Enriching rotifers with “premium” microalgae. *Isochrysis* aff. *galbana* clone T-ISO

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**A B S T R A C T**

The effect of semi-continuous culture on the nutritional value of microalgae was tested in the rotifer *Brachionus plicatilis* in short-term enrichment experiments. *Isochrysis* aff. *galbana* clone T-ISO was cultured semi-continuously with renewal rates from 10 to 50% of the volume of the culture per day and used to feed the rotifers. After 24 h, dramatic differences in dry weight and protein, lipid and carbohydrate contents were observed in the rotifers depending on the renewal rate applied to the microalgal culture. Rotifers fed T-ISO cultured with low renewal rates showed low dry weight and organic content, whereas rotifers fed microalgae from nutrient-sufficient, high renewal rate cultures showed higher dry weight and increases up to 60% in protein, 35% in lipid and 100% in carbohydrate contents. Feed conversion rate (FCR) and organic FCR decreased with increasing renewal rates, indicating a more efficient assimilation of the microalgal biomass obtained at high growth rates. The fatty acid profile of rotifers reflected that of T-ISO, with maximum content of polyunsaturated fatty acids (PUFAs), n-3 fatty acids and docosahexaenoic acid (DHA) being found in the rotifers fed microalgae from the renewal rate of 40%. Results demonstrate that the biochemical composition of *B. plicatilis* is strongly modified through the use of semi-continuous cultures of microalgae in short-term enrichment processes. This technique provides an excellent tool to improve the nutritional value of the live feed used in fish larval cultures.

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1. Introduction

Microalgae play an essential role in aquaculture since they constitute the basis of the food chain, being the main diet for molluscs and initial larval stages of some crustacean and also for the production of live feed which is used for first-feeding of fish and crustacean larvae. The conditioning of water in fish larval tanks, known as “green water” techniques, constitutes another important use of microalgae for aquacultural purposes. However, microalgae are considered the bottleneck for the culture of molluscs (Persoone and Claus, 1980) and a serious limitation for the production of larvae of diverse marine finfish species, as their production is expensive and laborious (Wikfors and Ohno, 2001). One of the major applications of microalgae in aquaculture is the enrichment of rotifers in order to improve their nutritional content before being fed to fish larvae. Although artificially enriching diets are widely used, there is a growing trend to include microalgae in enrichment procedures. The effect of different microalgal species on the proximate biochemical composition of *Brachionus plicatilis* has been reported (Ben-Amotz et al., 1987; Whyte and Nagata, 1990; Whyte et al., 1994) demonstrating the importance of the biochemical composition of the food algae in modulating the biochemical composition of the filter-feeder. Good quality microalgae constitute an excellent diet for enrichment since they provide rotifers with essential fatty acids (Dhert et al., 2001), but frequently these high quality microalgae are not available in large enough quantities. The traditional culture systems, consisting of tanks, large cylinders or polyethylene bags that are used in batch mode often result in low productivity (Borowitzka, 1997) and do not allow control of the biochemical composition of the obtained biomass (Otero et al., 2002).

Continuous and semi-continuous culture has been proposed as an alternative to the generally used batch culture methods for the production of microalgae (Borowitzka, 1997). These systems not only provide higher and constant supplies of microalgal biomass, but their controlled conditions permit manipulation of the biochemical composition of the algal cells (Suknien and Wahnon, 1991; Otero and Fábregas, 1997; Otero et al., 1997). Hence, selecting the most appropriate conditions in continuous or semi-continuous cultures can strongly enhance the nutritional value of the microalgae. This has been demonstrated in culture experiments with filter feeders, in which an increase in the dilution rate of the microalgal culture improved assimilation efficiency in *B. plicatilis* (Scott, 1980), and somatic growth, survival and reproductive rate in *Artemia* (Fábregas et al., 1998, 2001). Although the modification of the biochemical composition of rotifers through the use of different species of microalgae is widely documented (Ben-Amotz et al., 1987; Frolov et al., 1991; Carri et al., 1993; Whyte et al., 1994), very few studies have approached the application of controlled-composition microalgae from continuous or semi-continuous cultures to improve the nutritional value of live feed (Øie et al., 1994; Fábregas et al., 1998, 2001). In this work, we propose the use of semi-continuous cultured microalgae for the short-term enrichment of *B. plicatilis*, studying the effect of the renewal rate applied to semi-
continuous culture of the marine microalga *Isochrysis aff. galbana* clone T-ISO, a species commonly used in the enrichment of rotifers for fish larvae because of its good fatty acid profile, on the biochemical composition of the rotifers.

2. Materials and methods

2.1. *Isochrysis aff. galbana* clone T-ISO

The marine microalga *Isochrysis aff. galbana* clone T-ISO (CCMP 1324) was cultured in 30 mm diameter glass tubes under a circadian light:dark cycle (12 h:12 h) and a light intensity of 162 μmol photon m−2 s−1 at a temperature of 21 °C. Each tube contained 80 ml of culture medium, prepared with autoclaved seawater (salinity 35‰) enriched with nutrients (KNO₃, 4 mM; NaH₂PO₄, 0.22 mM; ZnCl₂, 2.29 μM; MnCl₂, 3.37 μM; Na₂MoO₄, 2.72 μM; CoCl₂, 0.38 μM; CuSO₄, 0.465 μM; ferric citrate, 212 μM; thiamin, 1 mg L⁻¹; biotin, 13.4 μg L⁻¹; vitamin B₁₂, 9.4 μg L⁻¹; EDTA, 14 μmol L⁻¹), modified from Fábregas et al., 1985). Continuous aeration (230 ml min⁻¹) was provided through glass capillaries (diameter 1 mm), supplemented intermittently with CO₂ in order to keep pH below 8. Cultures were inoculated at a cell density of 7×10⁶ cells ml⁻¹ and once early stationary phase was reached, the semi-continuous regime was started, with daily renewal of 10, 20, 30, 40 and 50% of the culture volume. Renewals were carried out with autoclaved seawater enriched at the same nutrient concentration. Three replicates were set for each condition. Cell density was assessed daily in the harvested cultures by microscope cell counting using a Neubauer haemocytometer. Once the steady-state was achieved, the cultures were maintained for 1 week before being used for enrichment of the rotifers and for biochemical analyses.

2.2. B. plicatilis

*B. plicatilis* were cultured routinely in soft-aerated 6 l flasks with autoclaved seawater (salinity 35‰) and fed a mixture of *Isochrysis galbana* T-ISO, *Tetraselmis suecica*, *Nannochloropsis gaditana* and *Rhodomonas lens*. Periodically, rotifers were filtered with a 45-μm mesh size nylon net, rinsed with distilled water and transferred to a new flask containing autoclaved seawater. Previous to the experiment, rotifers were filtered and deprived of food for 12 h in order to avoid interferences from the gut content on the results. To carry out the enrichment, rotifers were transferred to flasks containing 700 ml of autoclaved seawater, at a final density of 200 individuals ml⁻¹. The food ration was calculated on a cell number basis (27,000 cells of T-ISO per individual equivalent to a cell density of 5.4×10⁶ cells ml⁻¹). This ration was sufficient to feed the rotifers during 24 h, as evidenced by a slight residue of microalgal cells in the rotifer cultures after that period. Patiño (1995) concluded that a ration of 2000 cells of *T. suecica* per rotifer, equivalent to 400 ng of dry weight, was enough to sustain feeding and provided a constant ingestion rate avoiding the existence of periods of gut repletion followed by intervals of decreased filtration activity. An average dry weight of 18 pg T-ISO cell⁻¹ regardless of the culture conditions was assumed for the calculation of the food ration, even thought the increase of renewal rate causes a decrease in the volume and organic content of microalgal cells (Fábregas et al., 1996). Three replicates of rotifer cultures were set for each renewal rate of the microalga. After 24 h of feeding, rotifers were collected. A sample of rotifers was collected after the 12 h starvation period and before the enrichment as a control group.

2.3. Sampling procedures

For dry weight determination, samples of 5 ml of microalgal culture or 100 ml of rotifer culture were filtered on previously weighed precombusted Whatman GF/C fiberglass filters. Microalgal samples were dried three times with 5 ml of 0.5 M ammonium formate in order to remove salts and rotifer samples were rinsed with distilled water. Filters were dried overnight at 80 °C and dry weight determined gravimetrically. For biochemical analyses, microalgal samples (5 to 10 ml) were collected by centrifugation and immediately frozen at −20 °C. 80 ml of rotifer culture were collected on a sieve (45 μm mesh size), rinsed with distilled water and frozen at −20 °C for fatty acid analysis. The remaining rotifers were also collected on a sieve and freeze-dried for the rest of the biochemical analyses. Feed conversion rates (FCR) were calculated as the ratio between dry or organic (protein + lipid + carbohydrate) weight of T-ISO supplied and the equivalent increase in rotifer biomass.

2.4. Biochemical analyses

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

2.5. Statistical analysis

The statistical treatment of the data was performed with the non-parametrical Mann–Whitney *U* test. All analyses were conducted using SPSS 14.0 for Windows.

3. Results

Cell density in steady-state T-ISO cultures decreased as renewal rates increased, whereas productivity showed a parabolic-shaped development, reaching its maximal value with the renewal rate of 30% (Fig. 1). The different renewal rates modified nutrient and light availability, thus affecting the biochemical composition of the algal cells. The C:N ratio decreased to one half between the renewal rates of 10 and 40% (Fig. 2) and remained stable with higher renewal rates (Fig. 2). The stability in the C:N ratio indicates nitrogen saturation in the microalgal cultures (Otero et al., 1998). Higher nitrogen availability allowed microalgal cells to accumulate protein contents of 5.01 and 4.16 pg cell⁻¹ at the renewal rates of 40 and 50% respectively, while only 3.41 pg cell⁻¹ of protein was obtained with a renewal rate of 10%. The Mann–Whitney *U* test showed that these protein contents were significantly higher (*P*<0.05) than those obtained in nitrogen-limited conditions, i.e. renewal rates from 10
to 30% (Fig. 2). Culture conditions also modified carbohydrate and lipid contents. Carbohydrate was the fraction most affected by the increase of renewal rates, with cell content decreasing significantly ($P<0.05$) from 10.19 pg cell$^{-1}$ to 2.69 pg cell$^{-1}$ between the renewal rates of 10 and 40%, whereas lipids decreased significantly from 6.47 pg cell$^{-1}$ at the renewal rate of 10% to 4.23 pg cell$^{-1}$ at the renewal rate of 30% ($P<0.05$) and stabilized onwards. As a consequence, the total organic content of T-ISO cells decreased as renewal rate increased (Fig. 2).

Variations in the biochemical composition of T-ISO caused by the different renewal rates strongly modified its nutritional value for *Brachionus plicatilis*. Final rotifer dry weight was inversely proportional to the weight of microalgae supplied, since an equal number of cells per individual were provided but cell dry weight decreased with increasing renewal rates (Fig. 2). Consequently, feed conversion rate (FCR), calculated as the ratio between dry weight of T-ISO supplied and the increase in rotifer biomass, underwent a 2-fold decrease as nutrient availability in the microalgal culture medium became higher and reached its lowest value in nitrogen-saturation conditions, i.e. with renewal rates of 40 and 50%, indicating that rotifers assimilated more efficiently microalgae from the higher renewal rates. Organic FCR, calculated as the ratio between organic weight of T-ISO supplied and the increase in organic microalgae or rotifers, showed the same trend, but its decrease was even steeper, more than 3-fold between the renewal rates of 10 and 50% (Table 1).

Control rotifers, sampled just before starting the enrichment experiment, showed low dry weight, 115.3±16.3 ng individual$^{-1}$ (Fig. 2). After the enrichment, rotifers showed significant ($P<0.05$) increases in dry and organic weight with regard to the control individuals. The groups of rotifers enriched with T-ISO from renewal rates from 10 to 30% reached similar dry weights, around 300 ng individual$^{-1}$, whereas in rotifers fed microalgae from the higher renewal rates, the dry weight continued to increase, reaching 460.7±11.2 ng individual$^{-1}$ at a renewal rate of 50%. Dry weight was significantly different in all groups ($P<0.05$), despite the similar values observed in rotifers enriched with T-ISO from nutrient-limited renewal rates, i.e. 10 to 30% (Table 1). Organic content followed the same pattern as dry weight and remained almost constant in rotifers enriched with T-ISO cells from nutrient-deficient cultures, but increased markedly in rotifers fed nutrient-sufficient microalgae. This development contrasts with the drop in the organic content of T-ISO as renewal rate increased. No statistical differences were found between the protein, lipid or carbohydrate content of the rotifers enriched with microalgae from renewal rates of 10, 20 and 30%. In contrast, for rotifers fed T-ISO cells from nitrogen-sufficient cultures, the same three components, protein, lipid and carbohydrate, increased significantly with regard to nutrient-limited conditions, and significant differences between renewal rates of 40 and 50% were also observed ($P<0.05$). Protein content varied between 91 and 100 ng individual$^{-1}$ in rotifers fed microalgae from the renewal rates from 10 to 30% and increased to 158 ng individual$^{-1}$ in rotifers fed cells from the renewal rate of 50%. The increase in lipid content was less, from 33 ng individual$^{-1}$ at the renewal rate of 10% to 45 ng individual$^{-1}$ at the renewal rate of 50%, while carbohydrate content almost doubled, from 28 to 49 ng individual$^{-1}$ (Fig. 2). Despite the differences found in the gross biochemical composition expressed as individual content, percentages of protein, lipid and carbohydrate in the organic fraction of the rotifers did not change significantly, being 61–63% for protein, 17–21% for lipid and 17–20% for carbohydrate. Control rotifers showed a higher percentage of protein, 69.5%, and a lower percentage of carbohydrate, 11%, compared to enriched rotifers (data not shown). In agreement with the trend observed for FCR and organic FCR, percentages of recovery of the organic fractions tended to increase with the renewal rate. Percentages of protein recovery did not follow any particular trend and ranged from 58 to 67% between the renewal rates of 10 and 40%; however, the highest value, 95%, was obtained with the renewal rate of 50%. Lipid and carbohydrate recoveries underwent a continuous increase with renewal rate, from 13 to 27% for lipid and from 7 to 55% for carbohydrate (Table 1).

Culture conditions affected the fatty acid profile of the microalga and those changes were reflected in the rotifer. The increase in the renewal

![Fig. 2. Composition of the organic fraction of *Isochrysis aff. galbana* clone T-ISO cultured semi-continuously with different renewal rates (left) and *Brachionus plicatilis* enriched for 24 h with the same microalgal cultures (right), expressed as pg · cell$^{-1}$ and mg · rotifer$^{-1}$ respectively. Crosshatched bars, protein; hatched bars, lipid; open bars, carbohydrate; ○, C: N ratio. C: rotifer control sample, harvested before the start of the enrichment.](image-url)

**Table 1**

<table>
<thead>
<tr>
<th>Renewal rate</th>
<th>10%</th>
<th>20%</th>
<th>30%</th>
<th>40%</th>
<th>50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-ISO consumed (µg ml$^{-1}$)</td>
<td>92.94</td>
<td>97.79</td>
<td>75.17</td>
<td>61.77</td>
<td>76.76</td>
</tr>
<tr>
<td>Organic weight of T-ISO consumed (µg ml$^{-1}$)</td>
<td>91.38</td>
<td>79.99</td>
<td>60.33</td>
<td>50.37</td>
<td>46.19</td>
</tr>
<tr>
<td>Rotifer dry weight (ng individual$^{-1}$)</td>
<td>312.38±18.81</td>
<td>296.76±59.49</td>
<td>281.62±41.62</td>
<td>284.06±5.67</td>
<td>474.63±104.62</td>
</tr>
<tr>
<td>FCR (weight of T-ISO consumed/increase in rotifer weight)</td>
<td>3.1</td>
<td>2.9</td>
<td>2.1</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Organic FCR (organic weight of T-ISO consumed/increase in rotifer organic weight)</td>
<td>2.6</td>
<td>2.7</td>
<td>2.1</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>% Protein recovery</td>
<td>67.52</td>
<td>58.15</td>
<td>72.33</td>
<td>63.68</td>
<td>59.07</td>
</tr>
<tr>
<td>% Lipid recovery</td>
<td>13.00</td>
<td>13.61</td>
<td>15.02</td>
<td>23.48</td>
<td>26.68</td>
</tr>
<tr>
<td>% Carbohydrate recovery</td>
<td>7.70</td>
<td>11.50</td>
<td>24.24</td>
<td>45.44</td>
<td>54.82</td>
</tr>
</tbody>
</table>

Values in the same row with different superscripts are significantly different ($P<0.05$).
rate resulted in a strong increase in the percentage of polysaturated fatty acids (PUFAs) in T-ISO, from 29.5% of the total fatty acids with a renewal rate of 10% to 46.36% in the cultures with a renewal rate of 40% (Table 2). The main polysaturated fraction corresponded to n-3 fatty acids, which also reached its maximum percentage, nearly 35%, with a renewal rate of 40%. The most abundant PUFAs in T-ISO was 18:4n-3, that reached its highest percentage, 1.72%, in the renewal rate of 40% (Table 2). At the renewal rate of 40%, increasing from 6.28% to 9.88% between the renewal rates of 10 and 40%. Eicosapentaenoic acid (20:5n-3) content was very low in T-ISO, ranging between 0.41% and 1.65% of the total fatty acids in T-ISO cells, as algae with low C:N ratios and thus high relative content of assimilable nitrogen improved assimilation efficiency (Table 2). The increase in the percentage of PUFAs was consistent with the results of previous studies (Droop and Scott, 1978) and reinforced the hypothesis that microalgal species can be used as a feed source for rotifers. The increase in the percentage of PUFAs with increasing renewal rates, up to 5% in the rotifers fed microalgae from the renewal rates of 10% to 40% (Table 2). The percentage of DHA increased slightly with increasing renewal rates, up to 5% in the rotifers fed microalgae from the renewal rate of 40%. The percentage of EPA also reached its maximum value, 1.72%, in the renewal rate of 40% (Table 2). The increase in the percentage of PUFA cells, as algae with low C:N ratios and thus high relative content of assimilable nitrogen improved assimilation efficiency and somatic growth (Whyte and Nagata, 1990). However, no differences were observed in the composition of rotifers fed different microalgal species when the algae were cultured under the same conditions of semi-continuous regime (Oie et al., 1994). On the contrary, the results of experiments on short-term enrichment are more divergent. Although Scott and Baynes (1978) attributed only minor effects to the species of microalgae used on the biochemical composition of rotifers sampled at 24 h intervals, other studies found changes in the composition of the rotifers after 6 h of enrichment, and the existence of correlation between the composition of the rotifiers and that of the microalgae after feeding periods of 48 h (Frolow et al., 1991). Continuous increases in the individual protein and lipid contents and the dry weight of B. plicatilis enriched with microalgae up to 48 h have also been reported (Reitan et al., 1997). A change in the rotifer’s diet from yeast and emulsified oil to T-ISO modified the percentages of some fatty acids after a feeding period of 23 h (Lie et al., 1997). After 24 h of enrichment with T-ISO cultured in semi-continuous regime, rotifer dry and organic weight underwent up to 4-fold and 5-fold increases respectively, with increasing renewal rate. Droop and Scott (1978) calculated that the gut content from actively feeding rotifers constituted approximately 12% of the total rotifer carbon (Scott, 1980). The incorporation of nutrients into animal tissue is expected to be low in short-term feeding experiments, and modifications of the biochemical composition have been attributed mainly to the presence of feed in the digestive tract, whose contribution to the organism total weight, as seen above, is also limited. In contrast, our results exceed the figure proposed by Droop and Scott (1978) for gut content and hence suggest a rapid and high assimilation of microalgal nutrients into the rotifer body tissue, that strongly depends on the culture conditions of the microalgae. Consequently, even though it is generally accepted that rotifers act as “carriers”, transferring the content of their guts to fish and crustacean larvae which can easily assimilate the nutrients contained in the partially digested algal biomass (Lubzens et al., 1989; Wikfors and Ohno, 2001), changes generated in the weight and biochemical composition of the rotifer in only 24 h indicate that this might not be totally accurate.

Changes in the biochemical composition of the rotifers showed two different trends. Individuals enriched with T-ISO cultured under nitrogen limitation incorporated similar contents of protein, lipid and carbohydrate regardless of the renewal rate applied to the microalgal cultures and thus independently of both the degree of nutrient limitation and the variations in the composition of T-ISO cells. The nutritional status of these T-ISO cultures is similar to those of batch culture systems commonly used in aquaculture facilities, which are harvested at the end of the exponential phase after nutrient depletion. Once nitrogen sufficiency was reached, incorporation of nutrients from the microalgae to the rotifers increased with increasing renewal rates. On the contrary, differences in nutrient availability in continuous cultures of T. suecica did not cause modifications of the biochemical composition or the survival of tiger prawn Peneaus semisulcatus larvae (DSouza and Kelly, 2000), although the nutritional status of their microalgal cultures was not clearly established. The decrease in the content of storage compounds, carbohydrate and lipid, with high renewal rates probably enhanced the digestibility of T-ISO cells, as algae with low C:N ratios and thus high relative content of assimilable nitrogen improved assimilation efficiencies and somatic growth.
growth in Artemia (Sick, 1976). However, neither the changes in the biochemical composition of B. plicatilis nor the evolution of FCR are directly related to the increase in renewal rate, the change of the nutritional status of the algal cultures or the evolution of the biochemical components of T-ISO cells. Similarly, Fábregas et al. (1998, 2001) could not find a single biochemical parameter of Phaeodactylum tricornutum or T. suecica cultured in a semi-continuous system with different renewal rates to explain the effect on growth and survival of Artemia.

Protein and lipid contents underwent between 3-fold and 4.5-fold increases in relation to the values of initial control rotifers, higher than the data reported by Reitan et al. (1997) after 24-h enrichment with T. suecica and I. galbana, with increases between 50 and 70% with regard to the initial values. Particularly, the enrichment with T-ISO from the renewal rate of 50% resulted in individual protein content comparable to those obtained by enriching rotifers with a high-protein artificial diet (Øie et al., 1997). Experiments in turbot larvae rearing have indicated the importance of the protein content of live feed, as higher growth and survival were observed in larvae fed rotifers rich in protein (Øie et al., 1997). However, lipid emulsions proved to be more efficient than T-ISO to improve the lipid content of rotifers, as up to 100 ng lipid/rotifer was achieved after 24 h of enrichment (Øie and Olsen, 1997; Øie et al., 1997). The increase in DHA content of T-ISO with increasing variable growth and feeding condition. Hydrobiologia. 358, 251–258.


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