INFLUENCE OF CHANGING TEMPERATURE AND DIFFERENT N/P RATIOS ON THE GROWTH RATE OF THE MARINE DIATOM, *Skeletonema costatum* (Greville) Cleve 1873 *(Oana Vlas, Luminita Lazăr, Laura Boicenco, Elena Pantea, Marius Făgăraș)*	**"Cercetări Marine" Issue no. 50** **Pages 73 - 83**	**2020**

INFLUENCE OF TEMPERATURE AND NUTRIENTS ON THE GROWTH RATE OF THE MARINE DIATOM, *Skeletonema costatum* (Greville) Cleve 1873

Oana Vlas[1], Luminita Lazăr[1], Laura Boicenco[1], Elena Pantea[1], Marius Făgăraș[2]

[1]National Institute for Marine Research and Development 'Grigore Antipa', 300 Mamaia Blvd., 900 581 Constanta, Romania
[2]'Ovidius' University of Constanta, 124 Mamaia Blvd., Constanta, Romania
E-mail: ovlas@alpha.rmri.ro

ABSTRACT

Skeletonema costatum is a species of microalgae that often produces blooms worldwide, which have led to mortality among fish and imbalances in the ratio of nutrients in the water column. The effect of temperature on the growth of *S. costatum* was examined under temperatures between 8 and 20°C. Also, the effect of nitrogen to phosphorus ratios on the growth of *S. costatum* was examined under Redfield ratio, nitrogen limitation and phosphorus limitation ratios. The diatom was able to grow in all the tested conditions, but the highest growth rates were reached at a temperature range of 17.7-18.8°C and phosphorus limitation conditions. Our study highlighted the fact that this species, the main responsible for the most intense and longest annual process of massive phytoplankton development can tolerate a wide range of nitrogen and phosphorus concentrations (at both limits) and a wide range of temperatures. These results point out that *S. costatum* can be considered an opportunistic species, with high potential to cause harmful blooms worldwide.
Key-Words: *S. costatum*, N/P ratios, temperature effect, blooms

AIMS AND BACKGROUND

Skeletonema costatum (Greville) Cleve 1878 (WoRMS, 2020) is a cosmopolitan diatom, widespread in marine and brackish coastal waters, being the main species that produces phytoplankton bloom phenomena that occurred with a higher frequency between 1970 and 1980, period dominated by eutrophication and pollution (Petran, 1997; Abaza *et al*, 2018). *S. costatum* was firstly observed by Skolka in 1955-1956 (Skolka, 1958).

The species has been the subject of many studies due to its importance in aquaculture (Shamsudin, 1992; Hashimoto *et al*., 2008), due to its affinity

for a wide range of nutrients (Tantanasarit *et al*., 2013, Shaik *et al*., 2015) and it has also been frequently reported as the dominant species in phytoplankton in various ecosystems (Aké-Castillo & Vázquez 2008, Rajkumar *et al*., 2009, Lim *et al*., 2014). The abilities of this species to tolerate high levels of nutrients such as ammonium (Smayda, 2004) and nitrates (Li *et al*., 2009) and to survive a wide range of temperature and salinity (Ebrahimi and Salarzadeh, 2016) explain the potential of the species to cause harmful blooms worldwide. Often, the blooms produced by this species have led to mortality among fish (Huo & Shu, 2005, Li *et al*., 2009) and imbalances in the ratio of nutrients in the water column (Zhou *et al*., 2017). Heavy rains increase nutrients level and stimulate the rapid proliferation of *S. costatum* (Li *et al*., 2009). Similar to the effect of heavy rains, rainfall that extends over a longer period of time, although it tends to decrease the concentration of nutrients in the water column, has also been shown to stimulate the rapid growth of this species (Vasudevan *et al*., 2014). Both findings claimed that *S. costatum* is an opportunistic species that can survive and use both maximum and minimum levels of nutrients in the water column.

The study aims to assess the influence of temperature variation and the influence of four types of culture medium with different nitrogen to phosphorus (N/P) ratios on the growth rate of a *S. costatum* strain isolated from the Romanian Black Sea shallow waters.

EXPERIMENTAL

Culture and growth conditions

Monospecific cultures of *S. costatum* were obtained in 2013 by the method described in Culcea, 2017. From 2019, *S. costatum* cultures are maintained in a growth medium prepared with autoclaved (121°C for 20 minutes) 0.22 µm filtered natural seawater and nutrient solutions (ISO 10253:2016), in Erlenmeyer flasks (250 ml) at 20°C. The gas exchange and the suspension of the cells are stimulated by positioning the cultures on the orbital stirrer at 70 rotations/minute. Furthermore, the stirring gives the cells equal access opportunities to nutrients and light. The light is provided by fluorescent neon set at approximately 4500-5000 lx and a photoperiod of 14:10 light to dark. Subcultures are made every 3-5 days, during the exponential growth phase, in freshly prepared growth medium. The glassware is cleaned and sterilized at 180°C for 3 hours using a hot air laboratory oven. The stoppers (cellulose or silicon) are autoclaved at 121°C for 20 minutes.

Culture testing conditions

To observe the effect of temperature variations on *S. costatum* development, new cultures (100 ml culture in 250 ml Erlenmeyer flasks) were inoculated and incubated at temperatures between 8-20 ± 2°C, maintaining the initial conditions of illumination and shaking. The experiment consisted of

exposing the species to gradually lower temperatures, by setting the thermostat to decrease the temperature every 24 hours with about 1.5-2.0°C until it reached 8°C. At 24-48 ± 2 hours, a 1 ml sample was taken with a sterile Pasteur pipette. Every 2-4 days, subcultures were made from the initial stock culture.

In order to observe the effect of nutrient variation, *S. costatum* was exposed to four types of culture growth medium containing different N/P ratios: nitrogen limiting conditions (4.2), phosphorus limiting conditions (63.3), optimum conditions according to Redfield, 1958 (16) and the ratio recommended by ISO 10253:2016 (18.8) (Fig. 1).

Fig.1. Preparation of growth medium with different ratios of nutrients

0.5 ml of concentrated culture (in the exponential growth phase) was inoculated in 99.5 mL from each type of culture medium. 100 ml Erlenmeyer flasks were used, placed on the orbital stirrer at 80 rpm (to ensure a better homogenization of the culture), at a constant temperature of 20°C and illumination of 4500-5000 lx, 12:12 light:dark cycle. The experiment ended 9 days after inoculation, when the cultures reached the decline phase. Both growth studies were done in triplicates (R1, R2 and R3).

Estimation of density and growth rates

Every 24 ± 2 hours, samples (1 ml) were taken from each test container with a micropipette. The samples were placed in labeled tubes and fixed with 30 µL of 37% formaldehyde (Fig. 2). The cells were counted using a hemocytometer and an inverted microscope. Density was estimated according to (IOC UNESCO, 2010): density (cells/mL) = counted cells x 10^4/number of squares counted.

Exponential growth rates were calculated for each replicate according to (ISO 10253:2016): $\mu = \frac{\ln N_L - \ln N_0}{t_L - t_0}$, where: t_0 = start time of the experiment; t_L = is the time of completion of the test or the last measurement performed by the control in the exponential growth phase; N_0 = initial cell density; N_L = is the cell density measured at time t_L. Assuming exponential growth and zero mortality, the intrinsic growth of the population, *r* is equal to μ.

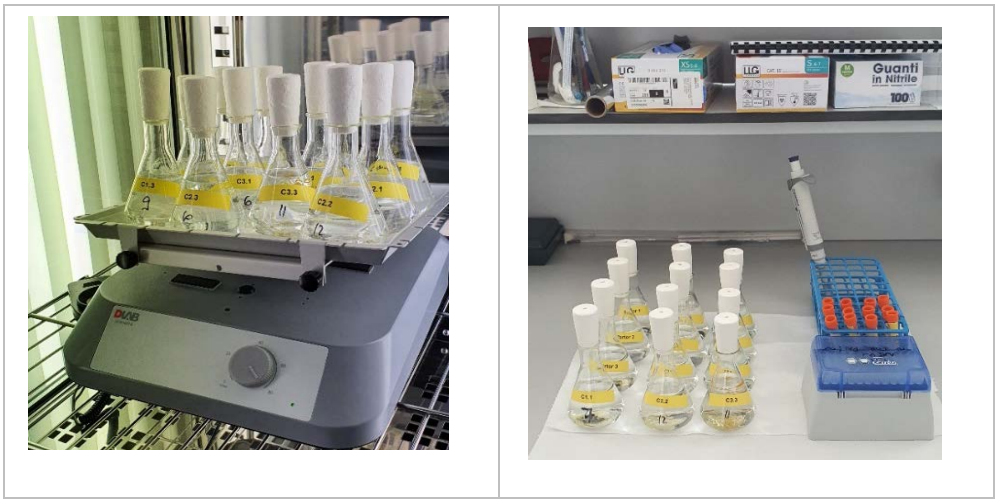

Fig. 2. Random positioning of the test containers on the 3D orbital shaker (left) and sampling (right)

Divisions per day (k) and population doubling time (T_2) were calculated according to: $k = r/\ln 2$, $T_2 = \ln 2/r$ (Andersen, 2005). The hours needed for doubling density were calculated by multiplying the doubling time with 24 hours.

Density and exponential growth rates were plotted on growth curves. For a better visualization of the growth phases, the Moving Average analysis tool was used.

RESULTS AND DISCUSSION

Temperature effect

Exponential growth rates were calculated for every replicate to observe the effects of temperature variations on the development of *S. costatum*. The highest growth rates (between 1.18-1.44 day^{-1}) were obtained in all three replicates at 18-19°C with an average of 1.28 day^{-1}. Also, high growth rates were obtained at temperatures between 12-13°C with an average of 0.90 day-

^{1} and between 8-11°C with an average of 1 day^{-1}. The lowest growth rates were observed at 16-17°C (average of 0.48 day^{-1}) (Fig. 3).

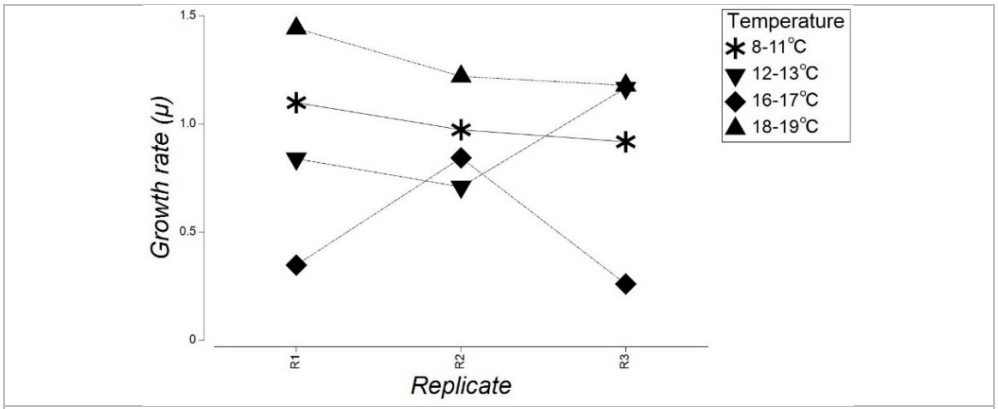

Fig. 3. *S. costatum* exponential growth rates at temperatures between 8-20°C

Therefore, the species seems to have a high tolerance for the temperature range 8-20°C, given that both the initial cultures and series of subcultures reached the exponential growth phase in the exposure period. However, the preference for the range of 18-19°C was highlighted compared to 8-17°C. Also, in other studies (Khan *et al*., 1998, Kaeriyama *et al*., 2011) it was observed that this species reached its maximum development at temperatures between 20-25°C (Fig. 4). This temperature range is in accordance with the optimum temperature obtained in Butterwick *et al*., 2005 (Table 1).

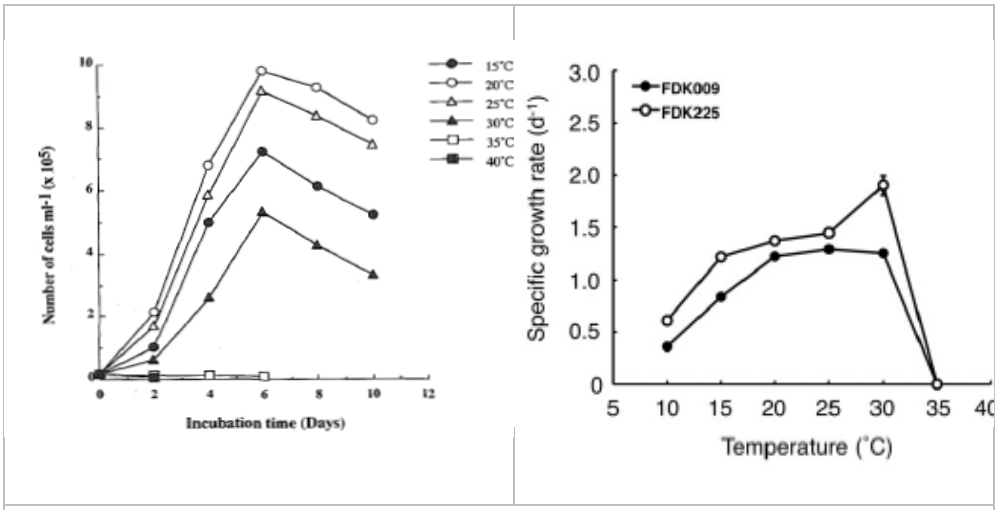

Fig. 4. *S. costatum* development at different temperatures (Khan *et al.*, 1998, left) and *S. costatum* (FDK009) and *S. pseudocostatum* (FDK225) growth rates at different temperatures (Kaeriyama *et al.*, 2011, right)

Table 1. *S. costatum* cardinal temperatures and optimal growth rate (Butterwick *et al*., 2005)

Species	T_{min}	T_{opt}	T_{max}	μ_{opt}
Skeletonema costatum	8	24.5	33	1

Nutrients effect

The growth curves were obtained for all growth mediums used (Fig. 5). The exponential growth phase started on the second day, in all flasks and ended on the 5th day. On the 6th day, it was observed the stationary phase, followed by a phenomenon which was also reported by other authors (Bolier *et al*., 1989). The phenomenon consisted in recording a second exponential growth phase, less intense than the first one.

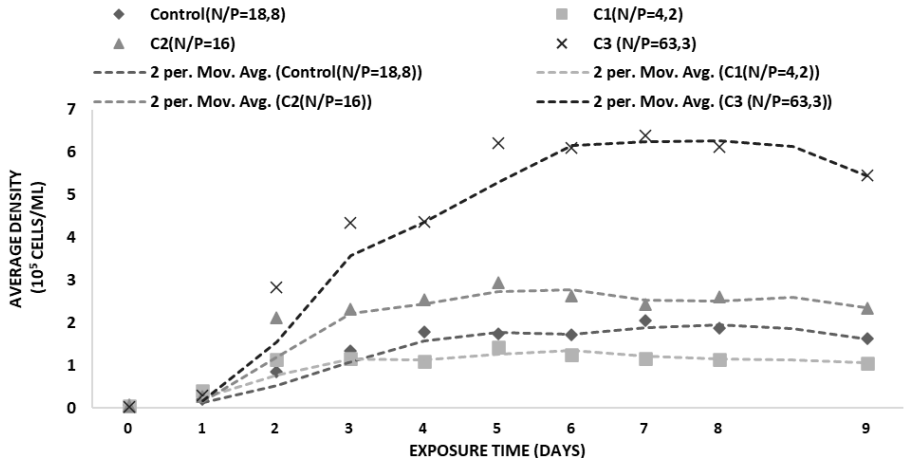

Fig. 5. *S. costatum* average density in growth medium with different N/P ratios

In the ratio considered **optimal** according to Redfield (N/P =16) *S. costatum* reached densities of up to $3.39 \cdot 10^5$ cells/mL and a specific growth rate (1.09 day^{-1}) higher than the one obtained in the control (0.78 day^{-1}).

Lower densities were recorded ($1.64 \cdot 10^5$ cells/mL) in the **N limiting** test cultures (N/P=4.2), but a specific growth rate similar to the control growth rate (0.80 day^{-1}) (Fig. 6).

In the **P limiting** condition (N/P=63.3), the development of microalgae was highly stimulated, reaching the maximum growth rate of 1.34 day^{-1}. In terms of density, the values were about 2-3 times higher ($7.5 \cdot 10^5$ cells/mL) than in the control and the optimal ratio test vessels and 5 times higher than

the maximum value obtained in N limiting test cultures.

Skeletonema costatum reached high intrinsic and specific growth rates (over 1 day^{-1}) in all test containers in the first day after start and between the first and the second day, which corresponded with the exponential growth phase. These rates decreased from the 3rd day to the end of the time exposure. The highest growth rates obtained in the first two days corresponded with a doubling in 11-16 hours in control, in 8-17 hours in N limiting conditions, in 8-10 hours in optimum condition and in 7-8 hours in P limiting conditions.

The doublings/day varied in these first two days between 1.45 in N limiting conditions and 3.22 in P limiting conditions. In control and optimum conditions, the doublings/day reached 1.53-2.06 and 2.34-2.95, respectively (Table 2).

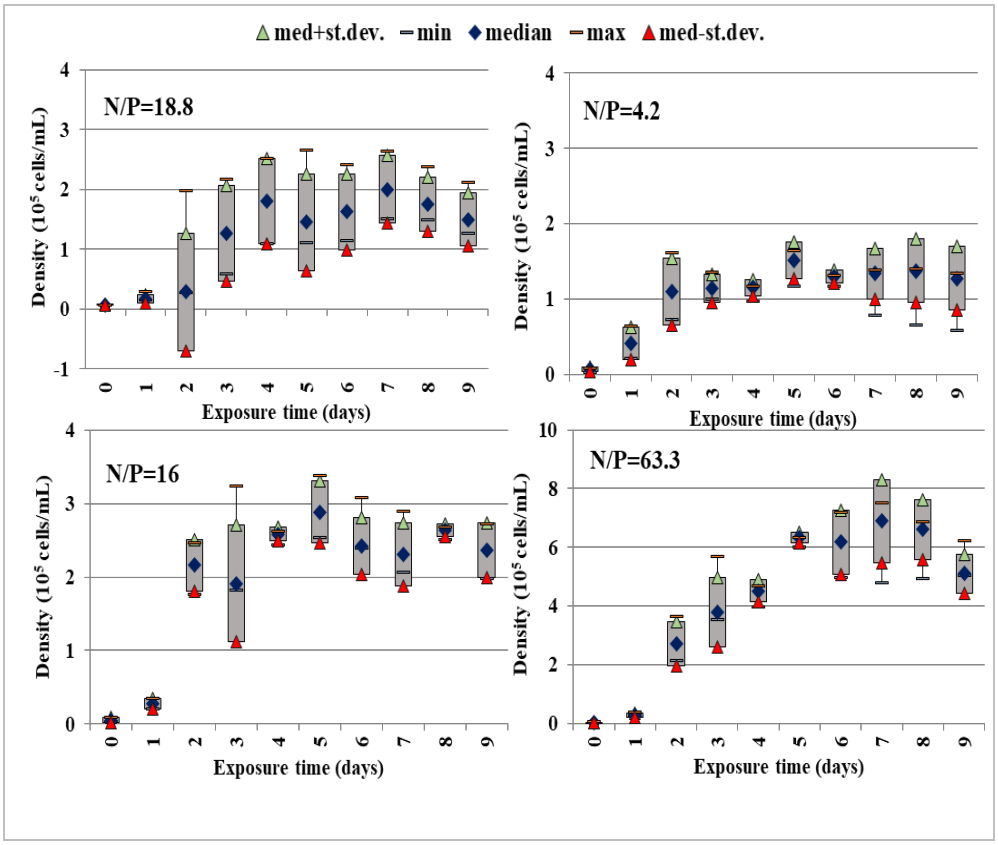

Fig. 6. *S. costatum* density variation in growth medium with different N/P ratios (3 replicates)

Table 2. Estimation of population growth rate (*r*), divisions per day (k), population doubling time (T_2) and hours for doubling density

Treatment	Time interval (days)	0-1	1-2	2-3	1-9	1-4	1-3
Control (N/P=18.8)	***r***	1.06	1.43	0.46	0.26	0.73	1.47
	k	1.53	2.06	0.66	0.38	1.05	2.12
	T_2	0.65	0.49	1.52	2.67	0.96	0.47
	hrs for doubling density	16	12	36	64	23	11
N limiting conditions (N/P=4.2)	***r***	2.05	1.00	0.02	0.12	0.32	1.53
	k	2.96	**1.45**	0.03	0.17	0.46	2.21
	T_2	0.34	0.69	38.47	5.99	2.18	0.45
	hrs for doubling density	8	**17**	923	144	52	11
Optimum conditions Redfield ratio (N/P=16)	***r***	1.62	2.05	0.09	0.27	0.74	1.88
	k	2.34	2.95	0.13	0.39	1.07	2.71
	T_2	0.43	0.34	7.88	2.58	0.93	0.37
	hrs for doubling density	10	8	189	62	22	9
P limiting conditions (N/P=63.3)	***r***	2.21	2.23	0.43	0.36	0.89	2.43
	k	3.19	**3.22**	0.62	0.52	1.28	3.51
	T_2	0.31	0.31	1.62	1.92	0.78	0.28
	hrs for doubling density	8	**7**	39	46	19	7

CONCLUSIONS

It was observed the high tolerance of *S. costatum* for the interval of 8 to 20 ± 2°C, affter exposure to temperature variations. However, the preference for the range 18-19°C was highlighted, compared to 8-17°C.

By increasing the *S. costatum* in four types of culture medium, a significant difference was observed in P limiting condition (N/P=63.3), which stimulated a higher development of microalgae. Densities were 2-3 times higher ($7.5 \cdot 10^5$ cells/mL) than those obtained in the control (N/P=18.8) and in the optimum ratio (N/P=16) and 5 times higher than the maximum value obtained in N limiting condition (N/P=4.2). This result highlights the impact of unbalanced N/P ratios on the development of microalgae with a high risk of starting intensive blooms.

Addressing the ecophysiology aspects of *S. costatum* studied in both natural environment and laboratory experiments, we can highlight that this species, responsible mainly for the most intense and longest annual process of massive phytoplankton development has impressive abilities to tolerate different levels of nutrients (at both limits) and to survive a wide temperature range. Thus, we can conclude that *S. costatum* can be considered an opportunistic species, with high potential to cause harmful blooms worldwide. It is important to note that often the blooms produced by this species have led to fish mortality and imbalances in the ratio of nutrients in the water column.

So far, the negative impact of blooms on the Romanian coast has been questioned only if they were of high intensity and took place during summer. As the evolutions of *S. costatum* occur in late winter-early spring season, they were not considered dangerous. These blooms can have negative effects consisting of asphyxiation of fish caused by the decreased oxygen concentration and the discomfort caused to tourists. Given the results of our experiments and the studies of other authors on the species' preference for temperatures of 20-25°C (specific for summer) and nitrogen-rich waters, it is necessary to draw attention to the need for further studies on the development of this species, both in terms of laboratory experiments and monitoring of the natural environment.

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