RESEARCH ARTICLE

Assessing the Viability of Commercial Media for the Mass Culture of *Chaetoceros muelleri*

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ABSTRACT

The microalgae *Chaetoceros muelleri* is considered a highly nutritious feed for the cultured larvae of the tropical sea cucumber *Holothuria scabra*. Due to the cost of analytical grade culture media used in the production of *C. muelleri*, there is a need to evaluate cheap alternative commercial media to decrease the cost of producing quality live microalgal food. In this study, two different indoor batch culture systems (1 L glass bottles and 10 L plastic carboys) were used to evaluate the effectiveness of two conventional (modified F/2 and Walne's) and one commercial (Epizyme AGP complete) microalgal culture media. Results of the 1 L glass bottle experiment showed that the peak cell density of *C. muelleri* in AGP (1,241 ± 116 x 10^4 cells ml^{-1}) was not significantly different from the modified F/2 (1,584 ± 41 x 10^4 cells ml^{-1}) and Walne's medium (1,319 ± 162 x 10^4 cells ml^{-1}) (Kruskal-Wallis test, p=0.78). Likewise, in the plastic carboy experiment, the maximum cell density of *C. muelleri* in Walne's medium (750 ± 144 x 10^4 cells ml^{-1}) and F/2 medium (653 ± 79 x 10^4 cells ml^{-1}) were higher, but not significantly different from AGP (496 ± 184 x 10^4 cells ml^{-1}) (Kruskal-Wallis test, p=0.43). The highest growth rate in the glass bottle cultures was the modified F/2 (0.38 div day^{-1}), while AGP was the lowest (0.34 div. day^{-1}). On the other hand, in carboy culture, AGP was higher (0.17 div.day^{-1}) compared to modified F/2 (0.15 div. day^{-1}) and Walne's medium (0.13 div. day^{-1}). The exponential growth phase was similar in the glass bottles, while in the carboy, the exponential phase was reached at a shorter time in the AGP treatment than those in the modified F/2 and Walne's media. The findings showed that AGP medium is an adequate alternative to replace the conventional media (modified F/2 and Walne's) during the secondary stock culture for *C. muelleri*. The viability of using cheaper and more readily available commercial AGP media for the indoor culture production of *C. muelleri* can contribute to cost-effective scaling-up of the hatchery production of quality *H. scabra* larvae and early juveniles.

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1. INTRODUCTION

The production of natural food for the different life stages of cultured marine invertebrates is a critical aspect of hatchery management (Lovatelli et al. 2004). For the culture of the highly valuable tropical sea cucumber *Holothuria scabra*, different microalgae species have been used as feed for larvae and early juvenile stages. A mixture of *Chaetoceros calcitrans, Tetraselmis chuii,* and *Isochrysis galbana* has been used in India (James 1999); *C. muelleri, C. calcitrans* and *Rhodomonas salina* in Vietnam (Pitt 2001); and *C. muelleri, C. calcitrans, I. galbana, R. salina,* and *Tetraselmis* spp. in New Caledonia (Agudo 2006). At the Bolinao Marine Laboratory in the northern Philippines, *H. scabra* larvae are fed with *C. calcitrans* and *I. galbana.* However, Knauer (2011) reported a significantly higher percentage of *H. scabra* larvae metamorphosed to the doliolaria stage when fed only with *C. muelleri* or with mixed species of live microalgae compared to other monospecific diets such as *C. calcitrans*, *I galbana*, or *P. salina.*

Chaetoceros muelleri is one of the most commonly used live feed in shellfish culture (Reis Batista et al. 2012; Pacheco Vega et al. 2010). This diatom species is known to have high levels of nutrients (30–40% protein, 10–20% lipids, and 5–15%

carbohydrates) and is relatively easy to culture in large volumes (Brown and Robert 2002; Brown et al. 1997; Renaud et al. 1999). Using *C. muelleri* as live feed may greatly benefit efforts to scale up the hatchery production of *H. scabra.*

Modified F/2 medium (Guillard and Rhyther 1962) and Walne's medium (Walne 1970) are commonly used to culture *C. muelleri* (Pacheco Vega et al. 2010; Martinez-Cordova et al. 2012; Reis Batista et al. 2012; Barros et al. 2014). However, the preparation of these conventional culture media requires chemicals that are quite costly. Additionally, some of these chemicals (e.g., sodium nitrate) are regulated and are difficult to obtain locally. Epizym-AGP Complete (hereinafter referred to as AGP) (Epicore Bionetworks Inc. 2015) is a commercial algal growth medium used by some hatcheries in the Philippines. It is cheaper and more readily available than conventional media (modified F/2 and Walne's) (see Table 4). In this study, two laboratory experiments were conducted using two different secondary culture systems. The first experiment used 1 L dextrose glass bottles (glass bottle) and the second 10 L plastic carboys (carboy). The experiment's objective was to assess the growth of *C. muelleri* using commercial media (AGP) compared to the two conventional media (modified F/2 and Walne's) to gain more insights on how to improve the mass production of *C. muelleri.*

2. MATERIALS AND METHOD

2.1 Experimental set-up

The experiments were conducted at the Phycology Laboratory of the UP MSI Bolinao Marine Laboratory under optimal conditions with temperatures ranging 24 - 25°C and 24 hours of light from 2 units of GE F40 watt (daylight 6500K) with an intensity of 93.37 μmole/s/m^2. All the glass bottles and carboys were constantly aerated during the duration of the experiments. Before conducting the experiments, the seawater used were filtered (10, 5, and 1μm cartridge filters), UV radiated, and salinity adjusted to 25 ppt (using distilled water). All glass bottles used in the experiment were initially filled with 800 ml seawater and sterilized using a manual autoclave at 15 psi for 15-30 minutes. The carboys used were filled with 7 L of chemically sterilized seawater (chlorination-dechlorination method) with 0.2 ml/ L concentration (Agudo 2006). There were three media tested: AGP, Walne's, and modified F/2. Table 1 shows the composition of the three media used in this study to which Walne's and AGP were supplemented with 1 ml L^{-1} of Silicate solution (same concentration with modified F/2).

Table 1. Chemical composition of Epizym-AGP complete, Walne's medium, and modified F/2 medium

Epizym-AGP complete (Epicore Bionetworks Inc (2015))

Solution	Reagent	Stock solution (g/1000ml)	Utilization per liter
1	Inorganic nutrients		
	Vitamins		
	Chelated trace metals		0.2 ml
	Microbial extracts		
	Marine algae extracts		

Composition of Walne's medium (Walne,1970)

Solution	Reagent	Stock solution (g/1000ml)	Utilization per liter
1	$NaNO_3$	100 g	
	Na_2EDTA	45 g	
	H_3BO_3	33.6 g	
	$NaH_2PO_4 \cdot 2H_2O$	20 g	
	$FeCl_3 \cdot 6H_2O$	1.3 g	1 ml
	$MnCl_2 \cdot 4H_2O$	0.36 g	
2	Vitamin B1	200.0 mg	
	Vitamin B12	10.0 mg	
	Distilled water	200.0 ml	

3	ZnCl2	2.1 g	
	$CoCl_2 \bullet 6H_2O$	2.0 g	
	$(NH_4)6Mo_7O_{24} \bullet 4H_2O$	0.9 g	
	$CuSO_4 \bullet 5H_2O$	2.0 g	
	Distilled water	100 ml	

Composition of Modified F/2 medium (Guillard, 1975)

Solution	Reagent	Stock solution (g/1000ml)	Utilization per liter
1	$NaNO_3$/1000 ml dH2O	75 g	1 ml
2	$NaH_2PO_4 \bullet 2H_2O$/1000 ml dH2O	5 g	1 ml
3	Na_2SiO_3	40 g	1 ml
4	Trace Metal		1 ml
	FeCl3	3.15 g	
	Na2EDTA	4.63 g	
	$CuSO_4 \bullet 5H_2O$/ 100 ml distilled water	0.98 g	
	$CoCl_2 \bullet 6H_2O$/100 ml distilled water	1.00 g	
	$MnCl_2 \bullet 4H_2O$/ 100 ml distilled water	18 g	
	$Na_2MoO_4 \bullet 2H_2O$/100 ml distilled water	0.63 g	
	Zn SO_4/100 ml distilled water	2.2 g	
5	Vitamins		1 ml
	Thiamine HCl	0.2 g	
	Biotin	0.001 g	
	B_{12}	0.001 g	
	Distilled water	1 liter	

2.2 Glass bottle experiment

For the first set-up, two liters of inoculum were initially prepared in two 1 L glass bottles four days before the experiment. Each glass bottle was then enriched with 1 ml Walne's medium and 1 ml Silicate solution. Then, 200 ml of a two-week-old primary stock culture of *C. muelleri* was poured into the glass bottles. Thus, at the start of the experiment, three replicate glass bottles were enriched with the three treatments (modified F/2, Walne's, and AGP medium). The prepared inoculum was then poured into each glass bottle to make 1 L. Initial cell density was counted after the aeration was provided. The initial cell density was not significantly different (one-way ANOVA, $p = 0.28$) (see Table 2).

Table 2. Peak cell density and growth rates of *C. muelleri* in glass bottles

Treatment	Initial density* (cells ml^{-1})	Peak cell density** (cells ml^{-1})	Growth rate (Div day^{-1})
AGP	$117 \pm 10 \times 10^{4\ a}$	$1,241 \pm 116 \times 10^{4\ a}$	0.34
Modified F/2	$114 \pm 4.5 \times 10^{4\ a}$	$1,584 \pm 41 \times 10^{4\ a}$	0.38
Walne's	$105 \pm 10 \times 10^{4\ a}$	$1,319 \pm 162 \times 10^{4\ a}$	0.36

* no significant difference (ANOVA, $p=0.28$)
** no significant difference (Kruskal-Wallis test, $p=0.78$)

2.3 Carboy experiment

For the second set-up, two 1L glass bottles were inoculated similarly to the first set-up to prepare the inoculum. After five days, 200 ml each of the prepared inoculum was poured into nine 1 L glass bottles which will be used as inoculum for each carboy. Three replicate carboys were enriched with each of the three media, followed by adding one bottle of the inoculum to 7 liters of seawater in the respective treatment per replicate. Initial cell density was counted after the aeration was provided. The initial mean cell density was not significantly different (one-way ANOVA, $p = 0.16$) (Table 3).

Table 3. Peak cell density and growth rates of *C. muelleri* in carboys

Treatment	Initial density* (cells ml^{-1})	Peak cell density** (cells ml^{-1})	Growth rate (Div day^{-1})
AGP	152±15 x 10$^{4\ a}$	496±184 x 10$^{4\ a}$	0.17
Modified F/2	165±10 x 10$^{4\ a}$	653±79 x 10$^{4\ a}$	0.15
Walne's	173±8.5 x 10$^{4\ a}$	750±144 x 10$^{4\ a}$	0.13

* no significant difference (ANOVA, p=0.16)
** no significant difference (Kruskal-Wallis test, p=0.43)

2.4 Data Gathering and Analysis

The average density of *C. muelleri* (cells ml^{-1}) per medium was calculated daily by taking samples from the containers (glass bottles and carboy) and counting three 1 ml aliquot per container under a microscope (Ken A vision) 10x magnification with a haemacytometer (Improved Neubauer). Cell density was counted and computed following the standard procedures of Creswell (2010). Good quality cells were noted in terms of size, shape (not disintegrated), and cell wall not ruptured. The population or cell density of *C. muelleri* was monitored until the culture reached the senescence or death stage.

The growth rate was estimated as $U = (lnX2–lnX1)/(t2-t1)$ where U is the growth rate, $X2$ is the microalgae density at any time, and $X1$ is the microalgae density at the beginning, while $t2$ is the period of microalgae culture since inoculation and $t1$ is the initial time (Schoen 1988).

The cost of the media was computed based on the price per ml of the chemical components used to prepare the necessary volumes for modified F/2 and Walne's media. For AGP, this was based on the price of the total amount used for AGP (0.2 ml).

Tests for normality (Shapiro-Wilk's test) and homogeneity of variance (Kolmogorov-Smirnov test) were conducted prior to testing for differences. In each experiment, one-way ANOVA was used to detect significant differences in the average initial cell density, and the Kruskal-Wallis test was used to detect significant differences in the average peak cell. All statistical data were analyzed using STATISTICA 7 (Stat Soft, USA).

3. RESULTS

The growth phase of the microalgae in both experiments using different media was compared. The cell density peaked at day 7 for those in the glass bottles reaching up to 1,584 ± 41 x 10^4 cells ml^{-1}, whereas those in the carboys peaked at different times, with Walne's medium reaching the highest peak of 750 ± 144 x 10^4 cells ml^{-1} at day 11. The growth rate was also noted to be higher in the glass bottles compared to the carboys (Table 2 and Table 3).

In the glass bottle experiment, *C. muelleri* cell density increased by more than 150% in all treatments by day 1, AGP (296 ± 58 x 10^4 cells ml^{-1}), modified F/2 (299 ± 5.1 x 10^4 cells ml^{-1}), and Walne's medium (287 ± 57 x 10^4 cells ml^{-1}) (Figure 1). In AGP, the exponential phase was from days 1 to 6, and cell density reached its maximum by day 7 (1,241 ± 116 x 10^4 cells ml-^{1}). The stationary phase was at days 7-11 (5 days), and then the cell density started to decline at day 12. In comparison, *C. muelleri* grown in modified F/2 showed rapid growth from day 1 until it reached its peak cell density on day 7 (1,584 ± 41 x 10^4 cells ml-^{1}) (Figure 1). After day 7, *C. muelleri* cultures underwent senescence. Similarly, *C. muelleri* grown in Walne's medium showed exponential growth starting at day 1, and maximum cell density was reached on day 7 (Figure 1). The stationary phase occurred over the next four days. The mean peak density attained in Walne's medium (1,319 ± 162 x 10^4 cells ml-^{1}) was slightly higher than AGP but lower than modified F/2. The mean peak cell density in three different media was not significantly different from each other (Kruskal-wallis test, p = 0.78) (Figure 1; Table 2). In terms of growth rates, the highest was with the modified F/2 at 0.38 div. day^{-1} followed by Walne's (0.36 div. day^{-1}). In comparison, AGP was the lowest growth rate with 0.34 div. day^{-1} (Table 2). However, in terms of the senescence or death phase, there was a gradual decline in all the culture media.

There was also no evident lag phase in the carboy experiment, and the growth rate and the number of days it took to reach the maximum peak cell density varied among the three culture media (Figure 2). In AGP, the exponential phase was observed to be shorter (days 1-6) compared to the other two media, and its maximum cell density was reached by day 7 (496 ± 184 x 10^4 cells ml-^{1}). The stationary phase was at days 7-9 (3 days), and then the density started to decline. In comparison, the exponential phase in the modified F/2 medium was from days 1-8, and the maximum cell density was reached on day 9 (653 ± 79 x 10^4 cells ml^{-1}) followed by a stationary phase of 5 days. For the Walne's medium, the exponential phase was from day 1-10, and the maximum cell density was on day 11 (750 ± 144 x 10^4 cells ml^{-1}), followed by a stationary phase of 3 days. However, the maximum cell densities in cultures enriched with the three media were not significantly different (Kruska-wallis test, p = 0.43). Growth rates of *C. muelleri* enhanced with AGP was higher (0.17 div. day^{-1}) compared to modified F/2 (0.15 div. day^{-1}) and Walne's medium (0.13 div. day^{-1}) (Table 3). However, cell densities declined sharply after the stationary phase in the AGP compared to the two other media (Figure 2).

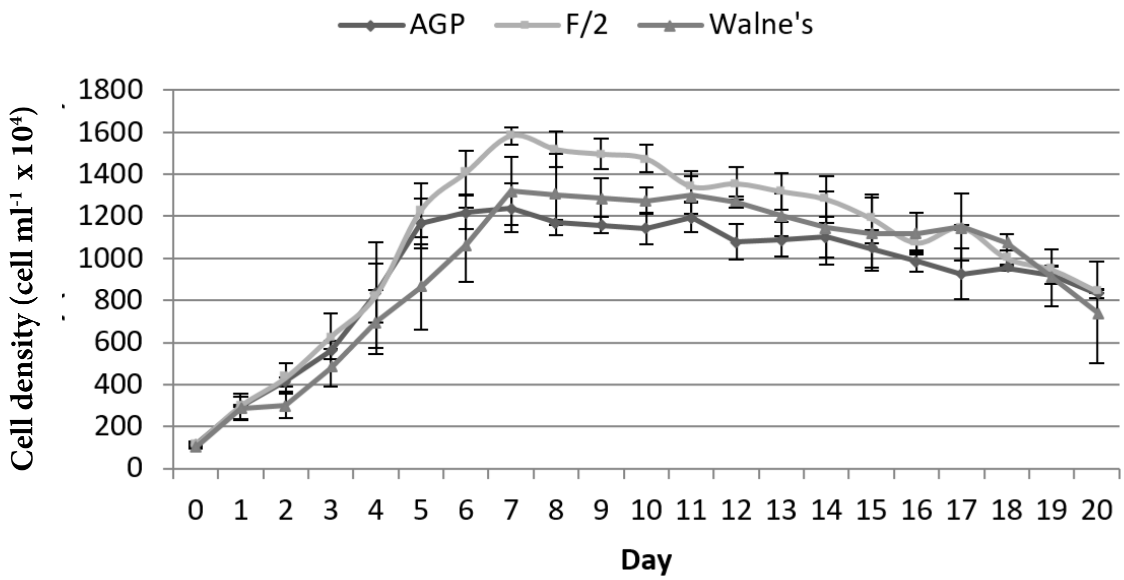

Figure 1. Growth of *C. muelleri* cultured in glass bottles using different nutrient media for 20 days

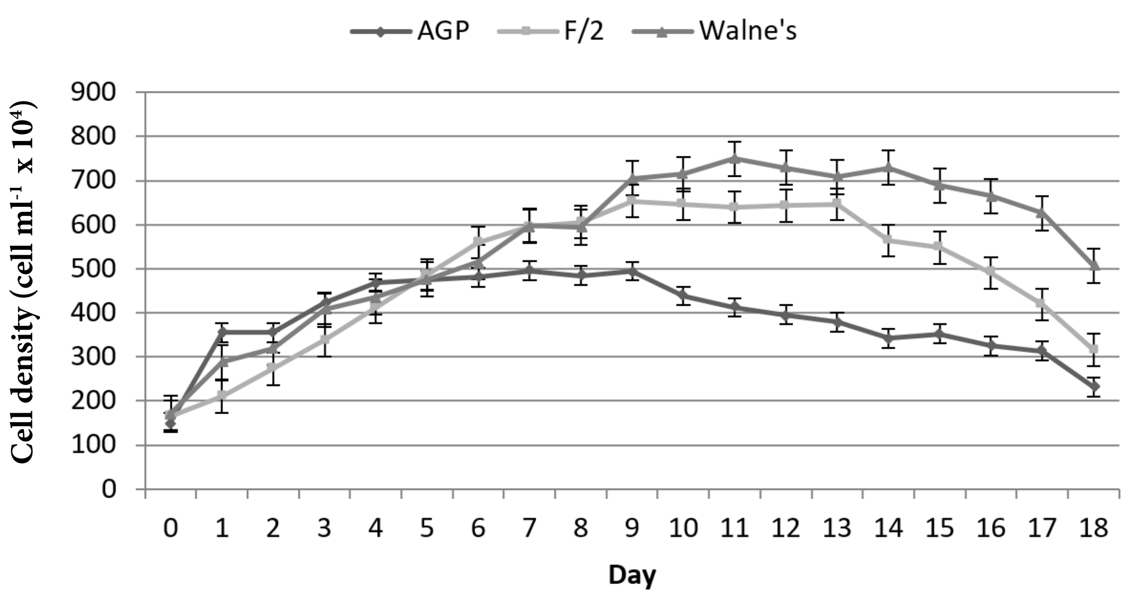

Figure 2. Growth of *C. muelleri* cultured in carboys using different nutrient media for 18 days.

4. DISCUSSION

In the bottle experiment, the growth rate ranged from 0.34 to 0.38 div. day^{-1} for the three culture media (Table 2). The result of the study was relatively similar to the study of Singh and Singh Priyanka (2015), where they investigated the effect of light intensity and different monochromatic light in a batch culture of *Skeletonema costatum*. The study showed that the highest growth rate of *S. costatum* is 0.3006 d^{-1} at 50 mmol m^{-2} s^{-1}. The growth rate of *S. costatum* increased with the increasing light intensity from 20 to 40 mmol m^{-2} s^{-1}, where the growth rate of algae is highly dependent upon the rate of photosynthesis.

Notably, growth rates in the carboys ranged from 0.13 to 0.17 div. day^{-1} for the three media. The lower range is comparable with Kumar and Saramma's (2018) study on the effect of salinity and pH in the growth of *Nannochloropsis salina* in a 1000 ml Erlenmeyer flask. At salinity 20 ppt, the growth rate of *N. salina* was 0.144/day. In this study, the growth rates of *C. muelleri* were similar to *N. salina*, wherein the temperature was maintained at 24–25°C. Notably, AGP has been reported to enhance the growth of other microalgae. For example, in a laboratory test that compared AGP's performance to the standard Guillard's F/2 media to culture *Tetraselmis chuii* in a flask at 250 ppm, the growth of *T. chuii* was 115% greater in AGP (Epicore Bionetworks Inc. 2015).

Furthermore, both experiments' peak cell density and growth rates were also not significantly different among treatments. These findings showed that AGP has the potential to produce high cell density and good cell quality as indicated by size (7–9 µm), shape (oval cylinder), and unruptured cell wall, which will improve the success in the mass production of *C. muelleri*. AGP contains all the primary nutrients of nitrogen, phosphorus, and potassium, as well as secondary and trace micronutrients (Table 1) that are essential to the synthesis of high-energy phytoplankton. Like Guillard's F/2 medium, AGP also contains microbial growth stimulants to enhance cellular function during the photosynthetic process (Epicore Bionetworks Inc. 2015).

Mass production of quality microalgae is a major challenge in every hatchery operation (FAO 1996). Outdoor cultures are prone to biological contaminants and other water quality problems, which may cause culture crashes. However, the good growth of the cultures indicates that the quality of the cultures was good and not confounded by contamination. At the Bolinao Marine Laboratory, indoor batch culture systems are applied because these are considered as one of the most efficient methods for a continuous supply of food for sea cucumber during the early stage of rearing. The first batch of culture is applied using 1 L glass bottles to produce good quality inoculum for the second batch culture system (carboy). During the first step of culture, all materials are sterilized to avoid contamination during the initial inoculation. Same with the first batch of culture, the second batch using carboy was also maintained for the preparation of good inoculum for scaling up. Cell quality or nutritional value is reportedly highest during the exponential phase (FAO 1996). However, the nutritional value of micro-algae can vary considerably depending on culture conditions (FAO 1996). In contrast, during the stationary phase, the cells rupture, and bacteria can proliferate. This condition renders the cultures unfit as starters or inoculum for scaling-up. Thus to maintain high-quality cultures, sub-cultures should be done during the exponential phase (Creswell 2010). Based on the results of both experiments, it is best to harvest *C. muelleri* cultures in the glass bottles and carboys during the exponential growth between days 4-6 for AGP. To further hasten the onset of exponential phase and perhaps increase logarithmic rates and prolong the stationary phase of growth, the use of higher density inoculum and increased concentrations of AGP for mass production of *C. muelleri* can be investigated.

There was no significant difference in the maximum cell densities and growth rates among the three media in the carboy experiment. Based on these results, the AGP medium has the potential to produce high cell density and good quality inocula for the indoor culture of *C. muelleri*. A study by Lopez-Elias et al. (2008) showed that *C. muelleri* cultured with F/2 in 250 L tanks gave a final cell yield that was 6.6 times higher than the initial cell density after three days. This result is similar to *C. muelleri* cultured in 90 L tanks using AGP in an outdoor culture of the Bolinao Marine Laboratory, wherein the use of high quantity inocula from carboys yielded 5.7 – 6.6 times higher cell yield than the initial cell density in the same number of days (UPMSI Sea Cucumber Research Program, unpublished data).

Moreover, AGP is substantially cheaper based on the estimated cost of the number of chemicals used to prepare a liter of modified F/2 and Walne's media (Table 4). The estimated cost of nutrient inputs per liter using AGP is only Php 0.351 compared to Php 1.945 and Php 2.861 for modified F/2 and Walne's, respectively (Table 4). Thus, the use of AGP will substantially reduce the overall cost of *C. muelleri* production.

Table 4. Estimated cost of commercial and conventional media used in secondary culture (glass bottle and carboy) of *C. muelleri*

Culture Medium	Chemical Composition	Price of working stock/L	Total Price/L
Walne's	$NaNO_3$	1.939	**2.861**
	Na_2EDTA	0.180	
	H_3BO_3	0.237	
	$NaH_2PO_4.2H_2O$	0.160	
	$FeCl_3.6H_20$	0.013	
	$MnCl_2.4H_2O$	0.003	
	Trace metal	0.001	
	Vitamin	0.027	
	Silicate	0.301	
F/2	$NaNO_3$	1.458	**1.945**
	$NaH_2PO_4.2H_2O$	0.053	
	Trace metal	0.063	
	Vitamin	0.070	
	Silicate	0.301	
AGP	In-organic nutrients		**0.351**
	Chelated trace metals		
	Vitamins	0.050	
	Microbial extract		
	Marine Algae extracts		
	Silicate	0.301	

This study clearly demonstrates that AGP is a suitable alternative to conventional media, considering the comparable growth rates, maximum peak densities, and cell quality. Moreover, because of AGP's substantially lower cost and accessibility, its use will certainly be essential for scaling the production of *C. muelleri*. Thus, it is a significant contribution to improving the cost-effectiveness in the hatchery production of quality *H. scabra* larvae and early juveniles.

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AUTHOR CONTRIBUTIONS

Cabanayan-Soy R: Conceptualization, Methodology, Data gathering, Writing-original draft preparation; **de Peralta GM:** Conceptualization, Writing-Analysis, Reviewing and Editing; **Juinio-Meñez MA:** Writing-Reviewing and Editing; Supervision, Resources.

ETHICS STATEMENT

No animal or human studies were carried out by the authors

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