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Picochlorum as an alternative to *Nannochloropsis* for grouper larval rearing

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ABSTRACT

Nannochloropsis oculata is widely used in grouper larvae hatcheries for the production of green water to stabilize water quality, provide shielding for light-sensitive fish larvae, and breed super-small rotifers (SS-rotifers), the first feed of grouper larvae. However, the practical use of *N. oculata* is hindered by an insufficient yield of this alga. This study evaluated *Picochlorum* strain S1b, an indigenous marine microalga capable of cultivation at high growth rates, to determine its suitability as an alternative to *N. oculata* in grouper larvae hatcheries. Feasibility was evaluated mainly according to two parameters: 1) the nutritive value of the microalga for SS-rotifers; and 2) the nutritive value of SS-rotifers for grouper (*Epinephelus coioides*) larvae. The nutritive value of algae was evaluated by comparing the proliferation rate and egg ratio of SS-rotifers. The nutritive value of SS-rotifers was evaluated by comparing the size and amino acid composition of SS-rotifers feeding on different microalgae. We also examined the "green water effect", i.e., changes in the vibrio community in the rearing tank following the introduction of microalgae, as well as its influence on the survival and growth rates of grouper larvae.

In these experiments, S1b provided the highest nutritive value for SS-rotifers, particularly when grown under mixotrophic conditions (S1b [mixo]). The cultivation of SS-rotifers using S1b [mixo] increased the percentage of small-size organisms, which are highly suitable as first feed for grouper larvae; and cultivation with S1b [mixo] altered the amino acid composition of SS-rotifers to more closely match that of grouper larvae, thereby enhancing their nutritive benefits.

Green water produced with either *Picochlorum* S1b or *N. oculata* was shown to inhibit the blooming of vibrio, an opportunistic pathogen of fish. Grouper larvae reared in green water produced with *Picochlorum* S1b had the same survival rate but a higher growth rate than those reared in green water produced with *N. oculata*. These results demonstrate the potential benefits of replacing *N. oculata* with *Picochlorum* S1b for the rearing of grouper larvae.

1. Introduction

Grouper is one of the most important warm-water aquaculture species in the world, particularly in Southeast Asia (Harikrishnan et al., 2010; Kohno et al., 1997). Global production of grouper has increased dramatically in recent years, from 60,000 metric tons in the early 1990s to nearly 200,000 metric tons in 2007 (Harikrishnan et al., 2010). Due to various breakthroughs in grouper larviculture, an increasing number of species are being produced on a commercial scale without the need to capture wild larvae (Harikrishnan et al., 2010; Liao et al., 2001; Tucker, 1999).

There are two different types of grouper larviculture: outdoor culture in mesocosm ponds, and indoor culture using tanks made by fiber-reinforced plastic (FRP) or concrete (Liao, et al., 2001). In the outdoor culture method fertilized eggs are introduced into open ponds with naturally occurring microalgae and zooplankton, which serve as feed for grouper larvae. The costs associated with this process are relatively low and the management easy; however, production of grouper larvae is very unstable, due to the damage caused by infectious diseases (Liao et al., 2001). Nerve necrosis virus (NNV) is a major viral pathogen infecting grouper in hatcheries, resulting in severe losses in many countries (Chi et al., 1997; Harikrishnan et al., 2010). Because indoor larviculture provides better protection against NNV infection, it has become increasingly popular in Taiwan (Liao et al., 2001). In modern grouper larvae hatcheries, fertilized eggs are disinfected using UV radiation or ozone to reduce the risk of vertical transmission of NNV from broodstock to fish larvae. Seawater and equipment are carefully

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treated with chlorine to kill pathogens, and live preys such as microalgae, rotifers (*Brachionus* spp.), and *Artemia* are cultivated on-site (rather than in open ponds) to ensure that they remain pathogen-free (Liao et al., 2001). Microalgae are also used to make green water systems in fish tanks to maintain water quality, provide shield effect, and maintain the nutritional quality of rotifers for fragile grouper larvae (Rimmer, 2000).

Nannochloropsis oculata (synonymous with marine *Chlorella*) is among the most popular microalga for indoor grouper larviculture (Nagano et al., 2007; Sugama et al., 2003; Yoseda et al., 2006), due to its high nutritive value for rotifers (Coutteau, 1996; Liao et al., 2001), the mouth-opening preys of grouper larvae. *N. oculata* also inhibits the opportunist pathogen vibrio (Sharifah and Eguchi, 2011), which infects grouper larvae (Liao et al., 2001) and retards the growth of rotifers (Tinh et al., 2007). Inefficient production is a major drawback of *N. oculata*, particularly during the summer and rainy season (Hirayama et al., 1989; Maruyama et al., 1997). *N. oculata* culture is also prone to contamination by protozoa, which has a detrimental effect on the stability of water quality, often leading to the crash of grouper larvae culture (Liao et al., 2001).

Various other microalgae have been used in grouper indoor larviculture. *Isochrysis* has proven effective in the production of green water to improve the growth and survival of grouper larvae (Su et al., 1997). *Tetraselmis chui* has been used to rear rotifers, demonstrating proliferation and growth rates exceeding those of rotifers fed on *N. oculata* (Su et al., 1994). However, the application of these two microalgae has also been restricted by slow growth rates. The freshwater microalga *Chlorella* (e.g. *Chlorella vulgaris*, K-22) is easily cultivated under mixotrophic or heterotrophic conditions to much higher densities than autotrophic microalgae (e.g. *Nannochloropsis*, *Isochrysis* and *Tetraselmis*). Condensed *Chlorella* paste has been developed specifically for application in larviculture to avoid the labor-intensive task of cultivating microalgae on-site (Dhert et al., 2001; Hirayama et al., 1989; Lee, 2003; Nagano et al., 2007). *Chlorella* is enriched with vitamin B_{12}, making it comparable to *N. oculata* in terms of nutritive benefit to rotifers (Hirayama et al., 1989; Maruyama et al., 1997). *Chlorella* provides a similar bacteriostatic effect on vibrio (Makridis et al., 2006), making it applicable to the production of green water for raising the larvae of gilthead seabream *Sparus aurata*, milkfish *Chanos chanos*, Mahimahi *Coryphaena hippurus* (Coutteau, 1996), seven-band grouper *Epinephelus septemfasciatus* (Thunberg) (Nagano et al., 2007), and dusky grouper *Epinephelus marginatus* (Russo et al., 2009). *Chlorella* represents the best candidate to replace *N. oculata* in larviculture. However, to the best of our knowledge, *Chlorella* paste is not used in many areas, due perhaps to the high price and short shelf life (less than one month) of this product.

This study evaluates the feasibility of using the local *Picochlorum* strain S1b for indoor grouper larviculture. This strain was selected for a number of reasons: 1. The growth rate exceeds that of *N. oculata* in local grouper larviculture particularly during summer months; 2. The size is similar to that of *N. oculata*, which facilitates consumption by super small rotifer (SS-rotifer) equipped with small mouth gape.

This study has two specific aims:

(1) To determine the suitability of S1b as bait for SS-rotifers. The nutritive value of S1b for SS-rotifers, and the nutritive value of S1b-fed SS-rotifers for grouper (*Epinephelus coioides*) larvae were compared by feeding SS-rotifers with *N. oculata* and commercial algal paste made from *Chlorella*.
(2) To determine the applicability of S1b in the production of green water for rearing grouper larvae. We compared changes in bacterial count in the water of fish tanks following the introduction of various microalgae and examined their influence on the survival and growth rates of grouper larvae.

2. Materials and methods

2.1. Algae used in this study

S1b is an axenic algal strain purified from a nearly dried-out fish pond in Tainan City, Taiwan, according to the methods described (Yang et al., 2010). The temperature and salinity of the water in the pond were 30 °C and 58 parts per thousand (ppt), respectively. For the purpose of identification, the 18S rDNA of this strain was cloned and sequenced as described in the above study. This 18S rDNA sequence then served as the query sequence to identify similar sequences in the public database on the website of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The basic local alignment search tool (BLAST) was used to perform this operation. The following identification was based on the construction of a phylogenetic tree with strains belonging to either *Picochlorum* or *Nannochloris* described by Henley et al. (2004), revealing a closer phylogenic relationship between this strain and *Picochlorum*, than with *Nannochloris*. The 18S rDNA sequence of S1b is registered in the NCBI database with the accession code JN191236.

The *N. oculata* strain TF7 was provided by the Tungkang Biotechnology Research Center, Fisheries Research Institute, Taiwan. Chlorella V12®, an algal product made from *C. vulgaris* specifically for the mass cultivation of rotifers, was purchased from Chlorella Industry Co. (Tokyo, Japan).

2.2. Cultivation of microalgae

The S1b and TF7 were scaled up using either phototrophic or mixotrophic cultivation systems developed in-house. The phototrophic system was semi-open, comprising 10 cylindrical transparent tanks (i.d. = 40 cm, volume = 120 L) of poly (methyl methacrylate). Each tank contained 90 L of fresh Walne's medium (Walne, 1970) prepared with full-strength seawater sterilized by the addition of chlorine (Coutteau, 1996). This was followed by the inoculation of 10 L of algal stock culture (0.5 g L^{-1}, cultivated in sterile Walne's medium). The mixotrophic system was a closed system comprising six individual 20-L polycarbonate carboys, each of which was filled with 9.8 L of fresh M3 medium (4.9 L Walne's medium + 4.9 L full-strength seawater, with glucose and yeast extract added to final concentrations of 6.0 and 4.5 g L^{-1}, respectively). This was autoclaved and then inoculated using 0.2 L sterile S1b culture (2.5 g L^{-1}, cultivated in a 500 mL flask using the same M3 medium). The inoculation was performed in a laminar flow hood to avoid contamination by microorganisms.

Each variety of microalgae was cultivated using three cylindrical tanks in a phototrophic system; and separately cultivated in a mixotrophic system using three carboys. These were maintained at 28 ± 1 °C, illuminated at 6000 lx with a fluorescent light for 12 h per day, and aerated at 0.1 vvm (gas volume flow per unit of liquid volume per minute) with air pre-filtered through a Millex-FG 0.2 μm syringe filter (Millipore, Billerica, MA, USA). Fifty mL of algal culture was collected from each tank or carboy every day, centrifuged (5000 × *g*, 5 min), rinsed using deionized water, lyophilized, and then weighed to determine the dry biomass.

After seven days of cultivation, the S1b and TF7 from each system were harvested using centrifugation (5000 × *g*, 5 min), rinsed with 20 ppt diluted seawater to remove residual medium, and stored in the form of algal paste at 4 °C prior to use.

2.3. Proliferation rate, egg ratio, and size distribution of SS-rotifers feeding different microalgae

The proliferation rate and egg ratio of *Brachionus* sp. were determined using the "individual culture method" described in previous