.3	T 2.8±0.5	T 2.6±0.2

(D = *D. salina;* B = *N. gaditana;* T = *I.* aff. *galbana;* Rot+ = wells with rotifers; Rot- = wells without rotifers).

Table 2. Initial hidrocarbon, protein and lipid contents (pg $cell^{-1}$), and total energy (Jul) of microalgal species used .

Algae	Hidrocarbons	Proteins	Lipids	Jul $(x10^6cells)^{-1}$
D. salina	54.23	33.68	35.18	0.3028
N. gaditana	1.77	1.66	2.55	0.1553
I. aff. *galbana*	4.36	3.93	11.73	0.6218

(Obtained from Lubian & Yúfera (1989), with permission).

Table 3. Initial energy per well (Jul), as function of microalgal composition.

Algae	DDD	BBB	TTT	DDT	DDB	DTT	DBB	BBT	BTT	DBT
J $well^{-1}$	0.454	0.680	0.435	0.446	0.530	0.443	0.604	0.596	0.519	0.378

(D = *D. salina;* B = *N. gaditana;* T = *I.* aff. *galbana*).

of *I.* aff. *galbana* and *N. gaditana*, due to the high values of highly unsaturated fatty acids (HUFA) used to feed *Sparus aurata* (Teleostea) larvae (Mourente & Odriozola, 1989). Segner and Braunbeck (1990) found that feeding with living food (Artemia) copper toxicity was lower to milkfish when compared with animals fed with artificial food. This reduction of toxicity could be due to a better nutrition status of fishes when fed with living food or to interactions of Artemia with copper that would imply detoxification processes. About this possibility, Barry *et al.* (1995) and Van Dam *et al.* (1995) exposed *Daphnia carinata* to different toxicants, in conditions of high food and low food (different cellular densities of green microalgae in media). Smaller body length as well as an increase of toxic effects in population were reported when animals were fed with low-food conditions. Levels of piretroid pesticide esfenvalerate decreases in media when microalgal density increases, due to accumulation processes, but toxicant must be inactivated in some way when accumulated by microalgae. Metabolism of microalgae are susceptible of modifying metal species too (turning them to inactive forms) or even producing chelating substances (Maeda & Sakaguchi, 1990). On the other hand, Absil *et al.* (1996) reported an increase of toxicity for the sediment-dwelling bivalve *Macoma baltica* when fed with copper-enriched microalgae. One solution to these problems could be given by Janssen *et al.* (1994). These authors described a 4-days toxicity test with rotifers involving a diary change of media. In these experiments, would be interesting to measure final concentration of toxicants each day, in order to determinate if bioaccumulation processes occur.

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