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ORIGINAL ARTICLE

Enriching Rotifers with "Premium" Microalgae. *Nannochloropsis gaditana*

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Abstract The nutritive quality of *Nannochloropsis gaditana* cultured semicontinuously with different daily renewal rates was tested as a diet for short-term enrichment of the rotifer *Brachionus plicatilis*. After 24 h, dramatic differences in the survival, dry weight, and biochemical composition of the rotifers depending on the renewal rate of microalgal cultures were observed. Survival after the feeding period increased with increasing renewal rates. Rotifers fed microalgae from low renewal rate, nutrient-deficient cultures showed low dry weight and organic contents very similar to those of the initial rotifers that were starved for 12 h before the start of the feeding period. On the contrary, rotifers fed nutrient-sufficient microalgal cells underwent up to twofold increases of dry weight and protein, lipid, and carbohydrate contents with regard to rotifers fed nutrient-depleted *N. gaditana*. Consequently, feed conversion rate decreased in these conditions, indicating a better assimilation of the microalgal biomass obtained at high renewal rates. No single microalgal biochemical parameter among those studied can explain the response of the filter feeder. Similarly to gross composition, EPA and n-3 contents in rotifers fed microalgae from nutrient-sufficient cultures were double than the contents found in rotifers fed nutrient-limited microalgae. In addition, very high positive correlations between the contents of EPA and n-3 in *N. gaditana* and *B. plicatilis* were observed. These results demonstrate that selecting the appropriate conditions of semicontinuous culture can strongly enhance the nutritional value of microalgae that is reflected in the growth and biochemical composition of the filter-feeder even in short exposure periods.

Keywords *Brachionus plicatilis* · Microalgae · *Nannochloropsis gaditana* · Nutrition · Semicontinuous culture · Biochemical composition

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Introduction

Microalgae are involved in the rearing of the majority of animal species object of mariculture production, including bivalve mollusks throughout all their life cycle and most crustacean, fish, and cephalopod species during some larval stages, directly in a few cases but mostly indirectly as the diet for the production of live preys which, in turn, constitute the diet for larvae. Microalgae culture may account, on average, for 30–40% of the exploitation costs in aquaculture industry (Borowitzka 1997). Many efforts have been, therefore, addressed towards the reduction of the use of microalgae in aquaculture processes and their substitution by artificial diets. Formulated feeds as solid microparticles or oil emulsions are used worldwide for the production of rotifers and *Artemia* as well as to boost their content in essential nutrients for larvae. However, these artificial diets may present some disadvantages. Oil emulsions may cause rotifer mortalities (Rodríguez et al. 1996) and reduce their hygienic condition (Dhert et al.

2001), and lipid residues can be transferred to larval tanks where they promote bacterial growth (Øie et al. 1997). In addition, solid microparticles can lose part of their nutrients due to leakage (Nordgreen et al. 2007). In that sense, manufacturers currently tend to include microalgal ingredients in their diet formulations for live feed. Moreover, low-bacterial, multispecific microalgal biomass remains the only available food source for bivalve culture (Muller-Feuga 2000) as alternative diets present deficiencies and are inadequate for a complete replacement of microalgal feed (Robert and Trintignac 1997; Ponis et al. 2003).

The major challenge to enhance the use of microalgae in aquaculture is the improvement of the reliability of cultures (Duerr et al. 1998) and the quality of the produced biomass. Traditional batch cultures are usually subjected to poor light supply, absence of CO_2 supplementation, and low nutrient concentrations, factors that result in low productivities. These culture systems are prone to crash unpredictably, and as they are usually harvested at the end of log phase, the nutritional value of microalgal biomass is often reduced and its biochemical composition cannot be controlled (Ponis et al. 2006). However, when the appropriate conditions of continuous or semicontinuous culture are used, high productivities can be sustained for long periods and the obtained microalgal biomass shows a constant biochemical composition that can be controlled by manipulating culture parameters in order to increase its nutritive value (Otero et al. 2002). Specifically, increasing the dilution or renewal rate in continuous and semicontinuous systems, respectively, leads to important variations in the nutritional quality of microalgae which strongly affect the performance of filter feeders, in terms of assimilation efficiency, growth, survival, and reproductive rates (Scott 1980; Fábregas et al. 1998, 2001; Ferreira et al. 2008). Microalgae cultured in controlled conditions are also particularly appropriated to tailor the biochemical composition of live feed, either in culture or in short-term enrichment procedures, in order to meet the nutritional requirements of larvae. *Artemia* juveniles enriched with microalgae cultured semicontinuously show high contents of protein and polyunsaturated fatty acids (Seixas et al. 2008); in addition, very high correlations between protein, lipid, and carbohydrate contents in 12-day-old *Artemia*, and their microalgal feed from semicontinuous culture have been reported (Fábregas et al. 2001). Likewise, the biochemical composition of the rotifer *Brachionus plicatilis* can also be strongly modified through the use of microalgae from semicontinuous culture. In a previous study (Ferreira et al. 2008), rotifers were enriched for 24 h with the prymnesiophycean *Isochrysis* aff. *galbana* clone T-ISO cultured semicontinuously with five different renewal rates, and dramatic differences in the gross biochemical composition and fatty acid profile of the rotifers depending on the nutritional status and the renewal rate of microalgal cultures were observed. The eustigmatophyte *Nannochloropsis* is widely used for rotifer production since it supports higher growth rates than baker's yeast (Lubzens et al. 1995), provides the rotifers with high amounts of EPA and it is easy to culture. In the present work, *Nannochloropsis gaditana* was produced in a semicontinuous system with different renewal rates and used for rotifer enrichment with the aim of comparing the results with those obtained with T-ISO and providing directions for the improvement of mass culture of microalgae in large-scale systems.

Materials and Methods

Nannochloropsis gaditana

The marine microalga *Nannochloropsis gaditana* Lubián (CCMP 527) was cultured in 30-mm diameter glass tubes containing 80 ml of culture medium consisting of autoclaved seawater (salinity 35 g l^{-1}) enriched with nutrients (KNO_3, 4 mM; NaH_2PO_4, 0.22 mM; $ZnCl_2$, 2.29 μM; $MnCl_2$, 3.37 μM; Na_2MoO_4, 2.72 μM; $CoCl_3$, 0.38 μM; $CuSO_4$, 0.465 μM; ferric citrate, 21.2 μM; thiamin, 1 mg l^{-1}; biotin, 13.4 μg l^{-1}; vitamin B_{12}, 9.4 μg l^{-1}; EDTA, 14 μmol l^{-1}, modified from Fábregas et al. 1985). Cultures were subjected to a circadian light/dark regime (12/12 h) at a light intensity of 162 μmol photon m^{-2} s^{-1} and a temperature of 21°C. Continuous aeration (230 ml min^{-1}), supplemented intermittently with CO_2 in order to keep pH below 8, was provided through capillary tubes of 1-mm diameter. Tubes were inoculated at an initial cell density of 32×10^6 cells ml^{-1}. Once cultures reached early stationary phase the semicontinuous regime was started by daily harvesting a 10%, 20%, 30%, 40%, and 50% of the volume and replenishing with the culture medium described above. Cultures were maintained for 1 week after steady-state was achieved before being used for enrichment of the rotifers and for biochemical analyses. The number of replicates for each culture condition ranged between three and six, enough to provide the number of cells necessary to feed the rotifers. Cell density was assessed daily before dilution by microscope cell counting using a Neubauer haemocytometer.

Brachionus plicatilis

Rotifers were cultured routinely in 6-l carboys containing autoclaved seawater (salinity 35‰) at a temperature of 21° C, and fed a mixture of marine microalgae (*Isochrysis* aff. *galbana* clone T-ISO, *Tetraselmis suecica*, *Nannochloropsis gaditana*, and *Rhodomonas lens*). Periodically, rotifers were filtered with 45-μm mesh size nylon net, rinsed with distilled water, and transferred to a new flask. Rotifers were filtered and

deprived of food for 12 h before the enrichment experiment in order to avoid interference from the gut content on the results. A sample of the starved rotifers was collected as a control group. For the enrichment, *B. plicatilis* were transferred to 1-l flasks containing autoclaved seawater, being the final volume and the final rotifer density 700 ml and 200 individuals ml^{-1}, respectively. Three replicates of rotifer cultures were set for each renewal rate of the microalga. Food ration was calculated on a cell number basis, and an average dry weight of 5 pg *N. gaditana* $cell^{-1}$, regardless the culture conditions, was considered to calculate the microalgae ration. 86,000 cells of *N. gaditana* (equivalent to a cell density of 60.2×10^6 cells ml^{-1}) were supplied per individual. The presence of remaining microalgal cells ($5\pm1.6\times10^6$ cells ml^{-1}) after the enrichment period confirms that the food ration was sufficient to feed the rotifers for 24 h. After this period, rotifers were collected.

Sampling Procedures

Dry weight was determined by collecting samples of 5 ml of microalgal culture on previously weighed precombusted Whatman GF/C fiberglass filters. Microalgal samples were washed three times with 5 ml of 0.5 M ammonium formate in order to remove salts. Rotifer samples (100 ml of rotifer culture) were also collected on Whatman GF/C filters and rinsed with distilled water. Filters were dried overnight at 80°C and dry weight determined gravimetrically. For biochemical analyses, 5 to 10 ml of *N. gaditana* cultures were centrifuged and immediately frozen at −20°C; 80 ml of rotifer culture were harvested on a sieve of 45-μm mesh size, rinsed with distilled water, and frozen at −20°C for fatty acid characterization. The rest of the rotifers were freeze-dried for other biochemical analyses.

Biochemical Analyses

Carbohydrate content was determined by the phenol-sulfuric acid method (Kochert 1978). Lipids were extracted (Bligh and Dyer 1959) and measured by the charring method of Marsh and Weinstein (1966). Protein was determined by the Lowry method (Lowry et al. 1951) as modified by Herbert et al. (1971). For the characterization of fatty acid content, lipid extracts were derivatized by methanolysis with 5% HCl in methanol at 85°C (Sato and Murata 1988), extracted with hexane, and analyzed with a gas chromatogram coupled to a mass spectrometer (MD-800, Fisons Instruments, Beverly, MA, USA). Triheptadecanoin (Sigma, St. Louis, MO, USA) was used as the internal standard. C–H–N determination was carried out on freeze-dried microalgal samples with an autoanalyzer (Carlo Erba EA 1108, Rodano, Italy). All the analyses were done in triplicate.

Calculations and Statistical Analysis

Feed conversion rate (FCR) was calculated as the ratio between dry or organic (protein + lipid + carbohydrate) weight of *N. gaditana* supplied and the equivalent increase in rotifer biomass. The statistical treatment of the data was conducted using SPSS 14.0 for Windows (SPSS, Inc.). Statistically significant differences in the biochemical composition between rotifers groups were detected by the non-parametrical Mann–Whitney *U* test at a significance level of 0.05.

Results

Semicontinuous Cultures of *Nannochloropsis gaditana*

The different renewal rates affected the nutrient input and effective irradiance in *N. gaditana* cultures, resulting in a decrease in steady-state cell density with increasing renewal rates, from 223×10^6 to 70×10^6 cells ml^{-1} (Fig. 1). C/N ratio decreased significantly ($P<0.05$) between the renewal rates of 10% and 30%, from 15.7 to 7.4, and remained close to that value in higher renewal rates (Fig. 2), indicating a shift from nitrogen-limited to nitrogen-saturated conditions at the renewal rate of 30% (Otero et al. 1998). This change in nitrogen availability caused strong modifications in the biochemical composition of microalgal cells. The percentage of protein in the organic fraction (sum of protein, lipid, and carbohydrate contents) of *N. gaditana* cells underwent a significant increase ($P<0.05$), from 36.19% to 60.39%, with increasing nitrogen availability in the range of nutrient-limited conditions, i.e., between the renewal rates of 10% and 30%. This increase in protein content was

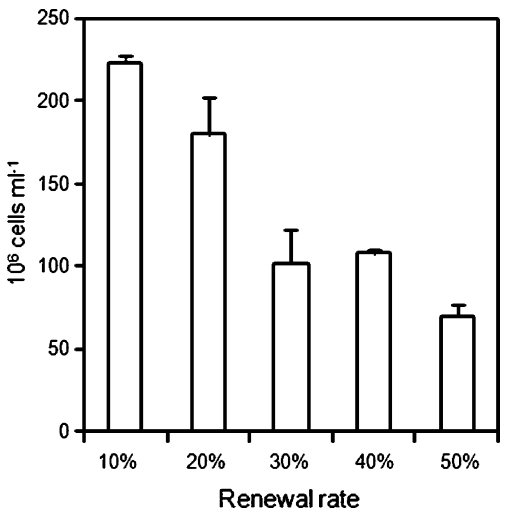

Fig. 1 Steady-state cell density (10^6 cells ml^{-1}) of semicontinuous cultures of *N. gaditana*

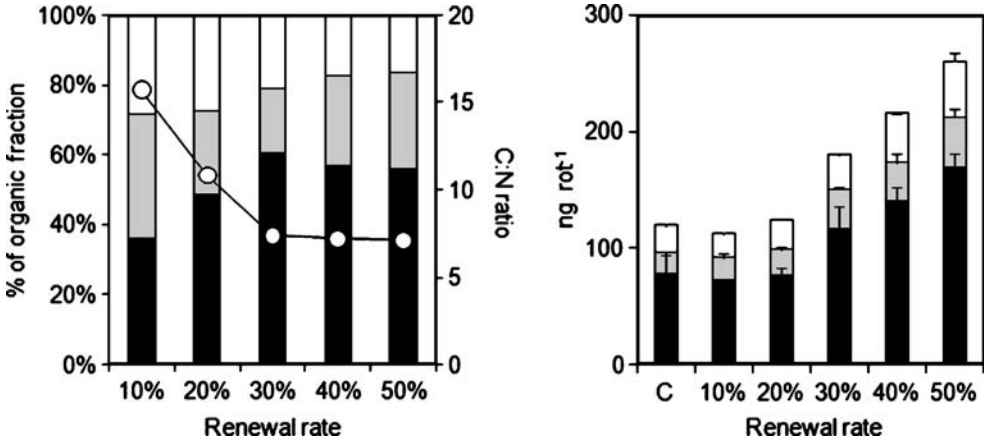

Fig. 2 *Left* Composition of the organic fraction and C/N ratio of *N. gaditana* cultured semicontinuously with different renewal rates. Protein, lipid, and carbohydrate contents are expressed as percentages of the organic fraction. *Right* Composition of *B. plicatilis* enriched for 24 h with those microalgal cultures, expressed as ng individual^{-1}. *Black bar* protein; *gray bar* lipid; *white bar* carbohydrates; *unfilled circles* C/N ratio; *C* rotifer control sample, harvested before the enrichment experiment

accompanied by decreases in the percentage of carbohydrates, from 28.47% to 21.01%, and particularly lipids, that decreased halfway, from 35.34% to 18.60% ($P<0.05$). In nitrogen-saturated conditions, between the renewal rates of 30% and 50%, the percentages of protein decreased slightly but remained high, whereas carbohydrates underwent a further decrease down to a value of 16.71% and lipids recovered partially, reaching values around 26% at the renewal rates of 40% and 50% (Fig. 2). Changes in the contents of protein, lipids, and carbohydrates were similar when these three components were expressed as pg cell^{-1}. Carbohydrate content decreased linearly with increasing renewal rates, from 1.26 to 0.67 pg cell^{-1}, being cellular contents significantly lower ($P<0.05$) in nutrient-sufficient than in nutrient-limited cultures. Protein cellular content increased significantly ($P<0.05$) from 1.60 pg at the renewal rate of 10% to 2.65 pg at the renewal rate of 30% and decreased at higher renewal rates, even though this decrease was not statistically significant ($P<0.05$). Lipid cell content followed the opposite trend of protein, decreasing from 1.57 to 0.82 pg cell^{-1} between the renewal rates of 10% and 30% and undergoing an slight but significant increase ($P<0.05$) at higher renewal rates (Table 1).

Changes in lipid content were accompanied by strong modifications in the fatty acid profile of *N. gaditana*. The highest total fatty acid content was found at the renewal rate of 10%, 1.18 pg cell^{-1}, which decreased almost by one half in nutrient-saturated conditions. The percentage of polyunsaturated fatty acids (PUFAs) increased dramatically with the increase in nutrient availability, from 12.71% at the lowest renewal rate to values around 40% of total fatty acids between the renewal rates of 30% and 50% (Fig. 3). Eicosapentaenoic acid (20:5n-3, EPA) was the main PUFA in *N. gaditana* and increased from 6.76% in the renewal rate of 10% to percentages around 27% in nutrient-sufficient cultures. Expressed as cellular content the increase

Table 1 Biochemical composition of *N. gaditana* and *B. plicatilis* (expressed as pg cell^{-1} and ng individual^{-1}, respectively) food supply and conversion efficiencies of rotifers enriched for 24 h with *N. gaditana* from semicontinuous cultures

	Renewal rate				
	10%	20%	30%	40%	50%
N. gaditana protein content	1.60±0.08	1.84±0.25	2.65±0.46	2.45±0.24	2.28±0.12
N. gaditana lipid content	1.57±0.20	0.89±0.10	0.82±0.17	1.12±0.06	1.04±0.12
N. gaditana carbohydrate content	1.26±0.19	1.04±0.16	0.92±0.23	0.74±0.03	0.67±0.01
N. gaditana Q_C	3.56±0.56	2.92±0.28	3.50±0.43	2.47±0.96	2.25±0.71
N. gaditana Q_N	0.28±0.06	0.33±0.00	0.55±0.06	0.40±0.16	0.44±0.02
N. gaditana supplied (μg ml^{-1})	530	427	425	448	404
Rotifer gross biomass production (μg ml^{-1})	35.37	38.36	56.15	74.17	84.72
Rotifer net biomass production (μg ml^{-1})	−8.1	−5.1	12.6	30.7	41.2
FCR (weight of *N. gaditana* supplied/increase in rotifer weight)	–	–	4.5	1.7	1.5
B. plicatilis protein content	73.32±0.41	77.02±6.96	116.66±1.57	140.83±11.80	169.74±10.64
B. plicatilis lipid content	18.92±3.06	22.03±2.21	33.56±1.71	32.37±7.99	42.84±6.57
B. plicatilis carbohydrate content	20.31±0.63	25.40±0.85	29.64±0.75	42.18±0.33	47.19±7.56
Protein recovery (%)	–	–	29.09	61.05	74.92
Lipid recovery (%)	1.37	9.93	38.27	30.71	44.60
Carbohydrate recovery (%)	–	4.86	14.22	61.38	67.52

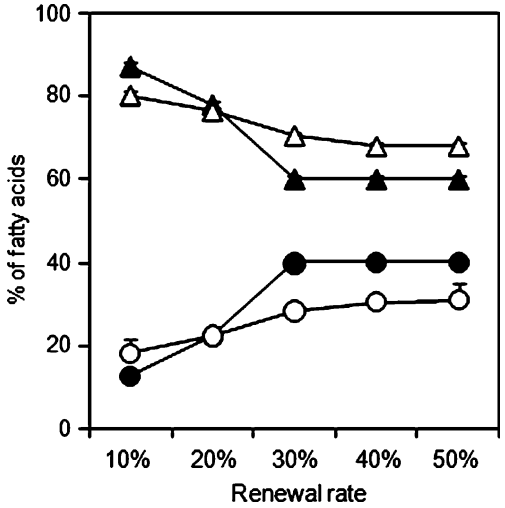

Fig. 3 Percentages of saturated + monounsaturated (*triangles*) and polyunsaturated fatty acids (*circles*) in *N. gaditana* and *B. plicatilis* enriched for 24 h. *Filled symbols N. gaditana*; *unfilled symbols B. plicatilis*

of EPA was however less steep, from 0.08 pg cell^{-1} at the renewal rate of 10% to values between 0.14 and 0.18 pg cell^{-1} in nitrogen-saturated conditions. Arachidonic acid (20:4n-6, ARA) was the second most important PUFA in *N. gaditana*. Its percentage increased with renewal rate in nutrient-limited conditions, from 3.56% to 6.55%, with a further slight increase up to 6.90% in the renewal rate of 50% (Table 2).

Short-term Enrichment of *Brachionus plicatilis*

The number of rotifers changed in relation to the initial value of 200 individuals ml^{-1} after 24 h of enrichment. The groups that received *N. gaditana* from the renewal rates of 10% and 20% presented a lower density, 180.7±17.6 and 183.3±6.7 individuals ml^{-1}, respectively. However, rotifer density increased over the initial value in the groups fed *N. gaditana* from renewal rates between 30% and 50%, reaching a maximum of 226±25.5 individuals ml^{-1} (Fig. 4). The presence of some microalgal cells after 24 h might suggest that rotifers did not consume all the feed supplied. The number of remaining cells increased up to the renewal rate of 40% and decreased again in the renewal rate of 50%; however, it is also possible that cells continued to divide after being transferred into the rotifer flasks, especially those from high renewal rates, since growth rate was high in these culture conditions.

Dry weight and biochemical composition of rotifers also underwent modifications related to the renewal rate of the microalgal culture used as feed. Enrichment with microalgal cells from nutrient-limited renewal rates, i.e., 10% and 20%, gave rise to the lowest protein, lipid, and carbohydrate contents (Fig. 2) and the lowest dry weight (Fig. 4), closely resembling those of control rotifers. Rotifers that received *N. gaditana* cells from nutrient-sufficient cultures reached higher dry weight values and higher organic

Table 2 Fatty acid composition (as percentage of total fatty acids) of *N. gaditana* cultured in semicontinuous regime and *B. plicatilis* enriched with those microalgae and total fatty acid content, expressed as pg *N. gaditana* cell^{-1} or as ng individual^{-1}

Fatty acids	Renewal rate									
	10%		20%		30%		40%		50%	
	Microalga	Rotifer	Microalga	Rotifer	Microalga	Rotifer	Microalga	Rotifer	Microalga	Rotifer
14:0	7.37±1.82	7.90±2.2	7.44±0.67	6.69±0.78	8.00±1.95	7.40±1.05	7.16±1.32	6.64±0.91	6.89±0.91	6.68±1.14
16:0	36.27±0.88	25.11±1.94	32.15±0.27	23.54±1.24	22.88±2.01	21.91±0.83	22.66±1.92	21.97±1.94	23.93±0.71	22.12±2.13
Other saturated	1.39±0.33	5.39±0.9	1.67±0.24	4.65±0.46	1.04±0.44	4.20±0.34	1.31±0.03	5.08±0.57	1.30±0.20	5.17±0.43
16:1 (n-7)	31.59±1.20	24.61±2.13	28.37±1.01	24.52±1.59	24.35±2.87	23.47±0.02	24.42±1.81	21.15±0.93	23.90±0.86	20.71±0.14
18:1 (n-9)	10.18±1.65	8.82±1.03	7.83±1.35	8.08±0.27	2.92±0.72	4.88±0.05	3.81±0.25	6.50±0.13	3.57±0.25	5.88±0.91
18:1 (n-7)	0.44±0.07	4.43±0.69	0.48±0.03	4.98±0.13	0.66±0.11	5.08±0.08	0.50±0.04	2.84±2.40	0.34±0.41	3.66±2.03
Other monoinsaturated	0.05±0.04	3.88±0.79	0.08±0.02	3.87±0.45	0.19±0.13	3.52±0.27	0.07±0.02	3.65±0.40	0.06±0.04	3.74±0.43
18:2 (n-6)	1.45±0.23	3.01±0.5	2.33±0.08	4.03±0.50	3.30±0.45	3.93±0.17	3.61±0.44	4.73±1.01	2.91±0.04	4.21±0.83
20:4 (n-6)	3.56±0.51	4.16±0.90	5.70±0.68	4.48±0.66	6.55±0.93	4.52±0.01	6.87±0.83	5.36±0.71	6.90±0.47	5.39±0.48
20:5 (n-3)	6.76±0.93	8.25±1.43	12.37±0.31	10.04±1.22	27.11±3.96	15.89±1.58	26.55±3.70	17.41±2.40	27.09±0.77	18.33±1.13
22:6 (n-3)	–	1.78±0.8	–	1.56±0.44	–	1.30±0.52	–	1.66±0.57	–	1.26±0.50
Other polyunsaturated	0.93±0.12	2.67±0.51	1.57±0.08	3.57±0.20	3.00±0.41	3.91±0.14	3.04±0.21	4.32±0.37	3.20±0.04	4.07±0.33
Sum n-3	6.84±0.97	11.02±1.72	12.49±0.34	13.06±0.78	27.15±3.49	18.54±2.19	26.70±3.70	20.59±2.91	27.22±2.37	20.93±1.71
Sum n-6	5.44±0.81	8.81±1.69	8.77±0.57	10.51±1.33	11.83±1.87	10.69±0.30	12.63±1.10	11.35±1.47	11.90±1.15	10.84±2.70
n-3/n-6	1.26±0.03	1.26±0.16	1.42±0.12	1.25±0.09	2.29±0.09	1.73±0.16	2.11±0.11	1.85±0.50	2.29±0.01	2.00±0.42
EPA/ARA	1.90±0.01	2.00±0.09	2.17±0.33	2.25±0.06	4.14±0.37	3.52±0.36	3.87±0.32	3.25±0.10	3.92±0.42	3.41±0.24
Total fatty acids	1.18±0.14	18.05±2.07	0.86±0.09	15.09±4.17	0.67±0.19	19.14±0.58	0.54±0.02	12.75±3.12	0.64±0.08	16.05±2.46

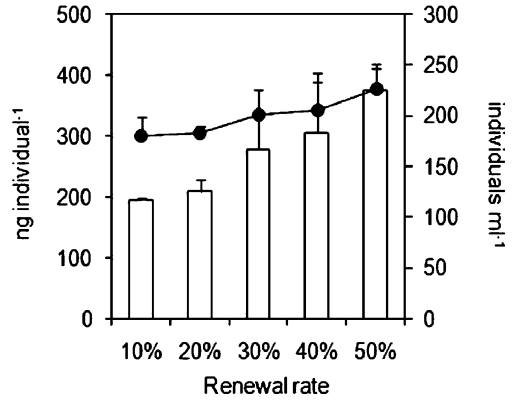

Fig. 4 Final density of rotifers (*filled circles*) and individual dry weight (*bars*) of rotifers after 24-h enrichment with *N. gaditana*

content, which increased with increasing renewal rate. The three components of the organic fraction were similarly affected by the conditions applied to microalgal cultures, undergoing an increase of approximately 2.3-fold between the renewal rates of 10% and 50% and reaching maximum values of 169.74 ng individual^{-1} for protein, 42.84 ng individual^{-1} for lipids, and 47.19 ng individual^{-1} in the case of carbohydrates (Fig. 2). Highest dry weight, 374.85 ng individual^{-1}, was obtained in the rotifers enriched *N. gaditana* from the renewal rate of 50%, nearly doubling the dry weight of rotifers enriched with nutrient-limited microalgae (Fig. 4).

As a consequence of the different final rotifer densities and individual dry weights, biomass productivity of rotifer cultures varied as well strongly depending on the renewal rate applied to *N. gaditana* cultures. The final biomass in rotifer cultures receiving microalgae from nutrient-limited conditions was lower than the initial biomass, so in those conditions there was no net productivity. Nevertheless, net biomass productivity rose in the rotifer groups supplied nutrient-sufficient microalgae, and increased with increasing renewal rates, up to 41.2 μg ml^{-1} (Table 1). FCR ratio between the dry weight of feed supplied and the increase in rotifer biomass could, therefore, be calculated only for rotifers enriched microalgae from nutrient-sufficient cultures. FCR values decreased more than threefold within that range, from 6.7 in the renewal rate of 30% to 1.9 in the renewal rate of 50% (Table 1). The recovery of the three components of the organic fraction increased with renewal rate. Net recovery of protein only occurred in rotifers fed microalgae from nutrient-sufficient cultures, whereas carbohydrates were recovered in rotifers fed *N. gaditana* from the renewal rate of 20% onwards and net recovery of lipids occurred in all enrichment conditions (Table 1).

Total fatty acid content in rotifers fluctuated with no particular trend, ranging between 12.75 and 19.14 ng individual^{-1}. However, fatty acid profile showed strong variations determined by the fatty acid composition of the microalga used as feed. Although modifications were not as dramatic as those observed in *N. gaditana*, the percentage of PUFAs in the rotifer increased with increasing renewal rates, from nearly 20% in the rotifers enriched with *N. gaditana* from the renewal rate of 10% to 32% in the rotifers fed microalgae from the renewal rates of 40% and 50% (Fig. 3). Among PUFAs, n-3 fatty acids increased from 11% in rotifers enriched with microalgae from the renewal rate of 10% to approximately 21% with highest renewal rates (Fig. 5). EPA was the most abundant n-3 in the rotifers, and its percentage increased more than twofold with increasing renewal rates, from 8.25% to 18.33% between the renewal rates of 10% and 50%. The evolution of n-3 and EPA contents in the rotifers perfectly mirrored the variations in the microalga, as shown by the strong correlations, R^2=0.955 and 0.951, respectively (Fig. 5). EPA content expressed as ng individual^{-1} doubled, from 1.47 ng individual^{-1} in rotifers fed microalgae from the renewal rate of 10% to 2.95 ng individual^{-1} in rotifers enriched with microalgae from the renewal rate of 50%. As for *N. gaditana*, ARA was the second most abundant PUFA

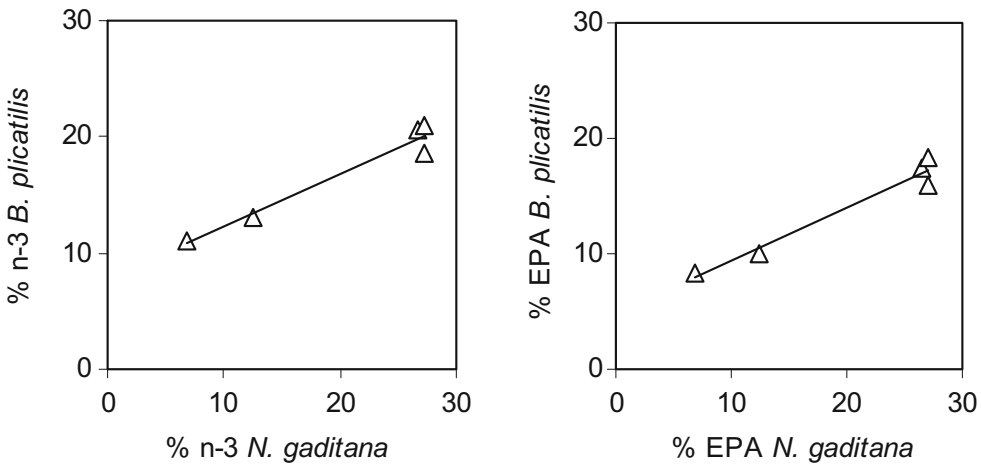

Fig. 5 Correlation between n-3 (*right*) and EPA (*left*) contents as percentages of total fatty acids in *N. gaditana* and *B. plicatilis*. R^2=0.955 and 0.951, respectively

in rotifers and followed a similar trend to that of EPA, though its increase was less marked (Table 2). EPA/ARA ratios were very similar in the microalga and the rotifer and increased with renewal rate in nutrient-limited conditions, stabilizing from the renewal rate of 30% onwards in values between 3.2 and 3.5 (Table 2).

Discussion

Semicontinuous Cultures of *Nannochloropsis gaditana*

Changes in nutrient and light availability caused by the application of different renewal rates modified productivity and biochemical composition of *N. gaditana* in a similar way to other microalgal species cultured in semicontinuous system. Steady-state cell density diminished with increasing renewal rates, a common feature of semicontinuous cultures (Fábregas et al. 1995a; Otero and Fábregas 1997; Otero et al. 1997; Ferreira et al. 2008). The decrease of C/N ratio in nutrient-limited conditions was reflected in an increase in the protein content of microalgal cells, that decreased slightly once nitrogen saturation was achieved, as reported by Fábregas et al. (1995a) for *Dunaliella tertiolecta*, Otero and Fábregas (1997) for *Tetraselmis suecica*, Fábregas et al. (1998) for *Phaeodactylum tricornutum* and Ferreira et al. (2008) for T-ISO. Simultaneously to the increase of protein content, a decrease of the storage compounds was observed. Whereas carbohydrate content tended to decrease with increasing renewal rates in all microalgal species studied (Fábregas et al. 1995a, 1998; Otero and Fábregas 1997; Otero et al. 1997; Ferreira et al. 2008), the evolution of lipids may vary among species. In *Nannochloropsis* sp., the increase of nutrient availability induces a decrease of total cell lipid content in chemostat continuous cultures (Sukenik et al. 1993); however, in the semicontinuous system lipid cell content in *N. gaditana* underwent a slight increase in nutrient-sufficient conditions that might be due to the synthesis and accumulation of non-acyl lipids such as pigments or sterols, as fatty acid content diminished with increasing renewal rates. The decrease of the saturated plus monounsaturated fatty acid fraction, from 70% to 56% of total fatty acids, suggests a shift in the acyl lipid profile, since saturated and monounsaturated fatty acids are mainly associated to triacylglicerides whereas polyunsaturated fatty acids are located in galactolipids included in the thylakoid membrane (Sukenik et al. 1989).

Short-term Enrichment of *Brachionus plicatilis*

After 24 h of enrichment with *N. gaditana*, a dramatic effect of the culture conditions of the microalga on the survival, dry weight, and biochemical composition of the rotifers was observed. The dry weight of the rotifers was clearly affected by the nutritional status of the microalgae, modulated through the culture conditions, remaining steady with nitrogen-limited microalgae and increasing up to twofold with nitrogen-sufficient microalgae (Figs. 2 and 4). A previous study demonstrated that the nutritional status of microalgal cells, modulated in our case through renewal rate, was the main factor affecting the nutritional value of semicontinuously cultured *Isochrysis* aff. *galbana* T-ISO for rotifer enrichment in the same conditions (Ferreira et al. 2008). Similar results were obtained with phosphorous or nitrogen-limited *Scenedesmus obliquus* when feeding *B. calyciflorus* after a period of 24 h (Jensen and Verschoor 2004). The present data clearly confirm those results. As observed for T-ISO, the enrichment of rotifers with *N. gaditana* cells from nitrogen-limited semicontinuous cultures, showing high C/N ratios (Fig. 2), gave rise to individuals with low and similar values of dry weight and protein, lipid, and carbohydrate contents, regardless the renewal rate of the culture and therefore regardless the degree of nutrient limitation and C/N ratio. Microalgae with high C/N ratios were demonstrated to have a deleterious effect on filter feeders leading to negative population growth, as observed for the copepod *Acartia tonsa* (Jones and Flynn 2005). Results obtained with T-ISO (Ferreira et al. 2008) and *N. gaditana* (present work) indicate that this negative effect is also observed after short feeding periods in animals with a shorter lifespan, such as *B. plicatilis*. In addition, enrichment with nutrient-limited *N. gaditana* produced a negative effect on somatic growth, since rotifers showed slightly lower dry weights and organic contents than control rotifers, which had been starved for 12 h before the feeding period. On the contrary, rotifers enriched with nutrient-limited T-ISO showed low dry weights and organic contents in comparison with those fed nutrient-saturated T-ISO, but higher than control individuals (Ferreira et al. 2008). This difference could be due to the different initial status of the rotifers used in both experiments.

On the other hand, rotifers enriched with microalgae from nitrogen-saturated cultures, as indicated by the stabilization of C/N ratio in values around 7, showed higher dry weight and organic contents. In this condition of nutrient availability, despite presenting very similar C/N ratios, the increase of the renewal rate applied to the microalgal cultures had a strong positive effect in the dry weight and the accumulation of protein, lipid, and carbohydrates in the rotifers, as observed in rotifers enriched with T-ISO and as reported by Coutinho (2008) in enrichment experiments with the cryptophyte *Rhodomonas lens*. Diverse literature is available regarding the importance of the C/N ratio of the prey on growth, feeding behavior, and biochemical composition of the filter feeder in long-term

experiments resembling ecological conditions (Andersen et al. 2004, Mitra and Flynn 2005). In our case, the C/N ratio of the microalgal cells remains stable with increasing renewal rates once the point of nitrogen saturation was achieved (Fig. 2) but growth measured as dry weight suffered a twofold increase. Our results, obtained with high prey concentrations and a very short exposure period, corroborate previous studies demonstrating that the prey quality cannot be evaluated solely on the basis of the C/N/P stoichiometry (Mitra and Flynn 2005).

The influence of the nutritional status of microalgal cells on the consumption by filter feeders may vary, since predators can adopt different strategies when food quality decreases, ingesting a higher number of prey to compensate the lower content of a limiting nutrient or on the contrary, cutting down prey consumption due to the production of mucus or toxins or the development of thicker cell walls (Mitra and Flynn 2005). Whereas ingestion of *Tetraselmis suecica* by *Artemia* sp. increased with the increasing renewal rates of the microalgal cultures as a response to lower C/N ratios in the microalgae (Fábregas et al. 2001), Rothhaupt (1995) determined that nitrogen or phosphorus limitation of microalgal cultures did not affect the ingestion rate of the rotifer *Brachionus rubens*. In the present work, no clear conclusion can be derived from the data of remaining cells after the feeding period since it cannot be discarded that cells continued to divide in the rotifer flasks.

The digestibility of cells defined as the predator's capacity to "break down" the cell and absorb the nutrients is another factor that may be affected by culture conditions and strongly modifies the nutritional value of a microalgal species. In fact, *Nannochloropsis* is a genus of poor nutritional value for molluscs (Brown et al. 1998) and *Artemia* (unpublished results) probably due to its hard cell wall. Carbohydrate content of *N. gaditana* decreased continuously with increasing renewal rates. This is a common response to the increase of nutrient availability in continuous and semicontinuous nutrient-limited cultures (Sukenik and Wahnon 1991; Sukenik et al. 1993; Otero et al. 1997) and can affect the nutritional value not only through the C:N ratio but through the digestibility of cell walls. Although it has been suggested that high carbohydrate contents in *Nannochloropsis* sp. could be associated to the accumulation of glucan reserves (Volkman et al. 1993), Sukenik et al. (1989) did not detect the presence of carbohydrate globuli in electron micrographs of *Nannochloropsis* sp. Hence, these polysaccharides are likely part of the cell wall, rather than intracellular storage compounds and can, therefore, affect seriously the digestibility of the microalgal cells. In fact, mature *Nannochloropsis* sp. cells show a 10–30-nm thick cell wall, whereas young cells, which must be predominant in semicontinuous cultures subjected to high renewal rates, appear naked (Rodolfi et al. 2003). The difficulty of lipid and pigment extraction in *Nannochloropsis* from stationary phase cultures compared to young cells (unpublished results), might reinforce this observation. Therefore, the negative effect of high C/N ratios in nitrogen-limited microalgal cultures can be partially attributed to the low digestibility of the cell wall in *Nannochloropsis*.

The number of rotifers fed microalgae from nutrient-limited cultures decreased after the enrichment period with regard to the initial density of 200 individuals ml^{-1}. On the contrary, no change or slight increase in rotifer numbers were recorded with nutrient-saturated microalgal cultures (Fig. 4).The decrease in rotifer numbers with nutrient-limited microalgae could be explained either by a high mortality rate or by a decrease in fecundity which would not compensate mortality, both due to the poor quality of the microalgal food produced in cultures with low renewal rates. Although egg ratio (number of eggs per female) was not measured, the low dry weight obtained in these conditions suggests a low egg production, since there is a positive correlation between egg ratio and individual dry weight (Yúfera et al. 1993). Likewise, the high dry weight of the rotifers enriched *N. gaditana* from the renewal rates of 40% and 50% could be partially attributed to a high egg production, rather than to the increase of body weight. It is unlikely that the effect of food quality on growth, measured as rotifer numbers, were noticeable after 24 h as an egg needs approximately 1–1.5 days to hatch after spawning at a temperature of 20°C (Fukusho 1989), but differences in dry weight after this period indicate that fecundity; therefore, population growth rate will be strongly affected by the nutritional status of the algal food in long term cultivation experiments.

Culture conditions strongly affected the efficiency of assimilation of the different biochemical fractions of the microalgal food. Under nitrogen-saturated conditions the protein content of microalgal cells decreased with renewal rate (Table 1), but total weight and protein content of the rotifers increased as the result of an increase in protein assimilation from 29% to 75% (Table 1). The improvement of carbohydrate assimilation was even higher, from 14% to 68% (Table 1). This fact corroborates the observations of Jones et al. (2002), who found that nitrogen assimilation by *Acartia tonsa* was much more efficient compared to carbon assimilation when the copepod was fed with microalgae cultured in nitrogen-deplete conditions. These changes in the assimilation efficiency allowed that carbohydrate and lipid contents in the rotifer increased in a similar way to proteins. Therefore, the ratio protein versus carbohydrates plus lipids remained constant for all renewal rates, indicating a compensatory mechanism of assimilation that enables the stability of the relative gross biochemical composition of the rotifers regardless the nutritional status

of the microalga, at least in short period exposures. The improvement of the assimilation of microalgal biomass with the increase of dilution or renewal rate in continuous and semicontinuous systems has been reported in previous studies on 24-h enrichment of *B. plicatilis* (Ferreira et al. 2008) and in long-term culture of *Artemia* sp. (Fábregas et al. 2001) with semicontinuous cultures of T-ISO and *Tetraselmis suecica*, respectively, and also in culture experiments with *B. plicatilis* (Scott 1980). It has been observed that the increase of protein and the decrease of carbohydrate content of the microalgal feed results in better somatic or populational growth of filter feeders (Sick 1976; Scott 1980; Fábregas et al. 2001; Ferreira et al. 2008). The ratio 20:5n-3/C of phytoplankton has been proposed as predictor of *Daphnia* growth rate and egg production (Müller-Navarra et al. 2000). In our case, none of the fatty acid derived indexes can explain the growth results, especially those obtained with nutrient saturated microalgae. In conclusion, as observed for C/N ratio, a direct and completely linear correlation between assimilation efficiency, growth or reproductive performance and the evolution of a single biochemical parameter could not be established, neither for the present study nor for previous works (Fábregas et al. 2001; Ferreira et al. 2008).

Implications of enrichment with *N. gaditana* cultured with different renewal rates for larval nutrition lie namely on the dramatic changes observed in the protein fraction and the fatty acid profile of the rotifers. Although enrichment diets and oil emulsions are widely used to increase the nutritional value of rotifers before supplying fish larvae due to the difficulty of obtaining constant supplies of good-quality microalgae, the use of *N. gaditana* cultured with high renewal rates notoriously increased nutrient content of rotifers. Protein content of rotifers fed microalgae from the renewal rate of 50% duplicated with regard to control rotifers and reached a value very similar to that achieved in rotifers enriched for 24 h with an artificial diet specifically formulated to enhance the protein content of rotifers (Dhert 1996); in addition, protein increased only 50% with regard to initial values in rotifers enriched with the artificial diet (Øie and Olsen 1997). Enrichment with *Tetraselmis chuii* and *N. gaditana* has been observed to provide rotifers with higher amounts of free amino acids than artificial diets (Aragão et al. 2004). Free amino acids are particularly valuable nutrients for fish larvae, as they are rapidly assimilated. Fish larvae have high amino acid requirements both for growth and as a source of energy, and free amino acids may constitute an important nutrient source during early life stages, when the digestive tract is not fully developed and protein digestion is incomplete (Rønnestad et al. 2003). As microalgae cultured in nitrogen-sufficient conditions contain much higher levels of free amino acids compared to starved cells (Flynn et al. 1992), it is possible that *N. gaditana* from high renewal rates provided rotifers with high contents of free amino acids, as well as with protein.

In contrast with the evolution of growth and protein content, the increase of renewal rate beyond the point of nutrient saturation did not result in a higher accumulation of fatty acids in the rotifers. The content of n-3 fatty acids was high in rotifers enriched with *N. gaditana* from nutrient-sufficient cultures, approximately 20% of total, suggesting an efficient accumulation of these nutrients after a feeding period of 24 h. Correlations between the percentages of EPA and total PUFAs in *N. gaditana* and the rotifer were much higher than those reported for different fatty acids in other short-term enrichment (Rodríguez Rainuzzo et al. 1989) or culture experiments (Frolov et al. 1991). The concentration of n-3 fatty acids in rotifers undergoes a fast increase in the first period of enrichment (Olsen et al. 1989; Rodríguez et al. 1996). Accumulation is particularly rapid during the first 20–30 min due to the filling of the gut; subsequently the increase of n-3 fatty acid content slows down, what is attributed to the accumulation into rotifer tissues. Consequently, enrichment periods of a few hours are not enough to provide rotifers with high contents of n-3 fatty acids (Ferreira 2007; Coutinho 2008); but after 24 h of feeding, the fatty acid profile of rotifers seems to stabilize (Olsen et al. 1989). EPA is an essential fatty acid in fish larval nutrition, whereas high contents of protein enhance nutritive value of rotifers for turbot larvae (Øie et al. 1997). Rotifers enriched *N. gaditana* cultured with high renewal rates contain high levels of both nutrients. The combination of *N. gaditana* and a source of DHA for the enrichment of rotifers, either consecutively or simultaneously, would probably result in a very suitable nutritional profile to be used in larval rearing.

The results obtained in this study are comparable to those from our previous study on the enrichment of *B. plicatilis* with T-ISO (Ferreira et al. 2008). Despite the differences in the dry weight of the initial rotifers, in both cases enrichment with microalgae from nutrient-deficient cultures resulted in rotifers with low dry weight and protein, lipid, and carbohydrate contents, which were similar regardless of the renewal rate and the degree of nutrient limitation; therefore, independent of the biochemical composition of the microalgal cells. On the contrary, rotifers enriched with nutrient-sufficient microalgae showed higher dry weight and organic content, which increased with increasing renewal rates and independently of the evolution of the biochemical composition of microalgae. Productivity of rotifer cultures also increased with the renewal rate of microalgal cultures, and feed assimilation became more efficient, leading to a decrease of FCR. The fatty acid profile of rotifers reflected that of the microalgal feed, in some cases with very high positive correlations. These observations

suggest the existence of some common patterns in nutrient assimilation from microalgae, which are independent of the initial status of the rotifers and probably of the structure of the population.

In conclusion, selecting the appropriate culture conditions is crucial for the production of microalgae for rotifer enrichment, since only microalgal cultures subjected to high growth rates and nutrient availability are suitable to improve the biochemical composition of rotifers. Additionally, semi-continuous cultures operated in these conditions generate high microalgal productivities (Fábregas et al. 1995a, b). Therefore, the simple manipulation of culture conditions increases the productivity of culture systems and improves the nutritional value of microalgal biomass, what is evident after an exposition period of only 24 h. Results can be easily extrapolated to any photobioreactor being operated continuously for the production of microalgae and should be taken into account for the establishment of optimal culture conditions in large scale systems for aquaculture applications.

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