

Feeding strategies of the copepod *Acartia clausi* on single and mixed diets of toxic and non-toxic strains of the dinoflagellate *Alexandrium minutum*

A. Barreiro^{1,*}, C. Guisande¹, M. Frangópulos², A. González-Fernández¹, S. Muñoz¹,
D. Pérez¹, S. Magadán¹, I. Maneiro¹, I. Riveiro¹, P. Iglesias¹

¹Facultad de Ciencias, Universidad de Vigo, Lagoas-Marcosende, 36310 Vigo, Spain

²Centro de Estudios del Cuaternario Fuego-Patagonia (Fundación CEQUA), Avenida Bulnes 01890,
6 200 000 Punta Arenas, Chile

ABSTRACT: The role of mesozooplankton grazers in the development of monospecific algal blooms has often been examined in a scenario in which grazers, depending on their abilities of recognition, select against toxic species and increase grazing pressure on non-toxic species. Here, we present a different ecological scenario in which grazers may select between different strains (toxic and non-toxic) of the same species, which may coexist in similar densities in natural environments prior to bloom initiation. The calanoid copepod *Acartia clausi* was fed with single and mixed diets of 2 strains of the dinoflagellate *Alexandrium minutum*, a producer of paralytic shellfish poisoning (PSP) toxins. One strain produced high, and the other low, quantities of PSP toxins. We examined feeding strategies and estimated copepod responses based on their food selection abilities, toxic effects on maintenance physiology and fitness, and benefits produced by a toxin-dilution strategy in a mixed diet. Copepods were found to feed selectively on *A. minutum* strains. Diet composition had a strong effect on parameters such as food ingestion, mortality, egg hatching, and egg production. The effect on copepod mortality and egg production was greatly reduced when a mixed diet (toxic + non-toxic) was provided to the copepods. However, the negative effects on egg hatching were dose-dependent, and this parameter was not recovered by toxin-dilution mechanisms. We conclude that copepods did not effectively reject the toxic strain and that the effect of *A. minutum* on mortality and egg production, but not on egg hatching, is reduced by dilution mechanisms. Therefore, we suggest that feeding pressure by grazers does not appear to be an important mechanism that favors toxic over non-toxic strains prior to bloom initiation.

KEY WORDS: Zooplankton · Phytoplankton · Toxins · Food selection · Toxin dilution · Fitness

Resale or republication not permitted without written consent of the publisher

INTRODUCTION

The role of zooplankton grazers in the development of monospecific toxic algal blooms has been mainly studied by examining the ability of grazers to select between toxic and non-toxic phytoplankton species, as well as the effect of toxins on the physiology of grazers. Zooplankton organisms are constrained by morphological features (body size and feeding appendages) to be either selective or generalist feeders (DeMott 1986, 1988, Gilbert 1990, Kirk & Gilbert 1992). The most

selective-feeding organisms are able to recognize and avoid the consumption of toxic phytoplankton species (Huntley et al. 1986, DeMott 1989, Gilbert 1990, DeMott & Moxter 1991, Kirk & Gilbert 1992, Teegarden 1999, Guisande et al. 2002a), which would otherwise negatively affect their survival and/or reproduction (Ives 1987, Lampert 1987, Sykes & Huntley 1987, Gliwicz & Lampert 1990, Bagoien et al. 1996, Dutz 1998, Frangópulos et al. 2000, Guisande et al. 2002a). It has been suggested that behavioral avoidance of toxic phytoplankton by selective grazers has been devel-

*Email: aldo@uvigo.es

oped by associative learning processes (Uye & Takamatsu 1990). That is, selective grazers would distinguish between markedly morphological and/or chemical features of phytoplankton species, and would then re-direct their grazing pressure towards non-toxic species (Guisande et al. 2002a). This may represent a competitive advantage for toxic phytoplankton species and a hypothetical mechanism that favors the onset of monospecific toxic algal blooms (Gilbert 1990, Guisande et al. 2002a).

The latter reasoning assumes that, before the bloom begins, the phytoplankton community consists of a mixture of toxic and non-toxic phytoplankton species, and that the population of each toxic species is composed of individuals with homogeneous toxic properties.

In this study, we suggest a more complex pre-bloom ecological scenario: toxic and non-toxic strains of the same species coexist within a population. This is more likely to occur in natural environments. Indeed, it has already been proposed for some cyanobacteria (Kirk & Gilbert 1992), has been documented for the genus *Nodularia* in the Baltic Sea (Laamanen et al. 2001), and may also be a consequence of ballast waters, which presents a global environmental problem to many species (Hallegraeff & Bolch 1992). In such a scenario, prior to the onset of a bloom, it would not be so easy for selective grazers to distinguish between toxic and non-toxic phytoplankton cells.

An attempt was made in this study to examine some aspects of the relationship between selective grazers and endotoxic phytoplankton in a simplified version of the scenario proposed above. To explore this relationship it was considered necessary to integrate different aspects involved in the feeding ecology of grazers. These were: optimal diet theory predictions about food selection; the toxin-dilution hypothesis as a feeding strategy to balance diets when toxic species are included; and the consequences of the feeding behavior on the fitness of grazers.

The calanoid copepod *Acartia clausi* is the most abundant mesozooplankton organism in the Galician Rias (NW coast of Spain). In its natural environment, toxic individuals of the dinoflagellate *Alexandrium minutum* are present, and they occasionally form small monospecific blooms (unpubl. data from the monitoring management program of the Galician Government). The genus *Acartia* and other calanoid copepods have been recorded as being relatively highly selective grazers (Wilson 1973, DeMott 1988, Kirk & Gilbert 1992, Guisande et al. 2002a).

The aims of this study were (1) to test whether *Acartia clausi* is able to distinguish between 2 strains of *Alexandrium minutum* with different toxicities. One strain was isolated from the same environment as the

copepods and produces high levels of paralytic shellfish poisoning (PSP) toxins, whereas the other strain was isolated from Mallorca (Mediterranean Sea) and was low in toxicity; (2) to analyze the consequences of the resulting copepod feeding strategies by estimating food ingestion, copepod mortality, egg production and egg hatching, in order to distinguish the toxic effects on maintenance metabolism from those on reproductive fitness, and; (3) to discuss the role of feeding strategies of grazers within the proposed phytoplankton community scenario in the framework of evolutionary relationships between grazers and microalgae.

MATERIALS AND METHODS

Zooplankton collection. Zooplankton was collected by vertically integrated tows from a depth of 20 m to the surface, at a field station of 39 m depth located in the Galician Ría de Vigo, Spain (42° 13.3' N, 8° 47.7' W). Samples were then transported to the laboratory and maintained for several days with air bubbling in a 25 l container and, prior to the beginning of the experiments, were fed with the green algae *Tetraselmis suecica* and the dinoflagellate *Heterocapsa triquetra*.

Strains of *Alexandrium minutum*. The toxic strain of *Alexandrium minutum* (AT) was isolated from the Galician Rias (northwestern Spain, Atlantic Ocean) and came from a long-established population cultured in the Instituto Español de Oceanografía (Vigo, Spain). The non-toxic strain of *A. minutum* (ANT) used in this study was isolated in Mallorca (eastern Spain, Mediterranean Sea) and also came from a long-established population cultured in the Instituto Español de Oceanografía (Mallorca, Spain). Both strains of *A. minutum* contain only gonyautoxins 1, 2, 3 and 4 (Franco et al. 1994). Cultures were maintained in K medium (ccmp.bigelow.org), but the AT strain was cultured without the addition of phosphate to the medium so as to achieve higher toxin production. *A. minutum* cultures grew with a maximum rate of approximately 0.25 d⁻¹ (ANT) and 0.15 d⁻¹ (AT). For a period of 2 mo, up to the beginning of the experiment, about 25% of the medium was renewed once a week, producing semi-continuous conditions.

Experimental design. There were 3 different experimental food treatments, with 160 replicates each: only AT, only ANT, and 50% mixed treatments with both AT and ANT. Each replicate consisted of 1 adult female *Acartia clausi* in a 25 ml beaker containing the experimental food suspensions. *Alexandrium minutum* cells stayed in suspension during feeding experiments because of their swimming behavior. For all experimental food concentrations, the abundance of *A. minutum* was approximately 500 cells ml⁻¹. The culture

medium was prepared with aged natural seawater (salinity of 33.6‰) filtered through GF/F Whatman filters. The experiment was run at 18°C under a 12 h:12 h light:dark cycle. The copepods were transferred to new food suspensions every day. Replicates were not taken into account if the copepods died. Cell size, cell carbon and nitrogen content, toxin content per cell, ingestion rates, egg production, egg hatching, and copepod mortality were measured on a daily basis. Toxin content per copepod was estimated every 2 d. The experiment was performed for 6 d.

Cell size, volume and carbon content. Cell size was measured daily in a flow cytometer (FACSCalibur, Beckton Dickinson), and scatter light-detector measurements were calibrated with a Size Calibration Standards Kit (Bangs Laboratories). Measurements were undertaken in triplicates with samples of 1000 to 3000 cells each. Biovolume was calculated by the most likely geometrical shape method (Edler 1979, Hillebrand 1999), with *Alexandrium minutum* cells considered to be spherical. Cell carbon content was measured daily for each strain in triplicate; algal cells were collected on pre-combusted 13 mm GF/F Whatman filters and analyzed in a Fisons EA 1108 CHN analyzer.

Toxin analysis. To estimate the cell toxin content of *Alexandrium minutum* on a daily basis, 3 samples of algal cells were collected on pre-combusted 13 mm GF/F Whatman filters, stored at -30°C in ultracentrifuge plastic tubes and lyophilized. A total of 500 µl of 0.05 M acetic acid was added to the lyophilized material and the sample was homogenized using a pipette tip adapted to fit the shape of the vial. The sample was shaken and frozen twice. Finally, the extract was centrifuged twice at 4000 rpm for 10 min, after which 200 µl of the supernatant were carefully collected with a Hamilton syringe and stored at -30°C.

To analyze the evolution of the content of toxin in the copepod throughout the experiment, around 10 to 15 copepods were transferred from each experimental food treatment to filtered seawater every 2 d. To assure that the recently ingested toxins were excreted, copepods were transferred to distilled water after 2 to 3 h and immediately collected in 40 µl of distilled water. This experimental design allowed measurement of only those toxins accumulated by the copepods. Samples of copepods were stored at -30°C in ultracentrifuge plastic tubes and lyophilized. Acetic acid (125 µl, 0.05 M) was added to the lyophilized material, and then the steps described above were followed.

Analysis of the PSP-related toxins by high-performance liquid chromatography (HPLC) with fluorescence detection was performed following a modification of the method of Oshima et al. (1989) described by Franco & Fernández (1993). Chromatographic profiles of *Alexandrium minutum* cells were determined by

quadruplicate injections of 30 µl of extracts (diluted with 0.05 M acetic acid, as necessary). Toxins from the National Research Council of Canada (Halifax) were used as toxin standards.

Ingestion rates. Daily ingestion rates of *Acartia clausi* were estimated for each experimental food treatment by counting cell concentration in 20 replicates from a pooled sample of all grazers, as well as in 9 control replicates and 3 initial replicates. The grazing period was 24 h. Samples were preserved with 4% formaldehyde and counted in a Sedgewick-Rafter chamber under an inverted microscope. In the mixed-strain food treatment, the percentage of abundance of each strain was estimated using a flow cytometer by means of a fluorescence labeling technique using a monoclonal antibody. Frost's (1972) equations were used to calculate ingestion rates. Preference for each strain in the mixed treatment was calculated with Manly's α index (Manly 1974):

$$\alpha_i = \frac{\ln \frac{e_i}{n_i}}{\sum_{j=1}^m \ln \frac{e_j}{n_j}}$$

where e is the number of prey at the end of the experiment, n is the number of prey at the beginning of the experiment and i, j are different preys. The value of α changes in relation to changes in the abundance of resources.

Antibody staining. The monoclonal antibody used in this work, the 90.3, was generated from a mouse immunized with the AT strain (M. Carrera unpubl. data), following the method described by Köhler & Milstein (1975) and further modified by Galfre & Milstein (1981). The algal staining was performed by indirect immunofluorescence: algal cells were incubated with concentrated supernatant containing the monoclonal antibody 90.3 for 1 h 45 min at room temperature and washed twice in PBS-BSA 1%. Cells were then incubated with secondary antibodies (Biotin-goat antibodies anti-mouse IgG and IgM, diluted to a ratio of 1:200 in PBS-BSA 1% [Caltag Laboratories]) for 45 min at room temperature. Subsequently, they were washed twice and re-suspended in 100 µl of Streptavidin-Phycoerythrin (PE) (ratio 1:20) (DakoCytomation) and incubated for 15 min in obscurity, at room temperature. Cells were analyzed in a flow cytometer (FACSCalibur, Beckton Dickinson). Fig. 1 shows the differential staining between AT and ANT strains by the monoclonal antibody 90.3. This antibody binds to the AT strain at higher intensity than to the ANT strain, allowing discrimination between the 2 strains.

In addition to antibody recognition, other parameters analyzed such as cell size, cell complexity and auto-

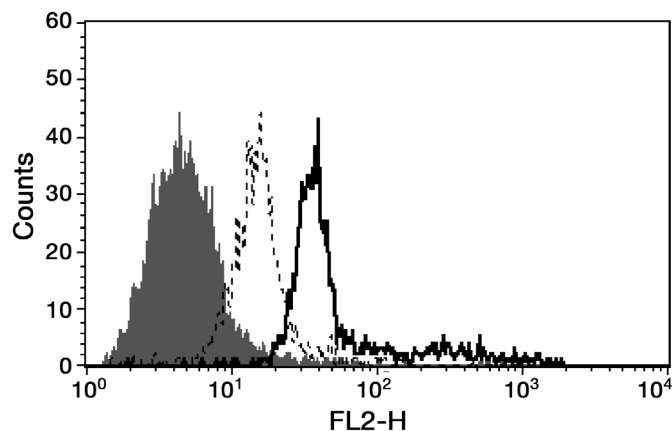


Fig. 1. *Alexandrium minutum*. Flow cytometric analysis of toxic and non-toxic strains incubated with and without monoclonal antibody. Shaded area: toxic strain of *A. minutum* without antibody; dashed line: non-toxic strain of *A. minutum* with antibody; solid line: toxic strain of *Alexandrium minutum* with antibody; x-axis: channel units of FL2 detector (585/42 nm fluorescence)

fluorescence measured at 530/30 and 670 nm were also useful in the discrimination between the 2 strains. All these parameters, measured by cytometer detectors, were introduced as independent variables in a discriminant analysis to achieve the best possible identification of strains. In each sample, between 1000 and 3000 cells were analyzed. For each daily ingestion experiment, we measured 7 replicates from beakers with copepods, 2 initial replicates and 3 control replicates. In order to have sufficient algal cells for the analysis, 2 replicates were placed together in a 50 ml centrifuge tube. They were then centrifuged and preserved with 1 ml of 4% formaldehyde for 2 d, and subsequently indirect immunofluorescence was performed (see above).

Strain identification. Discriminant analysis allowed the correct identification of most of the individuals for each strain in the mixed-strain food suspension (Table 1). The significance of the discriminant function was always <0.001 . Small errors in classifying individuals (AT or ANT) were randomly distributed between the 2 strains. For all analyses performed, the total correlations of each variable (cytometer detector measurements) with the discriminant function used in the analysis were in the following order of importance: 670+ nm fluorescence $>$ 585/42 nm fluorescence (antibody labeling) $>$ side scatter light (cell size) $>$ 530/30 nm fluorescence $>$ forward scatter size (cell internal complexity).

Table 1. *Alexandrium minutum*. Percentage of individuals of both strains correctly classified by cross validation in discriminant analysis

Day	Initial	Control	Grazers
1	88	90	90
2	90	86	86
3	83	89	89
4	89	88	88
5	93	91	91
6	94	97	97

Egg production and hatching success. For each experimental food concentration, the eggs produced by 15 copepods were gathered on a daily basis. Triplicates were maintained for 2 d at 18°C and then fixed with 4% formaldehyde. Subsequently, nauplii and unhatched eggs were counted.

RESULTS

Cell size and toxicity

Cell size was reasonably constant in both AT and ANT strains throughout the entire experimental period (Table 2). The AT strain was always larger than the ANT strain and there were approximately 2-fold differences in biovolume between the 2 strains (Table 2). However, the relationship between cell biovolume and carbon content was not straightforward (Table 2). As it was limited by phosphorous (see 'Materials and methods'), the AT strain had lower growth rates than ANT; this produced an increase in cell size in relation to the ANT strain which grew without limitations. AT toxicity was enhanced to a considerable extent by phospho-

Table 2. *Alexandrium minutum*. Cell parameters measured during experiment. Toxicity is total gonyautoxin (GTX) per cell (fg STXeq: saxitoxin equivalents). AT: toxic strain; ANT: non-toxic strain

Day	Strain	Size (μm)	Volume ($\mu\text{m}^3 \cdot 10^{-3}$)	Carbon (pg cell^{-1})	Toxicity (fg STXeq)	Toxicity (fmol)
1	AT	25.4	8.6		2451	5.6
	ANT	20.0	4.2		53	0.1
2	AT	21.3	5.1		3416	7.7
	ANT	17.7	2.9		51	0.1
3	AT	21.9	5.6		3664	8.4
	ANT	18	3.1		55	0.1
4	AT	25	8.2		4590	10.5
	ANT	18.3	3.2		61	0.1
5	AT	26.4	9.6		4219	9.5
	ANT	17.7	2.9		59	0.3
6	AT	26.6	9.9		4155	9.5
	ANT	17.2	2.7		60	0.1
Mean \pm SD	AT	24.5 \pm 2.3	7.8 \pm 2	429 \pm 38	3749 \pm 761	8.5 \pm 1.7
	ANT	18.2 \pm 0.9	3.2 \pm 0.5	517 \pm 100	57 \pm 4	0.1 \pm 0.1

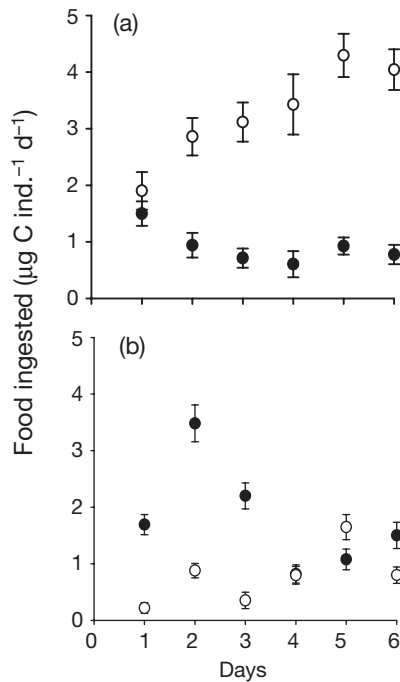


Fig. 2. *Acartia clausi*. Food ingested (mean \pm SD, $n = 20$) in food treatments with (a) only one of each strain or (b) with mixed toxic (AT) and non-toxic (ANT) strains of *Alexandrium minutum*. ●: AT strain; ○: ANT strain

rous limitation. The same strain cultured under nutrient replete conditions contained between 1.1 and 2.5 fmol toxin cell⁻¹ (Frangopulos et al. 2000, Guisande et al. 2002a,b), which was considered to be at the low end of the toxicity range for *Alexandrium minutum* (Chang et al. 1997). PSP content in other *Alexandrium* species reaches values of 250 fmol cell⁻¹ (*A. tamarense*, Frangopulos et al. 2004).

The ANT toxin content may have had no toxic effects or only weak toxic effects on our population of *Acartia clausi*, which had adapted to the presence of *Alexandrium minutum* in the environment. In an adapted population of *Acartia tonsa*, Colin & Dam (2002) did not find any effect at concentrations of 1.44 pg toxins cell⁻¹ (approximately 3.5 fmol cell⁻¹), at approximately the same cell concentration as in our experiment. In contrast, Dutz (1998), Frangopulos et al. (2000) and Guisande et al. (2002a,b) observed negative effects on *A. clausi* reproduction when cell toxicities were 1.1 to 2.5 fmol cell⁻¹.

Ingestion experiments

Mean cell densities throughout the experiment were 404 ± 72 cells ml⁻¹ in treatments with only ANT; 560 ± 84 cells ml⁻¹ in treatment with only AT; and 927 ± 226

total cells ml⁻¹ in the mixed-strain treatment with 538 ± 66 and 405 ± 89 cells ml⁻¹ for AT and ANT, respectively. Within this range of cell concentrations, copepods are not expected to be limited by food (Huntley et al. 1986, DeMott 1988). Fig. 2 shows the total ingestion rates for each experimental condition during the 6 d of the experiment. Feeding pressure was higher in the food suspension with ANT than in the food suspension with AT (Fig. 2a). For each experimental condition, daily grazing rates increased for ANT and decreased for AT. The amount of food ingested in relation to the food available was higher in the single cultures with ANT and lower in cultures with AT, while medium values were recorded in the treatment with a mixture of both strains (Fig. 3).

This reduction in grazing rates in the AT treatment may be due to physiological incapacitation or rejection due to 'bad taste'. Copepods in AT treatments looked unhealthy and exhibited erratic swimming behavior; in addition, as will be shown later, copepod mortality was higher in AT, which indicated that while rejection of toxic cells due to 'bad taste' could not be eliminated as a possibility, there was clear physiological incapacitation due to ingestion of toxins.

The maximum ingestion rates reported here (ANT treatment) are similar to those of Frangopulos et al. (2000) and Dutz (1998) for the same carbon concentration, with low toxicity strains of *Alexandrium minutum* and *A. lusitanicum*, respectively. Both authors stated that these values were lower than expected. Therefore, the ANT strain may also exhibit reduced ingestion rates to some extent, due either to 'bad taste' or cell toxicity.

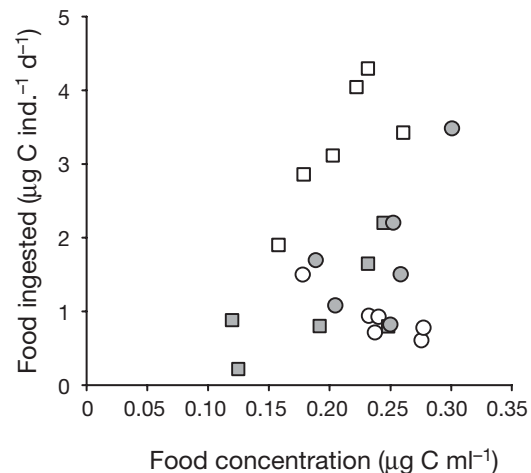


Fig. 3. *Acartia clausi*. Food ingested (mean, $n = 20$) as a function of *Alexandrium minutum* abundance. □: ANT diet; ○: AT diet; ■: ANT mixed diet; ●: AT mixed diet. SD of mean food ingested not shown, all lower than 0.6

Table 3. *Acartia clausi*. Food selection; Manly's α coefficients for strain selection in the mixed-strain treatment. AT: treatment with only AT; ANT: treatment with only ANT

Day	α AT	α ANT
1	0.92	0.08
2	0.65	0.35
3	0.92	0.08
4	0.54	0.46
5	0.36	0.64
6	0.59	0.41

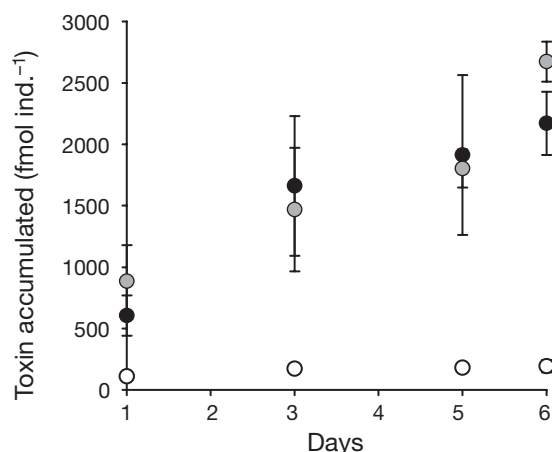


Fig. 4. *Acartia clausi*. Toxin accumulated (mean \pm SD, $n = 2$) for each treatment during experiment. ●: AT strain only; ○: ANT strain only; ○: mixture of both strains

Fig. 2b shows the results for the treatment with a mixture of both strains. There was an initial pattern (Days 1 to 3) that demonstrated preference for the AT strain. This could be expected if food was not limited and both strains were of the same nutritional quality, but AT was of a larger size. However, from Days 4 to 6, preference for the AT strain decreased to almost the same value as that for ANT (Day 4), and was lower than that for ANT on Day 5; however, there was again a slight preference for AT on Day 6. The α calculations for Manly's index of preference (Table 3) agreed with these observations, with α values from Days 4 to 6 close to a non-selective pattern.

Copepod toxin accumulation

Differences in toxin accumulation between experimental conditions are explained by the presence or absence of the AT strain (Fig. 4), and by total cells

ingested. There were no differences in toxins accumulated by the copepods between AT and the mixed culture (Table 4), which corroborated that the number of cells of the toxic strain ingested by copepods was similar for both the single culture and the mixed cultures that contained the toxic strain.

Other studies on toxin accumulation by *Acartia clausi* fed on *Alexandrium minutum* (toxicity 1.1 to 2.5 fmol cell⁻¹) have reported values of 0 to 500 pg toxin copepod⁻¹ after 1 d of feeding, and 1500 pg toxin copepod⁻¹ after 6 d of feeding on mixed diets with non-toxic species (Frangopulos et al. 2000, Guisande et al. 2002a,b); negative effects were also observed for ingestion rates, egg production and egg hatching, but not for mortality.

Copepod mortality

Mortality was assumed to be representative of treatment effects after Day 1, so the data for this day were omitted from Fig. 5. As expected, toxic AT cells caused a higher rate of mortality than the ANT cells, as is shown in treatments with only 1 of the 2 strains (Fig. 5). In the mixed-strain food suspension, the pattern of daily mortality was intermediate to that of the other 2, and there was a small delay (Day 2) before the mortality in the mixed-strain food suspension exceeded that of ANT (Fig. 5a). However, in this mixed treatment, the accumulated toxin in the copepods was the same as for the AT strain on Days 1 and 3 (Fig. 4). This suggested a mechanism of toxin dilution. The significant decline in mortality in the AT treatment during the last 2 d of the experiment could be due to a reduction in food ingestion (Fig. 2a). The 3 patterns of mortality can be well summarized by accumulated mortality (Fig. 5b). Although copepods accumulated the same amounts of

Table 4. *Acartia clausi*. ANCOVA results using time as a covariate. AT: treatment with only AT; ANT: treatment with only ANT; Mx: treatment with a mixture of both strains; df: degrees of freedom

Dependent variable: treatments compared	df			F	p
	Intersection	Error	Total		
Accumulated toxin					
AT-ANT	1	13	16	35.3	<0.001
AT-Mx	1	13	16	0.23	0.63
ANT-Mx	1	13	16	46.7	<0.001
Egg production					
AT-ANT	1	21	24	298.9	<0.001
AT-Mx	1	21	24	168.6	<0.001
ANT-Mx	1	21	24	0.03	0.86
Egg hatching					
AT-ANT	1	21	24	7.01	0.015
AT-Mx	1	21	24	0.04	0.83
ANT-Mx	1	21	24	17.5	<0.001

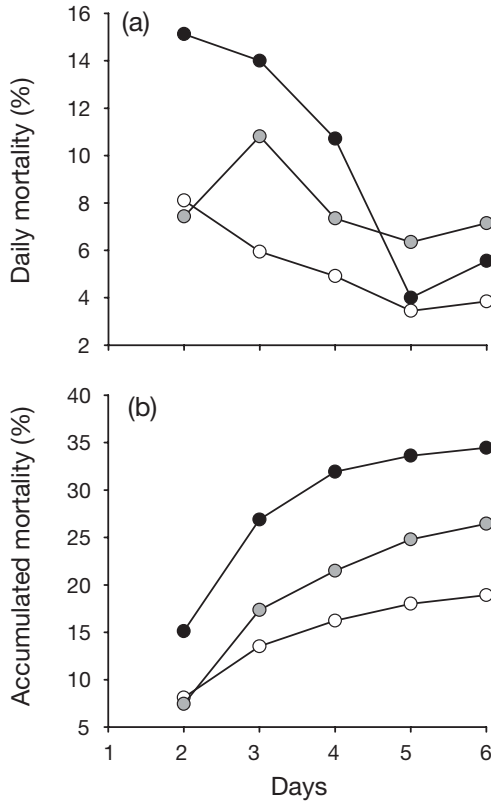


Fig. 5. *Acartia clausi*. (a) Daily and (b) accumulated mortality for each treatment during experimental period. Symbols as in Fig. 4

toxin in the mixed-strain treatment as in the single AT food suspension (Fig. 4, Table 4), copepods in the mixed-strain treatment showed an intermediate pattern of accumulated mortality and a delay prior to an increase in daily mortality. This clearly demonstrates a mechanism of toxin dilution.

Egg production and hatching success

Data from Day 1 were excluded from Fig. 6 and were not statistically analyzed, as it was assumed that they were not dependent on treatment effects but on recent nutritional life history in the field. Egg production is affected by total biomass ingested (Kiørboe et al. 1985). However, due to either the toxic effects of the AT strain or to reduced ingestion rates it was almost suppressed in the single diet with AT (Fig. 6). A toxic effect is more likely to explain the results we obtained when both strains were present, because egg production increased while total biomass ingestion was kept constant and AT ingestion decreased (Fig. 2). Although toxins accumulated in copepods were the same in the AT and mixed-strain diets, egg production values in the mixed-strain diet were the same as for the

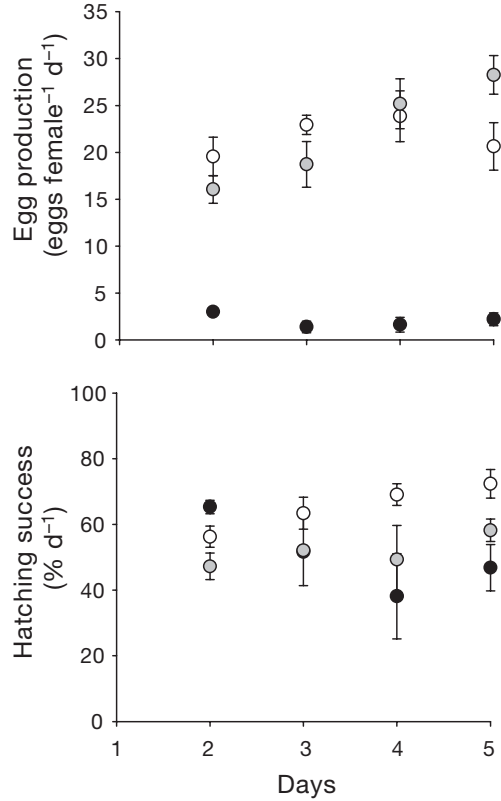


Fig. 6. *Acartia clausi*. Egg production and egg-hatching success during experimental period (mean ± SD, n = 3). Symbols as in Fig. 4

ANT diet (Fig. 6, Table 4). If reduced egg production was due to toxicity, this may be evidence of a toxin-dilution effect.

ANCOVA (where food ingested was a covariate [log transformed]) showed that there were significant differences in egg production (log transformed) among treatments ($F_{2,8} = 14.5, p = 0.002$) (Fig. 7). These results

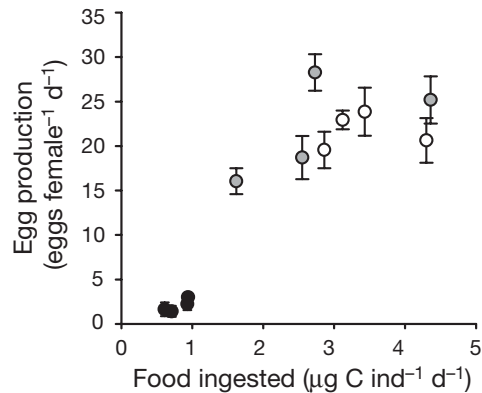


Fig. 7. *Acartia clausi*. Relationship between *Alexandrium minutum* ingested and egg production (mean ± SD, n = 3). Symbols as in Fig. 4. SD of mean food ingested not shown, all lower than 0.6

confirm that, in addition to reduced egg production due to reduced ingestion of food, there was an effect of toxins on egg production. However, when the AT treatment was excluded from the ANCOVA, there were not significant differences between ANT and the mixed diet ($F_{1,5} = 0.52$, $p = 0.502$), which supported the hypothesis of a dilution mechanism.

Highest egg production values (20 to 25 eggs female⁻¹ d⁻¹, ANT treatment) were lower than those reported by Kiørboe et al. (1985) for optimal conditions in *Acartia tonsa* (about 45 eggs female⁻¹ d⁻¹). This difference does not affect our conclusions and may be accounted for by the fact that (1) ingestion rates of the ANT strain in the present study were lower than optimal values and (2) ingestion rates were negatively affected by the toxins ingested.

Egg hatching was also affected in the AT treatment (Fig. 6) and did not present a toxin-dilution effect in percentage terms (Fig. 6), with no statistical differences between the AT and mixed-strain treatments (Table 4). Hence, regardless of any other food source consumed, egg-hatching success is related to toxins accumulated by copepods.

DISCUSSION

Experiments with zooplankton to test optimal diet theory predictions have shown abilities to optimize diet composition by selecting among different food categories (DeMott 1986, 1988, 1989, 1995). Prey size seems to be the main criterion when the foods offered have the same nutritional quality (Wilson 1973, Vanderploeg et al. 1984, Vanderploeg & Paffenhoffer 1985, Bogdan & Gilbert 1987, DeMott 1995). It has been demonstrated that differences in food quality can be detected between algal-flavored and unflavored microspheres, live algae and microspheres, live and dead algae, toxic and non-toxic algae (DeMott 1989), and even between algal cells in different growth phases (Cowles et al. 1988). These selective abilities were shown to be taxon-specific among zooplankton groups. Therefore, with predictions made by the optimal diet theory, selective behavior is dependent on total food concentration, which means that food selection appears to be stronger when the level of food is not restricted by limiting conditions (DeMott 1989, 1995).

The food selection behavior displayed (Fig. 2b, Table 3) showed an initial pattern of positive selection for the AT strain (Days 1 to 3). We hypothesize that this may be due to a size-selective behavior. Size-selective behavior is performed by scanning the particle size distribution in food suspensions, and is developed within the first 15 to 30 min of exposure to a particular experimental food treatment (Wilson 1973). The genus

Acartia and other copepods are known to be proficient at size selection among particles with equivalent nutritional quality (Wilson 1973, Vanderploeg et al. 1984, Vanderploeg & Paffenhoffer 1985, Bogdan & Gilbert 1987, DeMott 1995).

This initial size-selective pattern seemed to be disrupted from Day 4 to Day 6, when α values were close to the non-selective value of 0.5 (Table 2). Subsequently, grazing pressure was weakly redirected towards the non-toxic strain. We hypothesized that copepods were not able to effectively reject the toxic AT strain when mixed with the ANT strain because of difficulties in recognizing it. Fig. 2b shows that after Day 3, the decline in grazing pressure towards the AT strain was stronger than the parallel increase towards ANT. This led to α values close to a non-selective pattern (Table 3). In other words, copepods were no longer selecting cell size from Day 4 to 6, but this behavior was not adequate for an efficient rejection of the toxic strain. However, we should not reject the idea that the relatively low values of toxicity of our strain (see 'Results') may have influenced food selection, and it is possible that more toxic strains would have enhanced selective behavior of copepods against toxic cells.

The effects of PSP toxins on maintenance metabolism and physiology include reductions in swimming performance and survival rates and the inhibition of food ingestion (Ives 1987, Sykes & Huntley 1987, Bagoien et al. 1996, Colin & Dam 2002). All of these effects were observed in the present study (Figs. 2a, 3 & 5). It has been suggested that reduction in the rates of egg production is an indirect effect of reduced food intake (Colin & Dam 2002). This hypothesis can not be rejected in our study, but our results confirmed that there is a negative effect on egg production due to toxin ingestion by copepods (Fig. 7).

We considered egg-hatching success to be our best estimate of reproductive physiology and individual fitness. For this parameter, there was also a negative effect related to presence of the AT strain (Fig. 6), which has been previously described in the literature (Dutz 1998, Frangópulos et al. 2000, Guisande et al. 2002a,b). The pattern of hatching inhibition only appeared clearly after Day 3. This was probably due to a slow passage of the toxins and to accumulation in the gonads from the digestive system, affecting embryogenesis. Only 0.98% of the daily PSP toxins assimilated are allocated to the eggs (Guisande et al. 2002b).

The physiological effect of mixed diets which include non-toxic species has rarely been thoroughly examined (Turner et al. 2001, Colin & Dam 2002). In our experiments, for most of the parameters examined, a clear toxin-dilution effect was observed for the mixed-strain treatment for copepod mortality and egg production. In this treatment, with similar or even

higher amounts of the AT strain ingested and about the same level of toxin accumulated as in the AT treatment (Fig. 4), neither food ingestion nor egg production seemed to be affected by PSP toxins (Figs. 2b, 6 & 7). An additional toxin-dilution effect was demonstrated by mortality incurred from the mixed-strain treatment: higher mortality values were delayed until Day 3, and accumulated mortality was intermediate between those of the ANT and AT treatments. Our results are in agreement with those of Turner et al. (2001), who found that the consumption of alternative food items provided an amelioration of toxic effects.

Conversely, egg hatching did not exhibit such a toxin-dilution effect. Although overall fitness was enhanced with respect to the AT treatment, due to total egg production (Fig. 6), the percentage of eggs hatched was the same for both the AT treatment and the mixed-strain treatment (Fig. 6). These results for hatching success might indicate a dose-dependent effect.

Colin & Dam (2002) examined mixed diets by feeding a high-toxic strain of *Alexandrium minutum* and a non-toxic species (*Tetraselmis suecica*) to the copepod *Acartia tonsa*. They did not find any effect on hatching success, but found a dose-dependent effect that inhibited food ingestion and, indirectly, egg production. This meant that there was neither inhibition of embryonic development nor toxin-dilution effects. The presence of a different species as a non-toxic control in the study of Colin & Dam (2002) may make the interpretation of their results difficult, because of the possibility of unknown effects produced by this second species. In addition, their experimental period (3 d) was shorter than that of our study, and they only examined the results on the third day. Two days of acclimatization followed by sampling only on the third day seem insufficient to record clear patterns, especially for mortality and egg hatching. Major differences between the results from Colin & Dam (2002) and our study should, however, be species-specific relationships. The *Acartia tonsa* population in the former study was adapted to recurrent blooms of the toxic *Alexandrium minutum*, whereas our *Acartia clausi* population was adapted to the presence of this species but not to recurrent blooming events.

Feeding strategies to nullify the effects of toxic compounds have often been examined in invertebrates within the framework of the toxin-dilution and the non-additive hypotheses (Pennings et al. 1993, Bernays et al. 1994, Hagele & Rowell-Rahier 1999). Toxin dilution mechanisms may be a consequence of increasing excretion rates, enzymatic activity, and/or interference with other compounds. In such studies, estimates of fitness are often performed by measuring reduction in growth at juvenile stages (Pennings et al.

1993, Bernays et al. 1994, Hagele & Rowell-Rahier 1999). Although reduction in growth is assumed to have negative consequences on fitness, considered here as maintenance physiology, we showed that the effects of toxins on maintenance physiology can be different to those on reproductive physiology (a better estimate of fitness). Indeed, in our study, the toxic effects were not dose-dependent for maintenance physiology (because of toxin-dilution), but they were for reproductive physiology (egg-hatching success).

If, prior to the onset of a bloom, a phytoplankton community consists of a set of toxic or potentially toxic and non-toxic species, each with genetically different strains, grazers such as *Acartia clausi* do not appear to have sufficient selective abilities to achieve 100% distinction between these strains if there are not strong morphological differences between them. At this point, prior to the onset of a bloom, grazers may not yet experience any effects from favoring toxic strains of the same species over non-toxic ones. However, at least with PSP toxins, any feeding strategy that does not avoid consumption of toxic cells may produce a reduction in the fitness of the grazers, because the effects on egg-hatching success are dose-dependent and cannot be compensated for by any toxin-dilution strategy. This suggests that in evolutionary terms, individuals would be favored if they performed any feeding strategy that avoided consumption of toxic cells. Yet, as our results indicated, this selective feeding behavior may be of greater importance after the beginning of a bloom of a toxic species, when toxic cells dominate the community and grazers can effectively reject them, as has been shown in previous studies (Huntley et al. 1986, DeMott 1989, Gilbert 1990, DeMott & Moxter 1991, Kirk & Gilbert 1992, Teegarden 1999, Guisande et al. 2002a).

It has been suggested that the evolution of endotoxigenicity can be explained by the 'kin selection' theory (Kirk & Gilbert 1992). Toxic individuals may be captured by grazers, which will recognize them as toxic and will then avoid the consumption of close relatives that are also toxic. Subsequently, the inclusive fitness of the initially grazed individuals is enhanced. As Kirk & Gilbert (1992) also pointed out, this strategy may be susceptible to take-over by cheaters. To some extent, our findings support this possibility, because grazers are not always able to efficiently select between toxic and non-toxic strains; however, we should also consider that our strain was not of the highest possible toxicity, and that use of more toxic strains could produce different findings. Efficient selection can only be made when toxic and non-toxic strains coexist in similar numbers before a bloom. However, the 'kin selection' explanation is still accurate for the early and late phases of a bloom, when only a toxic strain becomes dominant in the phytoplankton community.

Acknowledgements. We are very grateful to A. Acuña for his helpful technical assistance. Funding was provided by the European Commission through the project FATE 'Transfer and Fate of Harmful Algal Bloom (HAB) Toxins in European Marine Waters' (contract no. EVK3-CT2000-00055), a project within the EUROHAB cluster, and by the Spanish government through an FPU Spanish Ministry of Science and Education grant to A.B. We also thank 3 anonymous referees for their very valuable comments.

LITERATURE CITED

- Bagoien E, Miranda A, Reguera B, Franco JM (1996) Effects of two paralytic shellfish toxin producing dinoflagellates on the pelagic harpacticoid copepod *Euterpina acutifrons*. *Mar Biol* 126:361–369
- Bernays EA, Bright KL, González N, Ángel J (1994) Dietary mixing in a generalist herbivore: test of two hypotheses. *Ecology* 75:1997–2006
- Bogdan KG, Gilbert JJ (1987) Quantitative comparison of food niches in some freshwater zooplankton. A multi-tracer cell approach. *Oecologia* 72:331–340
- Chang FH, Anderson DM, Kulis DM, Till DG (1997) Toxin production of *Alexandrium minutum* (Dinophyceae) from the Bay of Plenty, New Zealand. *Toxicon* 35:393–409
- Colin SP, Dam HG (2002) Testing for toxic effects of prey on zooplankton using sole versus mixed diets. *Limnol Oceanogr* 47:1430–1437
- Cowles TJ, Olson RJ, Chrisholm SW (1988) Food selection by copepods: discrimination on the basis of food quality. *Mar Biol* 100:41–49
- DeMott WR (1986) The role of taste in food selection by freshwater zooplankton. *Oecologia* 69:334–340
- DeMott WR (1988) Discrimination between algae and artificial particles by freshwater and marine copepods. *Limnol Oceanogr* 33:397–408
- DeMott WR (1989) Optimal foraging theory as a predictor of chemically mediated food selection by suspension-feeding copepods. *Limnol Oceanogr* 34:140–154
- DeMott WR (1995) Optimal foraging by a suspension feeding copepod: responses to short-term and seasonal variation in food resources. *Oecologia* 103:230–240
- DeMott WR, Moxter F (1991) Foraging on cyanobacteria by copepods: responses to chemical defenses and resource abundance. *Ecology* 72:1820–1834
- Dutz J (1998) Repression of fecundity in the neritic copepod *Acartia clausi* exposed to the toxic dinoflagellate *Alexandrium lusitanicum*: relationship between feeding and egg production. *Mar Ecol Prog Ser* 175:97–107
- Edler L (1979) Recommendations for marine biological studies in the Baltic Sea. *Phytoplankton and chlorophyll*. *Balt Mar Biol* 5:1–38
- Franco JM, Fernández P (1993) Separation of PSP toxins by reversed phase high performance liquid chromatography, with postcolumn reaction and fluorimetric detection. *Chromatographia* 35:613–620
- Franco JM, Fernández P, Reguera B (1994) Toxin profiles of natural populations and cultures of *Alexandrium minutum* Halim from Galician (Spain) coastal waters. *J Appl Phycol* 6:275–279
- Frangópulos M, Guisande C, Maneiro I, Riveiro I, Franco JM (2000) Short-term and long-term effects of the toxic dinoflagellate *Alexandrium minutum* on the copepod *Acartia clausi*. *Mar Ecol Prog Ser* 203:161–169
- Frangópulos M, Guisande C, de Blas E, Maneiro I (2004) Toxin production and competitive abilities under phosphorous limitation of *Alexandrium* species. *Harmful Algae* 3:131–139
- Frost BW (1972) Effects of size and concentration of food particles on the feeding behaviour of the marine planktonic copepod *Calanus pacificus*. *Limnol Oceanogr* 17:805–815
- Galfre G, Milstein C (1981) Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol* 73B:3–46
- Gilbert JJ (1990) Differential effects of *Anabaena affinis* on cladocerans and rotifers: mechanisms and implications. *Ecology* 71:1727–1740
- Gliwicz ZM, Lampert W (1990) Food thresholds in *Daphnia* species in the absence and presence of blue-green filaments. *Ecology* 71:691–702
- Guisande C, Frangópulos M, Maneiro I, Vergara AR, Riveiro I (2002a) Ecological advantages of toxin production by the dinoflagellate *Alexandrium minutum* under phosphorous limitation. *Mar Ecol Prog Ser* 225:169–176
- Guisande C, Frangópulos M, Carotenuto Y, Maneiro I, Riveiro I, Vergara AR (2002b) Fate of paralytic shellfish poisoning toxins ingested by the copepod *Acartia clausi*. *Mar Ecol Prog Ser* 240:105–115
- Hagele BF, Rowell-Rahier M (1999) Dietary mixing in three generalist herbivores: nutrient complementation or toxin dilution? *Oecologia* 119:521–533
- Hallegraeff GM, Bolch CJ (1992) Transport of dinoflagellate cysts in ship's ballast water: Implications for plankton biogeography and aquaculture. *J Plankton Res* 14:1067–1084
- Hillebrand H, Durselen CL, Kirschel D, Pollinger U, Zohary T (1999) Biovolume calculation for pelagic and benthic microalgae. *J Phycol* 35:403–424
- Huntley M, Sykes P, Rohan S, Marin V (1986) Chemically mediated rejection of dinoflagellate prey by the copepods *Calanus pacificus* and *Paracalanus parvus*: mechanism, occurrence and significance. *Mar Ecol Prog Ser* 28:105–120
- Ives JD (1987) Possible mechanisms underlying copepod grazing responses to levels of toxicity in red tide dinoflagellates. *J Exp Mar Biol Ecol* 112:131–145
- Kjørboe T, Moehlenberg F, Hamburger K (1985) Bioenergetics of the planktonic copepod *Acartia tonsa*: relation between feeding, egg production and respiration, and composition of specific dynamic action. *Mar Ecol Prog Ser* 26:85–97
- Kirk LK, Gilbert JJ (1992) Variation in herbivore response to chemical defenses: zooplankton foraging on toxic cyanobacteria. *Ecology* 73:2208–2217
- Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of redefined specificity. *Nature* 256:495–497
- Laamanen MJ, Gugger MF, Lehtimäki JM, Haukka K, Sivonen K (2001) Diversity of toxic and nontoxic *Nodularia* isolates (Cyanobacteria) and filaments from the Baltic Sea. *Appl Environ Microbiol* 67:4638–4647
- Lampert W (1987) Laboratory studies on zooplankton-cyanobacteria interactions. *NZ J Mar Freshw Res* 21:483–490
- Manly BFJ (1974) A model for certain types of selection experiments. *Biometrics* 30:281–294
- Oshima Y, Supino K, Yasumoto T (1989) Latest advances in HPLC analysis of paralytic shellfish toxins. In: Natori S, Hashimoto K, Ueno Y (eds) *Mycotoxins and phycotoxins*. Elsevier, Amsterdam, p 319–326
- Pennings SC, Nadeau MT, Valerie JP (1993) Selectivity and growth of the generalist herbivore *Dolabella auricularia* feeding upon complementary resources. *Ecology* 74:879–890

- Sykes PF, Huntley ME (1987) Acute physiological reactions of *Calanus pacificus* to selected dinoflagellates: direct observations. *Mar Biol* 94:19–24
- Teegarden GJ (1999) Copepod grazing selection and particle discrimination on the basis of PSP toxic content. *Mar Ecol Prog Ser* 181:163–176
- Turner JT, Ianora A, Miralto A, Laabir M, Esposito F (2001) Decoupling of copepod grazing rates, fecundity and egg hatching success on mixed and alternating diatom and dinoflagellate diets. *Mar Ecol Prog Ser* 220:187–199
- Uye S, Takamatsu K (1990) Feeding interactions between planktonic copepods and red-tide flagellates from Japanese coastal waters. *Mar Ecol Prog Ser* 59:97–107
- Vanderploeg HA, Paffenhof G (1985) Models of algal capture by the freshwater copepod *Diaptomus sicilis* and their relation to food-size selection. *Limnol Oceanogr* 30:871–885
- Vanderploeg HA, Scavia D, Liebig JR (1984) Feeding rate of *Diaptomus sicilis* and its relation to selectivity and effective food concentration in algal mixtures and inn lake Michigan. *J Plankton Res* 6:919–941
- Wilson DS (1973) Food size selection among copepods. *Ecology* 54:909–914

Editorial responsibility: Otto Kinne (Editor-in-Chief), Oldendorf/Luhe, Germany

*Submitted: August 12, 2005; Accepted: November 29, 2005
Proofs received from author(s): June 7, 2006*