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Nannochloropsis limnetica: A freshwater microalga for marine aquaculture

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ABSTRACT

Despite the availability of inert commercial feeds, microalgae are still the preferred feed for mass production and enrichment of the rotifer *Brachionus plicatilis*. Although good growth results can be obtained with different freshwater *Chlorella* species, marine species of the genus *Nannochloropsis* are most commonly used due to their high growth rate and content in polyunsaturated fatty acids, mainly EPA. In this work we explored the response of the freshwater species *Nannochloropsis limnetica* to different temperatures in semi-continuous cultures and compared its nutritional value for the rotifer *B. plicatilis* to that of the marine species *Nannochloropsis gaditana* both at laboratory scale and in hatchery facilities. *N. limnetica* could be cultured in the range 15–27 °C with highest dry-weight productivities at 22 °C. When compared with *N. gaditana* in semicontinuous cultures at laboratory scale, with a daily 40% harvesting rate, productivity of both species was similar, reaching a daily production of 0.64 g L^{-1} day^{-1}. Both species have an almost identical fatty acid profile. In laboratory-scale cultures with high algal rations, growth and egg-ratios of the rotifer *B. plicatilis* cultured with *N. limnetica* were more than twice than with the same doses of *N. gaditana*, while maintaining the same fatty acid profile in the filter-feeder, confirming the potential of this freshwater species for marine aquaculture. In hatchery-scale experiments in which *B. plicatilis* was fed with baker's yeast supplemented either with on-site produced fresh microalgal cultures or with concentrated algae, similar growth results were obtained for both microalgal species in 5-day batch cultures, although higher dry weights and slightly better egg ratios were observed with the freshwater species. Results indicate the potential of this freshwater species as a substitute of freshwater *Chlorella* in live-feed production protocols, due to its better fatty acid profile.

Statement relevance: The freshwater microalga *Nannochloropsis limnetica* promotes higher growth in the rotifer *Brachionus plicatilis* than the marine species *N. gaditana*. Results indicate the potential of this species as a substitute of freshwater *Chlorella* due to its better fatty acid profile. Moreover, no potentially pathogenic marine bacteria would be present in cultures of the freshwater species in comparison to its marine counterparts.

1. Introduction

Different marine species of the genus *Nannochloropsis* are used worldwide in the live food chain required for the cultivation of marine fish larvae, and are among the most commonly used microalgal species in many mariculture systems. Their main use is the cultivation of rotifers of the genus *Brachionus* that are used as live feed for fish larvae. Rotifer culture is an aquaculture process that demands high amounts of microalgae, since this is the only diet allowing the production of sustained continuous rotifer cultures at high densities (Yoshimura et al., 2003; Bentley et al., 2008) and provide better growth and biochemical composition compared to baker's yeast (Lubzens et al., 1995) and artificial diets (Aragão et al., 2004; Srivastava et al., 2006; Koiso et al., 2009). Fu et al. (1997) estimated that commercial concentrated microalgae represented up to 86% of the production costs even in efficient, high-density rotifer culture (James and Abu-Rezeq, 1988; Sukenik et al., 1993; Ferreira et al., 2009). Condensed freshwater *Chlorella* is being successfully used for maintaining dense cultures of *Brachionus* sp., but marine species of the genus *Nannochloropsis* have been demonstrated to produce equal or higher growth rates (Maruyama et al., 1997; Kobayashi et al., 2008). The main advantage of *Nannochloropsis* over other unicellular algae is its high content in eicosapentaenoic acid (EPA, 20:5n-3) that is transferred through the rotifers to fish larvae. The crucial role of highly unsaturated fatty acids (HUFAs) in the

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successful rearing of many marine aquaculture species has been extensively documented (Izquierdo, 1996; Tocher, 2010). In freshwater environments, the production of HUFAS by phytoplankton has been also identified as a key factor controlling food web interactions, (Müller-Navarra et al., 2000). At present different commercial products are available based on chilled, frozen, condensed or freeze dried marine *Nannochloropsis* that have been shown to produce good rotifer growth (Lubzens et al., 1995; Navarro et al., 2001).

Nannochloropsis spp. have been extensively studied as source of EPA for nutritional purposes (Sukenik, 1998; Chini Zittelli et al., 1999) and more recently a renewed interest was due to the potential of these species for biodiesel production (Rodolfi et al., 2008; Doan et al., 2011; San Pedro et al., 2013). Although most of the *Nannochloropsis* species that have been studied so far have been isolated from marine or saline habitats, the freshwater species *N. limnetica* was first isolated from a small inland pond in Germany, and seems to be present in many freshwater habitats (Krienitz et al., 2000). The fatty acid composition of *N. limnetica* is similar to that of marine species, with EPA contents close to 24% of total fatty acids, but with a higher content of total fatty acids (Krienitz et al., 2000). Little literature is available on the cultivation and nutritional value of *N. limnetica* (Krienitz and Wirth, 2006). The zebra mussel (*Dreissena polymorpha*) showed better reproductive performance and its larvae grew better when fed *N. limnetica* in comparison to the non LC-PUFA containing freshwater species *Scenedesmus obliquus* and the cyanobacterium *Aphanothece* (Wacker and von Elert, 2003; Wacker et al., 2002).

Marine *Nannochloropsis* spp. may be suitable for biofuel production since they would not compete with agricultural species for freshwater supply (San Pedro et al., 2013), whereas *N. limnetica* could be of interest for specific applications such as wastewater treatment. Moreover, since the presence of potentially pathogenic *Vibrio* spp. is common in the microalgal cultures used in hatcheries (Dubert et al., 2015), another possible advantage of the freshwater species is the absence of marine pathogens, which have been consistently related to low survival in fish larvae cultures and can be transmitted through live feed (Grisez et al., 1996). In this work we explored the viability of mass-cultivation of *N. limnetica*, from laboratory scale to pilot microalgal culture plant and hatchery facilities and compared the nutritional value of the freshwater species to that of the marine *N. gaditana* for the rotifer *Brachionus plicatilis*, both at laboratory scale and in large scale cultures using standard hatchery protocols.

2. Materials and methods

2.1. Microalgal cultures

Nannochloropsis limnetica Krienitz (SAG 18.99) and *Nannochloropsis gaditana* Lubián (CCMP 527) were cultured semi-continuously in 30 mm diameter glass tubes containing 80 mL of culture medium, with continuous aeration (200 mL min^{-1}) enriched with CO_2 to maintain pH between 7.5 and 7.8. Cultures were submitted to circadian light/dark regime (12/12 h) with an irradiance of 245 μE m^{-2} s^{-1} at a temperature of 20 ± 1 °C. Cultures where inoculated and allowed to reach early stationary phase before the semi-continuous regime was started by harvesting 40% of the volume of the culture daily during the first hour of the light cycle and replacing with fresh culture medium (Otero and Fábregas, 1997). Once the steady-state was achieved, samples were collected for biochemical analysis and biomass was used to feed the rotifers. All cultures were carried out in triplicate.

The fresh water microalga *N. limnetica* was cultured in OHM culture medium (Fábregas et al., 2000 adjusted to a N concentration of 10 mM KNO_3 (Fábregas et al., 2000). *Nannochloropsis gaditana* was cultured in autoclaved seawater (salinity adjusted to 35 g L^{-1}) enriched with modified Algal medium with 10 mM $NaNO_3$ (Fábregas et al., 1984).

N. limnetica was also cultured at different temperatures at laboratory scale keeping the culture tubes in polycarbonate (Makrolon®) water baths. The low temperature (15 °C) was obtained with a cooling unit frigedor. Reg (Selecta), and submersible heaters (Tetral T01212) were used for 22, 27 and 32 °C. Effective irradiance in the temperature experiment was 187 μE m^{-2} s^{-1} due to the absorption of containers and water used to maintain the desired temperature.

Industrial large scale cultures of *N. limnetica* and *N. gaditana* were carried out in the facilities of AlgaEnergy S.A. (Barajas, Madrid) during May and June 2013. Cultures were carried out in a greenhouse in either 100 L columns (diameter 25 cm) or flat panel photobioreactors with natural irradiance. Cultures were carried out with a nutrient concentration equivalent to 10 mM $NaNO_3$ with a proprietary formulation and industrial-grade reagents. pH was maintained between 7.8 and 7.9 by CO_2-enriched aeration. No temperature control was applied reaching maximum of 30 °C during the period monitored. Reactors were maintained under semi-continuous regime by harvesting 30% of the cultures daily. The biomass was concentrated by centrifugation and adjusted to a final concentration of 10 g L^{-1} and kept refrigerated at 4 °C until use.

Additionally, both species were cultured in standard 400 L plastic bags with aeration in the hatchery facilities of the Spanish Oceanographic Institute (Murcia). Cultures were maintained in a greenhouse with natural lighting, inoculated with 25 L of culture obtained in smaller plastic bags maintained under continuous artificial light (40 μE m^{-2} s^{-1}). A commercial nutrient solution was used through all phases of the culture (Nutriphyt®, Fitoplancton Marino SL, Spain). These cultures were used to feed the rotifers once a cell density of 25–40 × 10^6 cells mL^{-1} was reached in the bags.

2.2. Rotifer rearing experiments

At laboratory scale the rotifer *Brachionus plicatilis* Müller was routinely cultured in 6-L carboys containing autoclaved seawater (salinity 35 g L^{-1}) at a temperature of 20 ± 1 °C and fed a mixture of microalgae (*Nannochloropsis gaditana*, *Rhodomonas lens*, *Tetraselmis suecica*, *Isochrysis galbana* and *Isochrysis* aff. *galbana* clone T-ISO). Rotifers were concentrated by sieving, rinsed and transferred to a new carboy and starved during 12 h to minimize the effect of previous feeding. *B. plicatilis* was cultured in 1 L flasks (three replicates) containing 500 mL of autoclaved seawater with gentle aeration. The initial rotifer density was 50 individuals mL^{-1}. Rotifers were fed *N. gaditana* and *N. limnetica* at a rate of 120,000 cells per individual per day for a period of 5 days. This ration was calculated on the basis of previous experiments (Ferreira et al., 2009) in order to avoid food limitation during the experiment (more than 10% of cells remaining available after 24 h).

For the large-scale experiments, cultures were carried out in 550 L tanks. Three replicates were set for each condition. The routine used in the hatchery for standard cultivation of rotifers was applied for the comparison of fresh microalgal cultures of *N. limnetica* and *N. gaditana*. Cultures (initial volume 175 L: 90 L of seawater, 35 L of freshwater and 50 L of microalgal culture) were inoculated at a rotifer density of 200 rotifers mL^{-1}. The volume of the cultures was increased daily by adding: 25 L of algal culture the 2nd day and 25 L of algal culture and 12.5 L of freshwater the 3rd and 4th days. Cultures were harvested on the 5th day, with a final volume of 275 L. Additionally, the cultures were supplied daily with 0.3 g of freeze-dried *S. cereviseae* per 10^6 rotifers. The daily ration of microalgae supplied to the rotifers was between 70,000–500,000 cells per rotifer the first day, decreasing to 20,000–100,000 cells per rotifers from day 2 to 4. Daily, temperature (22.5 ± 0.2 °C) and dissolved oxygen (4–5.5 mg L^{-1}) was measured in the tanks and the number of rotifers and egg-bearing females was counted. Due to the addition of significant volumes of freshwater or marine microalgal cultures, final salinities in the rotifer cultures were 30–34‰ in the *N. gaditana* tanks and 22–25‰ in the *N. limnetica* tanks. The experiment was repeated with twice the volumes. In this case cultures were initiated with 350 L and reached 550 L on the 5th day of culture, following the same dilution procedure explained above. The same procedure was followed in the experiment in which algal concentrates