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ISOLATION AND MAINTENANCE METHODS FOR *SKELETONEMA COSTATUM* IN LABORATORY CULTURES

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ABSTRACT

Skeletonema costatum is an important species that frequently cause microalgal blooms in the Black Sea waters especially during spring. This study was aimed to use different techniques to isolate the species from their natural habitat. The first stage was the identification of the period when the species was dominant in the samples collected from the Mamaia station. Then the sea water sample was filtered (through a 60 µm multi-layer filter) to remove most of the phytoplankton and zooplankton species present in the sample. Successive dilutions followed until only the species of interest remained in the culture. The next stage was to observe *Skeletonema costatum* growth rate under laboratory conditions and to maintain the culture viable for future experiments. These experiments will imply the response of the species to a controlled variation of some important physico-chemical parameters, which may trigger some limitations in their development.

Key-Words: *Skeletonema costatum*, Black Sea, microalgal cultures, growth rate

AIMS & BACKGROUND

The purpose of this study was to isolate *Skeletonema costatum* from its natural environment and to maintain the species in laboratory cultures as a source of biological material of known origin and characteristics.

The steps followed in this paper were:

- Identification of the species that caused significant blooms in the Romanian Black Sea coastal waters from NIMRD phytoplankton database.

- Collecting several water samples from Mamaia to observe the optimal moment of species development.
- Species isolation from its natural habitat by filtration of the sample through 60 microns multilayer filter (to remove zooplankton and other larger species of microalgae) and by Allen & Nelson's, 1910 dilution method.
- After 20-30 days the microalgae can be transferred every 3-5 days during exponential growth, from 10 ml tubes in bigger recipients (Creswell, 2010).
- Culture maintenance by monthly check of the population situation and periodic refreshment of the nutrient medium (Cărăuș, 2007).

The selected species was *Skeletonema costatum* (Greville) Cleve 1878 (Fig. 1) due to its massive development, especially from the end of spring to the beginning of autumn, being a species that often recorded densities above 10^6 cells/L, accounting for up to 98% of the phytoplankton community (Han et al., 1992).

The species belongs to the group of diatoms (Bacillariophyceae), cosmopolitan, unicellular, colonial or solitary algae, which is characterized by the presence of the external siliceous cell wall named frustule. It is the most numerous group of algae in species (about 9000 on a planetary scale) and the most important in quantitative terms in the phytoplankton and microphytobenthos aquatic basins composition (Godeanu et al., 1995).

In the Black Sea, *Skeletonema costatum* recorded the first significant densities in March and April 1976, of 73 and $97.2 \cdot 10^6$ cells/L respectively. But on April 17, 1984, in the shallow waters of Mamaia, *S. costatum* reached the highest density that the species had known at the Romanian seaside up to that date, of $141.4 \cdot 10^6$ cells/L. *Skeletonema* produced again many high intensity blooming phenomena, also in spring (April, May), of 46 and $59.3 \cdot 10^6$ cells/L, respectively, but never reached the densities recorded in 1984 (Boicenco, 2010).

Fig. 1. *Skeletonema costatum* (in laboratory culture).

The main commercial use of Bacillariophyceae is as feedstock for larval cultures in aquaculture, with the following genera being most widely used: *Chaetoceros, Nitzschia, Phaeodactylum, Skeletonema* and *Thalassiosira*. Due to their high lipid and fatty acids content, the Bacillariophyceae are also under consideration in the production of biofuels and essential fatty acids for human consumption (Heimann et al., 2015). *Skeletonema costatum* (Greville) Cleve is widely used for the hatchery rearing of penaeid shrimp larvae and postlarvae and bivalve molluscs larvae and spat being identified as the best monospecific diet for Sydney rock oyster spat by O'Connor et al. (Heasmann et al., 2001).

EXPERIMENTAL

Sea water samples were collected in glass bottles (500 ml) from the surface layer at Mamaia station at the end of February 2013. The samples were analyzed using an inverted microscope and it was observed that the dominant species was *Skeletonema costatum* among other diatoms, dinoflagellates, flagellates and zooplankton species. Most of the other species were removed by **filtering** the culture through 60 microns sieve (in 2-6 layers). After a while, there were noticed some ciliates and rotifers in the culture (Fig. 2). The culture was filtered again until zooplankton species were no longer observed at the microscope.

Fig. 2. Rotifers in *Skeletonema costatum* culture.

Skeletonema needed a period of recovery (around 30 days) because most of the chains were broken or even distroyed. In this period, besides the target species, it was noticed the development of nanoflagellates in the culture. Thus, to obtain a **monoalgal culture**, it was used the **dilution** method (Allen & Nelson, 1910). Inoculations were done in 10 test tubes in 10 dilutions: 10 ml, 1, 0.1, 0.01, 0.001 - 0.000000001 ml (Fig. 3).

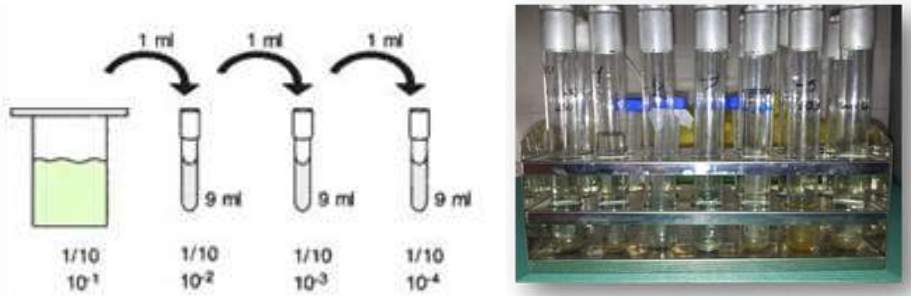

Fig. 3. Successive dilutions.

After 21-30 days, the water in the test tubes had a light brown shade, indicating a considerable development of the species. Following microscopic observation, culture lacked the presence of other microalgae or zooplankton.

Numerical density data were obtained by referencing to one milliliter the results of the algal cell counts in the analyzed sample fraction. The phytoplankton biomass in each sample was calculated according to the formula: $B = d \cdot (g \cdot 1000)$, wherein: B = biomass/$m^3$, d = density cells/L), g = mean weight of the algae in the sample. Calculation of the average species weights consists of assimilating their appearance with a geometric shape and calculating the volume which then is converted to average weight (Edler, 1979).

For a better illustration of the growth phases, it was used the Moving Average Excel Trendline. A moving average is commonly used with time series data to smooth out short-term fluctuations and highlight longer-term trends or cycles. In order to determine the type of growth of a population, estimates are made from one moment to the next showing the trend of numerical growth.

In order to **maintain** the culture, the following conditions were ensured:

- The test tubes with concentrated culture are kept in the laboratory, on a shelf without direct light, at room temperature (23°C, ±2°C). It is necessary to multiply the culture to obtain a biological selection reserve of the material. Thus, 10 ml of pure culture can be inoculated into 250 ml of nutrient medium (Fig. 4). Every 4-5 days the initial culture can be gradually transferred in 500, 1000, 2000 ml flasks or any volume is needed [2].

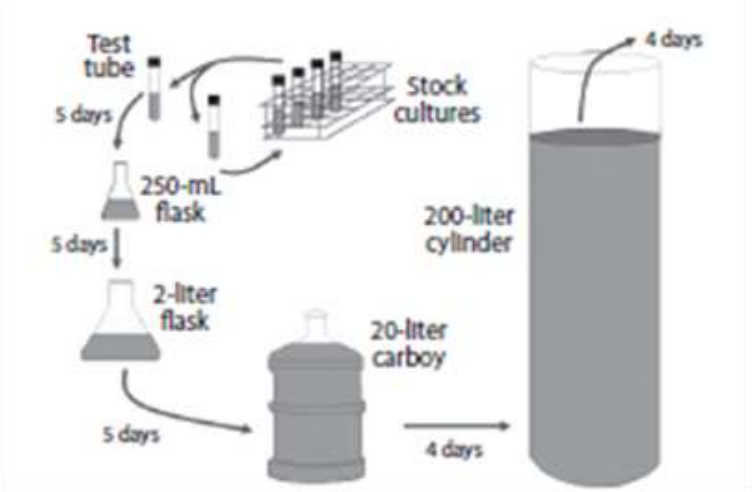

Fig. 4. Schematic representation of the method of inoculum gradual transfer (Creswell, 2010).

- All materials used must be sterile and carefully handled to avoid contamination. All test tubes were autoclaved for sterilization at 180°C, for 1 hour.
- Temperature and salinity were maintained at about 23-25°C and 18 PSU, agitation was done manually for containers of less than 1 liter at least 2 times a day to ensure good nutrient distribution and culture homogenization. For containers larger than 1 liter the mixing was provided by bubbling air into the culture using an air pump. Mixing is necessary to prevent sedimentation of the algae, to ensure that all cells of the population are equally exposed to the light and nutrients, and to improve gas exchange between the culture medium and the air (Lavens et al., 1996).
- The nutrient medium must be periodically refreshed. It contains sea water (filtered, sterilized at autoclave and then cooled) with a mixture of PO_4, K, N and a solution of microelements (silica, manganese, boron, cobalt, lithium, molybdenum, copper, tin, nickel, fluorine, iodine, aluminum, zinc, selenium and vanadium), important substances for development.
- The population situation should be monthly checked for a potential contamination.

RESULTS AND DISCUSSION

Growth dynamics can be determined by inoculating cells from a monoalgal culture in a sterile nutrient medium. In order to appreciate the multiplication dynamics, small samples are analyzed at regular intervals and the values of density and biomass are recorded on curves whose changes show the transition from one growth stage to the next one. Under these conditions the process evolves in four

successive phases (Lavens et al., 1996): the lag phase, the exponential growth phase, the stationary phase and the declining phase.

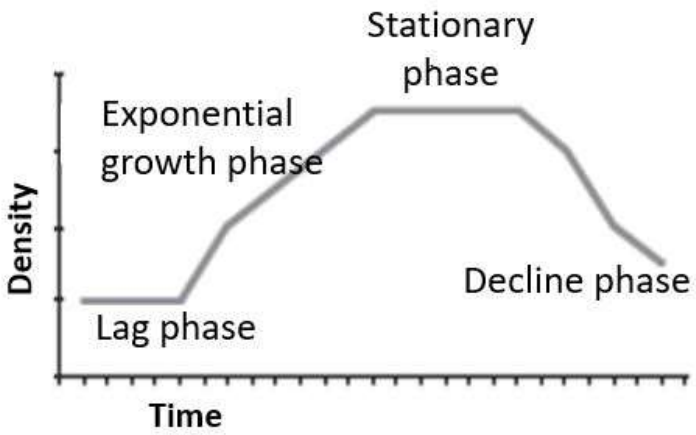

Fig. 5. Microalgal growth phases in a laboratory culture (Lavens et al., 1996).

The lag phase or zero growth, where there is no increase in cell counts, the number of cells in the inoculum remains unchanged or even decreases temporary. The phase of logarithmic growth or exponential multiplication is the phase in which the number of cells or the biomass doubles in the time unit. At the end of the logarithmic phase, a maximum amount of biomass accumulates, the substrate is quickly exhausted and the culture passes into the stationary growth phase.

The stationary growth phase corresponds to the period when, as a result of the gradual exhaustion of nutrients, the reduction in oxygen concentration and the accumulation of catabolism compounds that may have inhibitory effect on the cells, the increase takes all intermediate values between a maximum rate and zero. At this stage a balance is established between the number of cells that are formed by reproduction and that of the cells that are autolysing. The amount of biomass can remain constant, although the composition of the cells changes. Due to cell lysis, new substrates are released to serve as nutrients for viable cells. This phase can be prolonged when the scope is to keep the culture pure by modifying factors that decrease the rate of cellular metabolism.

The decline phase occurs as a result of the following factors: lack of nutrition and energy sources; denaturation of cellular components in the presence of accumulated substances (alcohols, acids, etc.); the percentage of autolysed cells. The consequence of these actions ultimately leads to the death of all cells, respectively to the loss of the culture.

For the observation of *Skeletonema costatum* growth dynamics in laboratory cultures **two experiments** were performed. The purpose of **the first experiment** was to observe the species growth dynamics in minimum laboratory conditions by using a small culture volume (1 liter), natural light source and by stirring twice a day by

hand. Thus, only two phases of growth were observed: a prolonged lag phase and the logarithmic growth phase (Fig. 6). In the lag phase, the values of density and biomass oscillated between 44-70·10^3 cells/ml, suggesting a period of accommodation of the species with the conditions provided and the culture medium. In the next 3 days, there was a significant increase of the density and biomass values of two and even three times higher than in the first days, reaching 152·10^3 cells/ml.

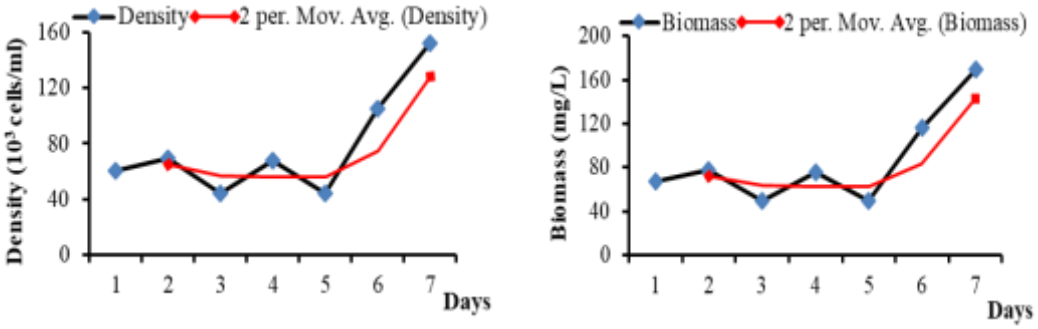

Fig. 6. Growth dynamics of *Skeletonema costatum* in lab culture by density (left) and by biomass (right) recorded in the first experiment.

In the second experiment the growth conditions were improved by providing a larger volume of both culture medium and inoculum (2 liters of inoculum at 4 liters of culture medium). A dark-light cycle of 12:12 hours was provided using two neon lights with cold light; the balloons were positioned 15-20 cm away from the light source. The mixing was achieved by aerating. Therefore, were recorded higher values of both density and biomass (approximately 906·10^3 cells/ml and 1133 mg/L) (Table 1).

Table 1. Density and biomass values recorded in the two experiments.

Days	1st Experiment		2nd Experiment	
	Density (10^3 cells/ml)	Biomass (mg/L)	Density (10^3 cells/ml)	Biomass (mg/L)
1	60	66.98	392	489.38
2	70	77.33	830	1038
3	44	49.51	749	936
4	68	75.66	906	1132.5
5	44	49.24	867	1083.75
6	105	116.55	837	1046.25
7	152	169.13	411	513.75

During the second experiment (Fig.7), four growth phases were observed:
1. The lag phase, in the first 3 days, with oscillating values of both density and biomass; there was a rapid increase by doubling the values in the first 2 days (from 392·103 to 830·103 cells/ml and from about 490 to 1038 mg/L), then moving towards a period of accommodation with some lower values (749·103 cells/ml and 936 mg/L).

2. The exponential growth phase, which showed an increase of approximately 20% compared to the previous day and more than 50% compared to the first day, when the culture reached the maximum point (906·103 cells/ml and 1132.5 mg/L).
3. On the 5th and 6th day, it was observed the stationary phase of the culture, a stagnant, slowing growth phase in which both cells and culture changed their appearance and density and biomass values gradually decreased (from 867·103 to 837·103 cells/ml, respectively, from 1083 to 1046 mg/L).
4. On the 7th day, it was recorded a sudden drop in values (from about 840·103 cells/ml to about 411·103 cells/ml) corresponding to the start of the decline phase.

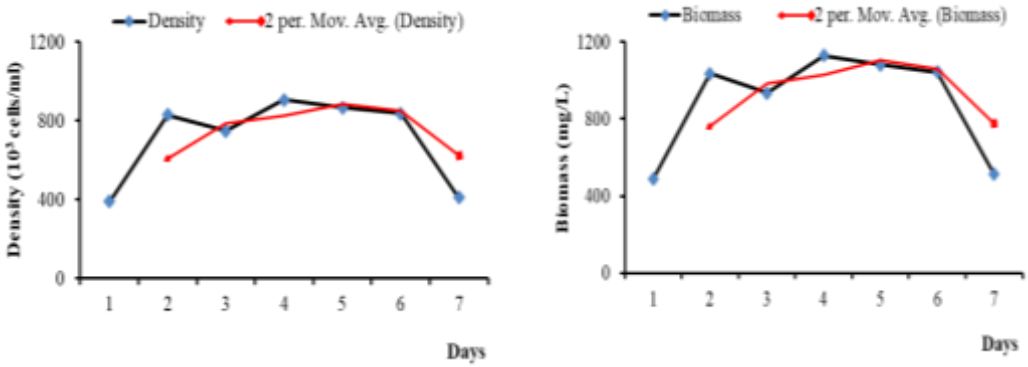

Fig. 7. Growth dynamics of microalgae by density (left) and by biomass (right) recorded in the second experiment.

As shown in other studies regarding *Skeletonema costatum* cultures, it can be observed the same growth trend in an 8-day study period (Fig. 8), identifying the four growth phases (Puskaric et al., 2003). In the first 3 days the lag phase is present with oscillating values, then the exponential growth phase is outlined over the next 3 days with values that increase significantly, followed by the stationary phase and finally the phase of decline.

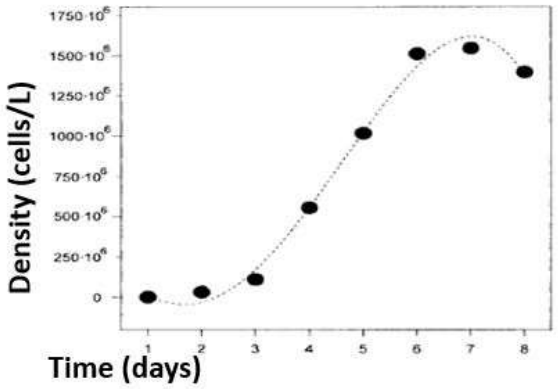

Fig. 8. Development of the species *Skeletonema costatum* in laboratory cultures.

With respect to the variation of microalgal density according to the concentration of nutrients available in culture (Fig. 9), it was observed a rapid increase of microalgae corresponding to the high nutrient concentration (Mata et al., 2010). With the massive development, microalgae consume nutrients that begin to decrease until they become a limiting factor. The end of the exponential growth phase and the beginning of the stationary phase correspond to a decrease in the nutrient concentration.

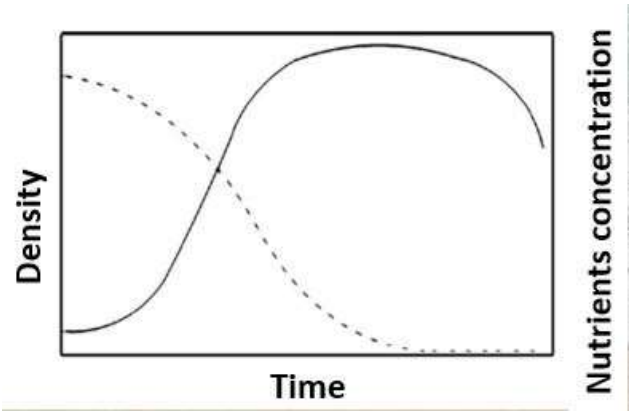

Fig. 9. Schematic representation of microalgal density (continuous line) and nutrient concentration (interrupted line) variation in microalgae cultures.

CONCLUSIONS

Skeletonema costatum is a dominant species in the phytoplankton community, especially during spring and summer, when reaches its maximum development. It is widely used in aquaculture as a feedstock for bivalve molluscs and shrimp larvae, but also in biodiesel production due to the high lipids concentration in dry biomass (12-51%).

Following the collection of water samples from Mamaia Station at the end of February 2013, it was identified the beginning of the period of development of the species *Skeletonema costatum*. The species isolation was reached by successive filtrations and dilutions and over time it was initiated a monospecific culture.

For the observation of *Skeletonema costatum* growth dynamics in laboratory cultures two experiments were performed. In the first experiment were provided only minimum laboratory conditions, thus only the first two growth phases were observed. By improving the conditions in the second experiment, all the growth phases could be observed:

- The lag phase, with oscillating values of both density and biomass; from $392 \cdot 10^3$ to $749 \cdot 10^3$ cells/ml and from about 490 to 936 mg/L).
- The exponential growth phase, when the culture reached the maximum point ($906 \cdot 10^3$ cells/ml and 1132.5 mg/L).
- The stationary phase of the culture, which showed a gradual decrease both in density (from $867 \cdot 10^3$ to $837 \cdot 10^3$ cells/ml) and biomass (from 1083 to 1046 mg/L). Starting with this phase the nutrient concentration decreases gradually until it becomes a limiting factor in the next phase.
- The decline phase with a sudden decrease, showing values twice as low as in the previous phase (from about $840 \cdot 10^3$ cells/ml to about $411 \cdot 10^3$ cells/ml) corresponding to the start of the decline phase.

In order to maintain the culture, the most important steps were:

- to study the growth dynamics of the species
- to multiply the culture to obtain a biological selection reserve of the material
- to keep the test tubes with concentrated culture on a shelf without direct light, at room temperature (23°C, ±2°C).
- to check the population situation monthly for a potential contamination.
- to refresh the nutrient medium periodically.
- to sterilize and to handle carefully the materials used.

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