

## The Effects of Different Culture Medium on the Growth of a Dinoflagellate (*Cochlodinium polykrikoide*) in Laboratory Condition

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### Abstract

Cultivation of *C. polykrikoide*s was carried out using different culture media including f/2 medium (without silicate), modified f/2 medium, Conway, TMRL and ESM in the Iranian Shrimp Research Center in 2013. *C. polykrikoide*s was cultured in test tubes, 100 ml, 250 ml, 500 ml Erlenmeyer flasks and in a 16 l aquarium, using 32 ppt seawater (27 ± 2°C) and with light intensity of 5000 lux, under cool-white fluorescent illuminations, and in a 12:12 h light-dark cycle. The best result was obtained using modified f/2 medium followed by TMRL medium. With ESM, cultivation was continued, in 100 ml Erlenmeyer flasks but it was not possible in more than 100 ml container. *C. polykrikoide*s cultivation was unsuccessful with the f/2 medium and Conway medium even in the test tube. The results of the study showed that mass cultivation of *C. polykrikoide*s was possible with modified f/2 medium even in an aquarium, but did not have favorable results with TMRL medium in the aquarium.

**Keywords:** *C. polykrikoide*s; Culture medium; Cell density; Laboratory condition

### Introduction

Of more than 5000 species of marine planktons, only 80 species are able to produce strong poison [1]. *Cochlodinium polykrikoide*s is a dinoflagellate species and its bloom in coastal areas is associated with fish kills in many parts of the world [2-4]. This dinoflagellate is a mixotrophic algae [5,6] with 30-40 µm long and 20-30 µm width [7]. They multiply through binary fission and contain pigments creating what is known as the red tide [7]. It has also been reported that, in the natural condition and on sunny days, *C. polykrikoide*s migrate to deeper area to adjust the intensity of the light received [8] and this algae can produce H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> which could be one of the reason for fish mortality [4]. In areas where *C. polykrikoide*s occur, fish are suffocated as the result of oxygen depletion [9]. The ichthyotoxic properties of *C. polykrikoide*s blooms have also been described by authors [4,10-12]. Pacific oyster larvae, *Crassostrea gigas* undergo metamorphosis during *C. polykrikoide*s bloom [13]. Mortality increase of American oyster larvae, *Crassostrea virginica* during exposures to *Cochlodinium* has been reported [14]. The first report of *C. polykrikoide*s induced red tide in Iran (Persian gulf) was made in 2007 [15]. Richlen et al. [16] reported long term *C. polykrikoide*s bloom in the Persian Gulf and Oman Sea that caused high fish mortality, affecting fishing activity and damaging coral reefs. In 2008, *C. polykrikoide*s bloom lasted 8 months which resulted in the loss of 34 tons of aquatic organisms [1]. Nevertheless, there has been little research concerning the causes and impacts of *C. polykrikoide*s blooms in the Persian Gulf. Dinoflagellate blooming has been linked to the washing of cobalamin (vitamin B1) from the soil and its entry into the sea [17]. It is also reported that, the increase in sunlight intensity

and nutrients contributed to excessive *C. polykrikoide*s bloom [18]. *C. polykrikoide*s has been found in cold and hot water and in salinity of 30-34 ppt [19].

Since ecological factors are changing continuously, it is necessary to find out the underlying causes of blooms and the impacts on ecosystems. Thus, close examinations and laboratory studies may be needed to unravel some of the reasons underlying such blooms. Therefore, it is important to have intensive culture of *C. polykrikoide*s under laboratory condition. Cultivation of *C. polykrikoide*s under laboratory conditions was reported using modified f/2 medium (f/2-Si) [20] and f/2 medium (without silicate) [21,22]. The effects of temperature, salinity and irradiation on *C. polykrikoide*s growth under laboratory condition and using modified f/2 medium (without silicate) have been investigated [23]. Also, the ESM medium has been used for the cultivation of this alga under laboratory condition [24]. The purpose of this study was to determine the proper conditions for the mass cultivation of *C. polykrikoide*s in laboratory condition.

### Material and Methods

Water samples were prepared from coastal waters of the Persian Gulf (salinity 39 ± 2.82 ppt, pH=7.95 ± 0.35) along Hormozgan province where *C. polykrikoide*s bloom occurred. The samples were then, passed through 70 µm plankton net and the materials left, were immediately transported to the laboratory where samples were transferred to 1 liter beakers, 3/4 of which were covered with black paper and placed under two fluorescent lamps. Light attraction was used [25] in order to separate, and the *C. polykrikoide*s attracted to the light were separated by sterile micropipette and transferred to the test tube containing modified f/2 medium without silicate [26]. This was repeated several times for more purification. Five culture media were used for the experiments, including f/2 medium without silicate

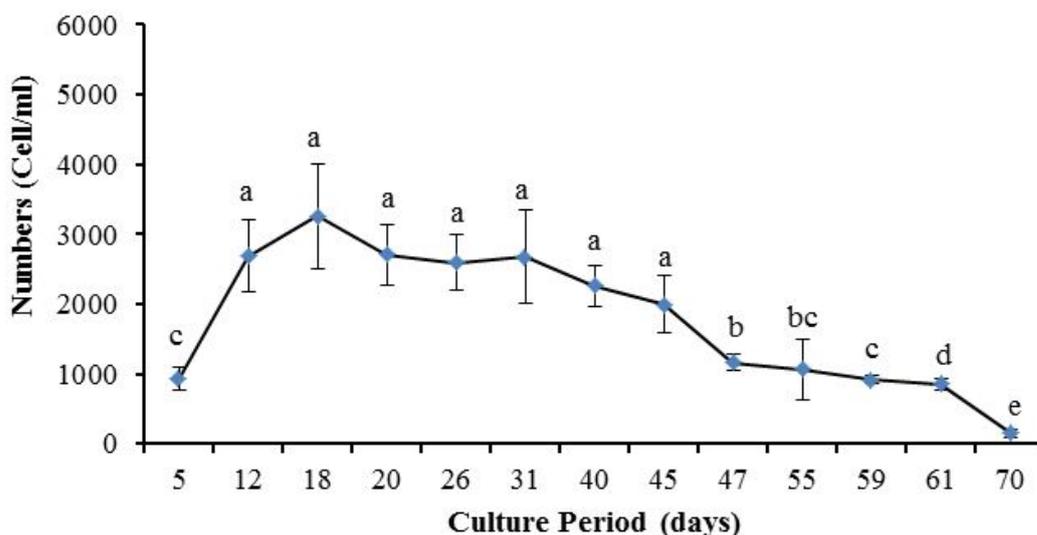
(includes  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{Na}_2\text{EDTA}$ ,  $\text{NaNO}_3$ ,  $\text{NaH}_2\text{PO}_4$ ,  $\text{H}_2\text{O}$ , Biotin and Thiamine.HCl) [27], Conway (includes  $\text{KNO}_3$ ,  $\text{NaH}_2\text{PO}_4$ ,  $\text{FeCl}_3$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{H}_3\text{BO}_3$ , vitamins B1 and B12) TMRL (includes  $\text{KNO}_3$ ,  $\text{NaH}_2\text{PO}_4$ ,  $\text{FeCl}_3$  and  $\text{SiO}_2\text{Na}$ ), ESM (includes  $\text{NaNO}_3$ ,  $\text{KH}_2\text{PO}_4$ , vitamins B1, B12 and Biotin,  $\text{EDTA-Fe}^{2+}$ ,  $\text{EDTA-Mn}^{2+}$  and Tris Aminomethane) [6] and modified f/2 medium (includes  $\text{NaNO}_3$ ,  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ,  $\text{Na}_3\text{EDTA}$ ,  $\text{FeEDTA}$ , Tris,  $\text{EDTA}$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{ZnCl}_2$ ,  $\text{H}_2\text{SeO}_3$ , Ampicilin, Kanamycin and Neomycin) [26] without silicate [15]. Culture was done in sterile test tubes (100 × 16 mm), each containing 10 ml of modified f/2 medium, 100, 250, 500 ml Erlenmeyer flasks and in a 16 liter aquarium (each containing 10 l of culture medium). The research was carried out, with 10 treatments and 3 replications in each treatment. The cultures were inoculated with 50 cells/ml [23]. To prepare the culture medium, sea water (salinity  $40 \pm 2$  ppt) was first entered to the sedimentary basin and then passed through a sandy filter. In the next step, water was passed through a 0.45 micron filter. To obtain the required salinity (32 ppt), water was diluted with distilled fresh water. Then they were placed in an autoclave (Iran Tab Zaeem-87419) for 15 minutes at a temperature of  $121^\circ\text{C}$ . All the tubes and flasks were covered with aluminum foil. Cultures were performed in controlled laboratory conditions, with  $27 \pm 2^\circ\text{C}$  and light intensity of 5000 lux, under cool-white fluorescent illuminations, on a 12:12 h light–dark cycle. *C. polykrikoide* cells were counted using a Sedgwick-Rafter counting chamber, under optical microscope.

This experiment was conducted based on a completely randomized design. All statistical analyses were done using SPSS statistical package

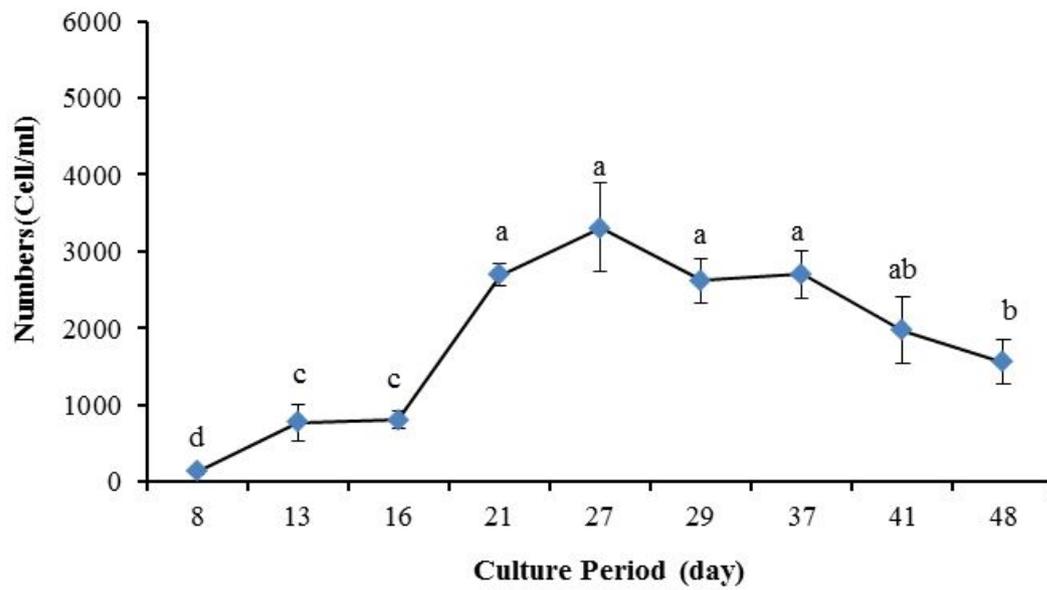
(version 16.0). One-way analysis of variance (ANOVA) and Duncan's multiple comparison tests were used to identify significant variations at 0.95 confidence limits ( $P < 0.05$ ).

## Results

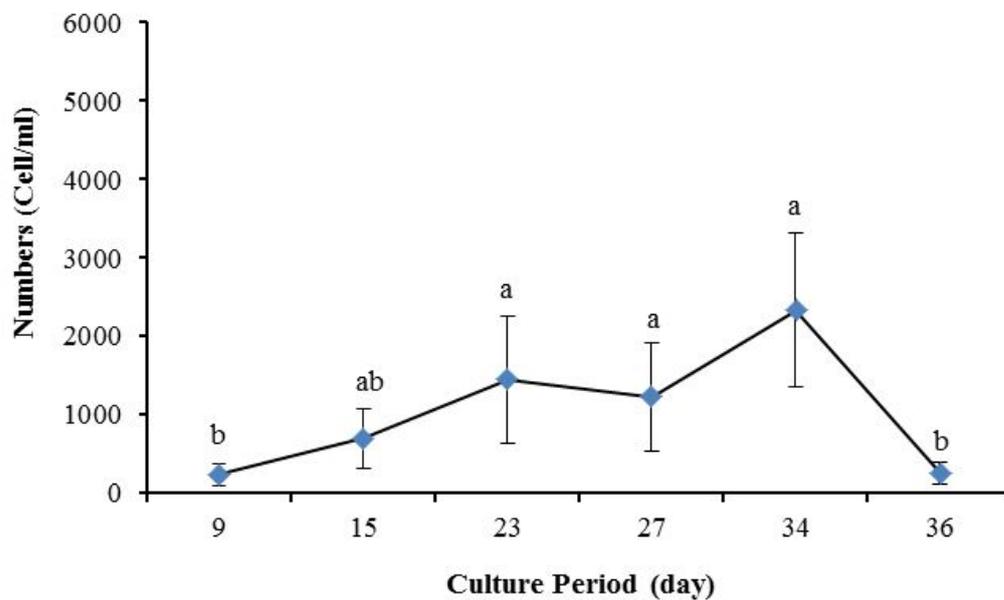
No results were obtained for cultivation of *C. polykrikoide*, using the Conway and f/2 medium. With ESM medium, *C. polykrikoide* culture was, to some extent successful in the test tubes and 100 ml Erlenmeyer. The result showed that, these mediums were not suitable for *C. polykrikoide* culture. By the use of TMRL, the highest cell density was obtained in test tubes and 250 ml Erlenmeyer flask, and cell density reduced in 500 ml Erlenmeyer flask and in aquarium. The average cell densities in test tubes, 100 ml, 250 ml and 500 ml Erlenmeyer flasks and in aquariums are presented in Figures 1 to 4 respectively. *C. polykrikoide* possessed the highest density in test tube and 250 ml Erlenmeyer. But the maximum density in the test tube was occurred earlier (Figures 1 and 2). In the 500 ml Erlenmeyer, the number of cells was lower than that of test tube and 250 ml Erlenmeyer, and the maximum density was occurred later (Figure 3). Cells density in the aquarium was lower than the test tube and Erlenmeyers, but the maximum density of *C. polykrikoide* was occurred earlier than 500 ml Erlenmeyer (Figure 4). Also, number of *C. polykrikoide* in the test tube, 250 and 500 ml Erlenmeyer and aquarium reached to 3260, 3310, 2326 and 366 cells/ml after 18, 27, 34 and 27 days respectively (Figures 1-4).



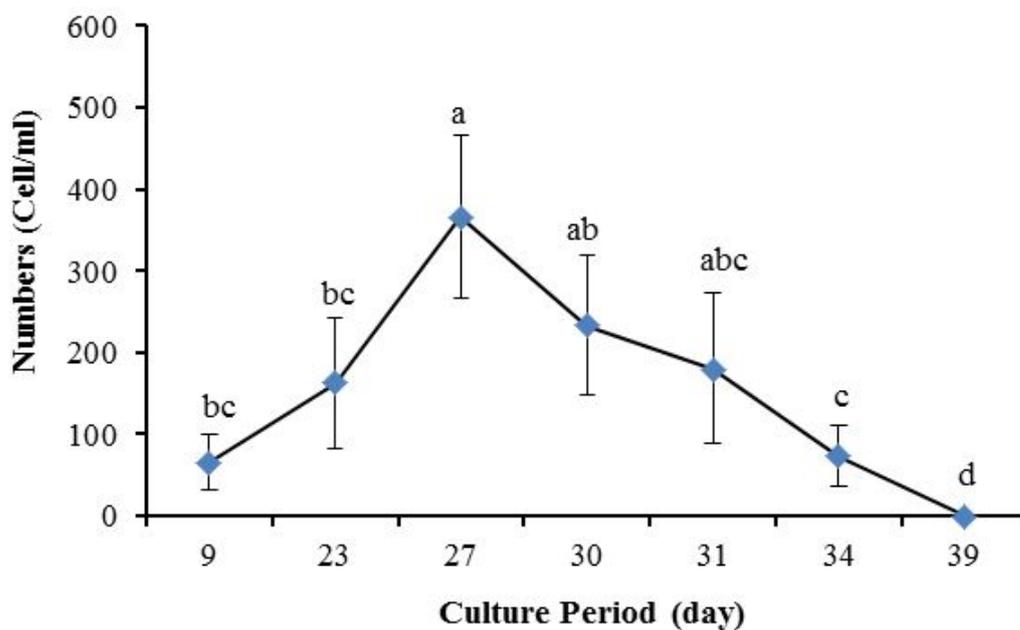
**Figure 1:** Average number of *C. polykrikoide* in test tube using TMRL medium. Each value is mean  $\pm$  SE of 3 individual observations. Different letters is mean significant difference ( $p < 0.05$ ).



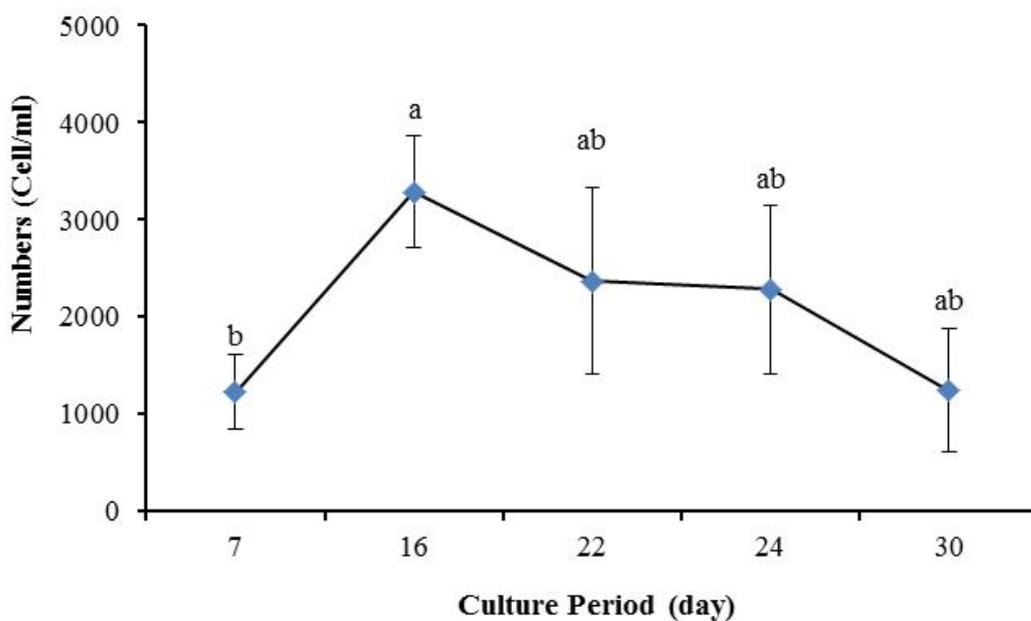
**Figure 2:** Average number of *C. polykrikoides* in 250 ml Erlenmeyer using TMRL medium. Each value is mean  $\pm$  SE of 3 individual observations. Different letters is mean significant difference ( $p < 0.05$ ).



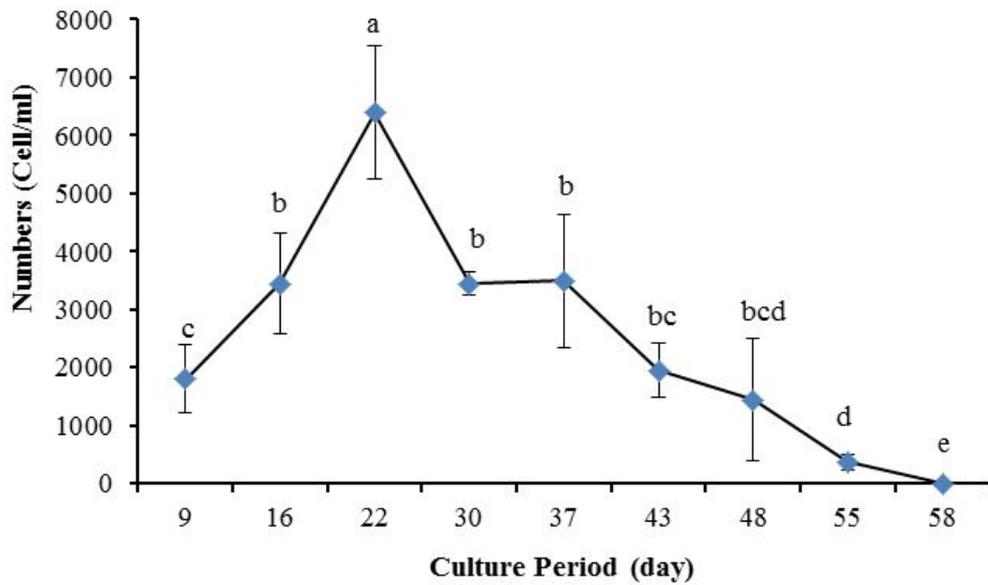
**Figure 3:** Average number of *C. polykrikoides* in 500 ml Erlenmeyer using TMRL medium. Each value is mean  $\pm$  SE of 3 individual observations. Different letters is mean significant difference ( $p < 0.05$ ).



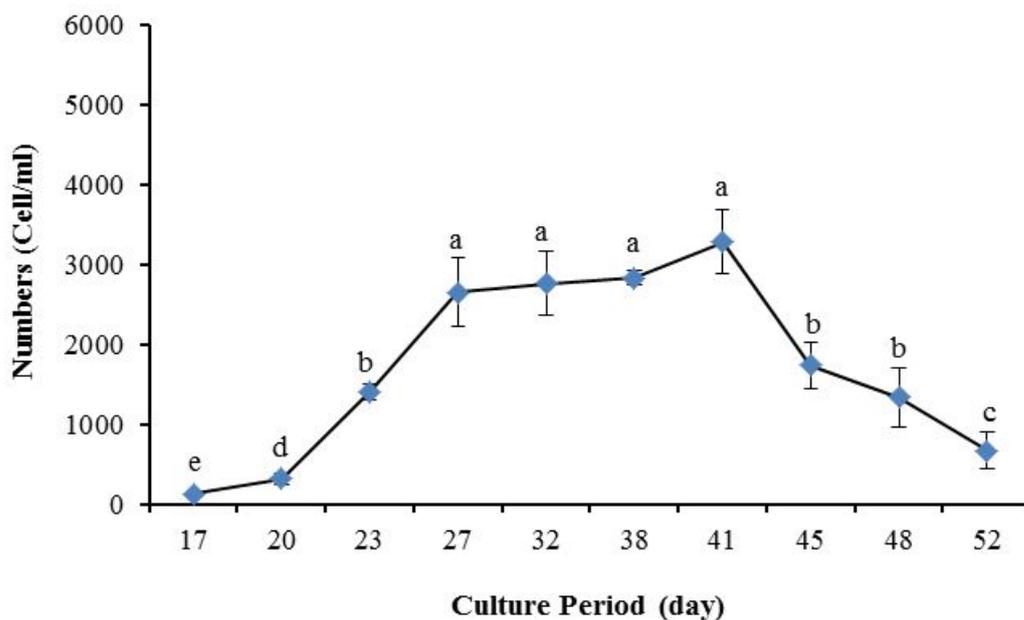
**Figure 4:** Average number of *C. polykrikoides* in 16 l aquarium using TMRL medium. Each value is mean  $\pm$  SE of 3 individual observations. Different letters is mean significant difference ( $p < 0.05$ ).



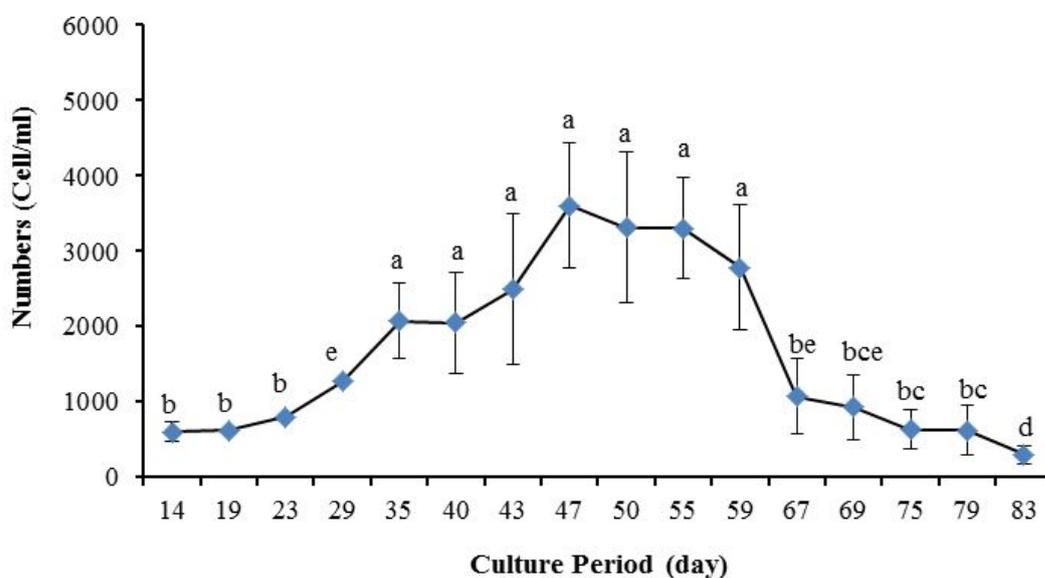
**Figure 5:** Average number of *C. polykrikoides* in test tube using modified f/2 medium. Each value is mean  $\pm$  SE of 3 individual observations. Different letters is mean significant difference ( $p < 0.05$ ).



**Figure 6:** Average number of *C. polykrikoides* in 250 ml Erlenmeyer using modified *f/2* medium medium. Each value is mean  $\pm$  SE of 3 individual observations. Different letters is mean significant difference ( $p < 0.05$ ).



**Figure 7:** Average number of *C. polykrikoides* in 500 ml Erlenmeyer using modified *f/2* medium medium. Each value is mean  $\pm$  SE of 3 individual observations. Different letters is mean significant difference ( $p < 0.05$ ).



**Figure 8:** Average number of *C. polykrikoides* in 16 l aquarium using modified f/2 medium. Each value is mean  $\pm$  SE of 3 individual observations. Different letters is mean significant difference ( $p < 0.05$ ).

By the use of modified f/2 medium, Cells density in 250 ml Erlenmeyer was significantly more than that test tube, 500 ml Erlenmeyer and aquarium (Figure 5-8). But the maximum density of *C. polykrikoides* in the test tube was occurred earlier. *C. polykrikoides* density in the aquarium was higher than test tube and 500 ml Erlenmeyer, but maximum density of *C. polykrikoides* was occurred later. The maximum *C. polykrikoides* density obtained through the use of modified f/2 medium in the test tube, 250 ml and 500 ml Erlenmeyer and aquarium were 3283, 6393, 3283 and 3600 cells/ml after 16, 22, 41 and 47 days respectively (Figures 5-8).

## Discussion

In the present study, no bloom occurred using f/2 medium (f/2-Si) and this was in agreement with that components of the f/2 medium (N, P, Fe, Mn, Co, Cu, Zn, Mo, B12, biotin, thiamine) did not seem to trigger the occurrence of *C. polykrikoides* blooms [28]. In the modified f/2 medium, different antibiotics (Ampicilin, Kanamycin and Neomycin) were used to reduce the activity of a wide range of bacteria. This factor, along with the addition of other nutrients to the modified f/2 medium, created suitable condition for *C. polykrikoides* blooms. As mentioned earlier, the cultivation of these algae requires special condition, which only supply with a limited number of culture medium. In this regard, in an investigation, *C. polykrikoides* was cultivated, using the f/2 medium (without silicate) and surface seawater taken between the South Sea of Korea and Jejudo [20]. Since *C. polykrikoides* could not be cultured using coastal seawater of South Sea of Korea, except during the bloom period [20]. Therefore, as noted in the present research, the role of water compounds in the bloom of *C. polykrikoides* is of great importance. The area where water is used, may affect the growth of algae [20]. Also, reported that, the cultures of *C. polykrikoides*, were inoculated with modified f/2 medium [20]. The use of this medium for the massive cultivation of *C. polykrikoides* is

consistent with the present study. Therefore, it can be expected that, in sea waters, the bloom of these algae occurs during the special conditions. In the present study, the maximum algae density reached by using modified f/2 medium was 6393 cells/ml after 22 days in 250 ml Erlenmeyer. But, it was in 250 ml Erlenmeyer after 27 days by using TMRL (3310 cells ml<sup>-1</sup>).

Due to the suitability of the first culture medium, the maximum number of algae was obtained in less time. In this phase, algae use the nutrients for multiplication. Therefore, as shown in Figures 1 to 8, the total duration of growth phases (included Lag phase, exponential phase, Stationary phase and Decline phase) was longer than TMRL by using modified f/2 medium (except test tube). In a study concerning *C. polykrikoides* culture in f/2 medium, the total Lag phase, exponential and stationary phases, lasted 22 days [28]. In the mentioned study, the Maximum *C. polykrikoides* density in the exponential phase was 1500 cell/ml and the culture vessels were 50 ml sterilized polystyrene flasks, containing 20 ml of f/2 medium. In the present study, the minimum and maximum duration were between 31 to 45 days for TMRL medium (Figures 1-4) and 24 to 59 days (Figures 5-8) for modified f/2 medium.

It has been reported that, under laboratory conditions and in the sea, *C. polykrikoides* forms chain at progressive growth [29]. In the present study, cells were formed in chain consisting of 2 to 8 cells. Maximum *C. polykrikoides* density, created in laboratory condition was 850 cells/ml [30]. Also, high densities of this algae,  $8 \times 10^6$  cells/ml [31],  $48 \times 10^3$  [23] and  $6 \times 10^6$  cells/ml have been reported [1]. In the present study, the highest density of this algae was  $5 \times 10^4$  cells/ml at it's accumulation site in the aquarium (with modified f/2 medium). In relation to the effect of *C. polykrikoides* cell density on fish mortality, it is reported that, fish survived in water containing less than  $1 \times 10^3$  cells/ml of *C. polykrikoides* [10].

The results of the present study showed that modified f/2 medium is the best medium for mass culture of *C. polykrikoide* followed by TMRL medium in laboratory condition.

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