*International Journal on Algae, 2009, **11**(3): 211-221*

Role of phosphorus in regulation of *Emiliania huxleyi* (Lohm.) Hay et Mohl. (*Haptophyta*) blooms in the northeastern Black Sea*

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ABSTRACT

A role of phosphorus in regulation of mass development of *Emiliania huxleyi* (Lohm.) Hay et Mohl. in the northeastern part of the Black Sea was investigated in laboratory experiments with use of bath culture and mathematic methods. It was shown that low nitrogen/phosphorus ratio does not necessarily point to nitrogen as limiting factor for coccolithophorids growth. The reaction of the phytoplankton community to the nitrogen and phosphorus supply depends on species composition of dominating complex. The supplement of nutrients to the base culture with phytoplankton community where dominating complex included diatoms resulted in their absolute dominance due to higher specific growth rate (maximum growth rate of diatoms was 1.53 day^{-1}, coccolithophorids – 0.53 day^{-1}). Deficiency of nitrogen and phosphorus caused acceleration of the coccolithophorids cells degradation rate from 0.08 to 0.20 day^{-1}.

KEYWORDS: coccolithophorids, phosphorus, limitation, phytoplankton, *Emiliania huxleyi*.

INTRODUCTION

In recent years coccolithophorids attract special attention due to their ability to form coccosphere composed of $CaCO_3$, one of the main sources of the oceanic ground sediments (Paasche, 2001). The rise of CO_2 concentration in atmosphere and its negative

* Originally published in *Algologia*, 2009, **19**(2), pp. 135-144 ISSN 1521-9429

consequences calls the interest to all aspects of coccolithophorids vital functions (Engel et al., 2005). Observations on coccolithophorids in natural conditions, and experiments in lab cultures and mesocosms allowed revealing environmental conditions caused their blooms (Egge & Aksness, 1992; Egge & Hiemdall, 1994; Riegman et al., 2000; Paasche, 2001; Iglesias-Rodrigez et al., 2002). They include, first of all, stratification of water column, increased insolation, and low concentrations of nutrients. Coccolithophorids were considered to be able to abundant growth at low phosphorus concentration and, consequently, the high nitrogen and phosphorus ratio (Egge & Aksness, 1992; Egge & Hiemdall, 1994). This ability was considered to be the main condition of high competitiveness of coccolithophorids comparing to other algae, and was taken in account in mathematical models (Aksness et al., 1994). However later it was proved that high nitrogen and phosphorus ratio is not necessary prerequisite for coccolithophorids bloom (Lessard et al., 2005).

Intensive coccolithophorids blooms are typical for the Black Sea, as it is known both from aerospace observations data (Cokacar et al., 2001; Burenkov et al., 2006) and direct measurements of cell concentration (Sukhanova, 1995). *Emiliania huxleyi* is dominating species of coccolithophorids in the Black Sea like everywhere in the Northern Hemisphere.

However the question about mechanisms and limiting factors of growth regulation in coccolithophorids of the Black Sea still remains open. Recently we have investigated intensive coccolithophorids bloom in the northeastern part of the Black Sea in early summer period (Mikaelyan et al., 2006), and the structure of phytocenoses in early and late summer in the shelf zone of this area (Pautova et al., 2007). In present study, which continues cited above investigations, our purpose was to reveal regularities of coccolithophorids development and formation of plankton community structure at nitrogen and phosphorus concentration gradient in marine water during early summer period.

MATERIALS AND METHODS

For investigation of the effect of various concentrations of basic nutrients (nitrogen and phosphorus) on the structure of phytoplankton community from the northeaster part of the Black Sea on primary phases of *E. huxleyi* bloom five experiments were carried out during May-June 2005 and 2006. Experiments N 1-3 took place in 2005, and N 4 and N 5 in 2006. In experiments we used mixed cultures of phytoplankton isolated from natural marine environment. For this sea water was taken from surface layer (0-0.5 m) at the stations situated in sites with sea depth over 10 m (central part of Golubaya Bay, Caucasian Coast near the town of Gelendjik) and over 50 m (middle Gelendjik shelf area). In the

experiments N 1, N 3-5 collections from the middle Gelendjik shelf were used, and in experiment N 2 we studied samples of water collected in open bay.

Mixed cultures were prepared in the next way. Two liters of sea water immediately after collecting were filtered through the reverse filtration camera for concentration and identification of phytoplankton species (Sukhanova, 1983). The other part of samples was filtrated through 2 layers of 180 μm-diameter mill net in order to extract mesozooplankton, then bottled into 5 L plastic bottles and delivered to the coastal laboratory of the Southern Branch of P.P. Shirshov Institute of Oceanology (Gelendjik), where experiments were carried out.

In the experiment N 1 nine transparent plastic bottles 5 L in volume containing four liters of cultural medium were used. All other experiments were carried out in 500 mL Ehrlenmeyer glass vessels containing 200 mL of cultural medium.

In the experiments N 1 and N 3 natural light with dark-light cycle typical for May-June was applied. Neutralizing filters were used to prevent light inhibition caused by direct sun rays. The temperature of cultural medium in these experiments varied from 18 to 22 °C during the day. In the experiments N 2 (2005), N 4 and N 5 (2006) thermoluminostat was used keeping temperature of the medium on the same level with sea water in sample-taking area (17-21 °C). The intensity of incident light was 58-61 μM/m^2 FAR. Light:dark regime was 16:8.

In all experiments periodical (accumulating or bath) regime of cultivation was applied. Nutrients (KNO_3 as nitrate source and Na_2HPO_4 as phosphates) were supplied one time at the beginning of experiment according to the scheme given in Table 1.

The volume of nutrients was calculated in such way that nitrogen concentration would increased from 12.1 μM to 14.3 μM, and phosphorus from 0.81 to 1.0 μM. We have designed our experiments according to the pattern of complete factorial experiment (CFE) 2^2 (Maksimov & Fedorov, 1969). It allowed us to apply methods of mathematical planning of experiment, and to present the results as regression equations. The number of cells in stationary phase N_{st} of bath culture was used as main parameter for counting regression equations.

The statistical data processing was made for 5% significance level.

Cultures were checked during the whole period of experiment to control their algological purity and the absence of organisms of higher trophical levels (especially infusorians and amoebae).

Cell number was counted under light microscope every day, in living state, using 0.05 mL Nojotte counting camera. Picoplankton cells (1-2 μm in size) were not counted.

Re-calculation of wet biomass in carbonic units was done according to the formulas counted for each taxonomic group of algae (Menden-Deuner & Lessard, 2000).

TABLE 1. The schedule and the results of the experiments N 1-5 on study of the effect of nitrate and phosphate supply on number cells of *Emiliania huxleyi* (10^6 cells/L) in the stationary phase of the growth of bath culture

Variant	NO_3	PO_4	Number of experiment				
	(X_1)	(X_2)	N 1 (22.05.2005)	N 2 (23.05.2005)	N 3 (22.05.2005)	N 4 (19.05.2006)	N 5 (06.06.2006)
1	-	-	3.6±0.84	5.2±1.2	0.11±0.1	9.6±0.86	11.75±2.1
2	+	-	1.27±0.44	3.64±1.1	0.48±0.44	7.85±2.47	9.75±3.18
3	-	+	15.2±10	11.65±2.6	5.45±1.06	24.8±2.3	27.1±4.8
4	+	+		62.55±14.07	11.09±2.56	20.0±7.92	15.0±4.24
Note. (-) – nutrients were not supplied; (+) – nutrients were supplied.							

RESULTS

In initial cultures dominating complex of phytoplankton was represented by either coccolithophorids by themselves (experiments N 1 and N 2) or by coccolithophorids + diatoms (experiments N 3-5) (Table 2).

TABLE 2. Cell number and biomass (units and %) of main the phytoplankton taxonomic groups in 2005 and 2006 (with reference to initial cultures of experiments N 2 and N 4)

Systematic group	*N*, cells/L	% of total	*B*, mg/m^3	% of total	*B*, mg C/m^3	% of total
23.05.2005						
Diatomea	41600	3.39	2.41	0.50	0.31	0.52
Dinoflagellates	765	0.06	2.21	0.46	0.31	0.52
Coccolithophores	1190000	96.55	474.37	99.04	59.46	98.96
Total	1230000	100	478.98	100	60.08	100
19.05.2006						
Diatomea	105622	9.26	171.65	44.63	1.20E+01	29.16
Dinoflagellates	14179	1.24	29.25	7.60	4.18E+00	10.14
Coccolithophores	1020800	89.50	183.74	47.77	2.50E+01	60.70
Total	1140600	100	384.64	100	41.21	100
Note. Small flagellates and piloplankton (cells 1-2 μm) were not take in account.						

In all experiments the number dynamics of *E. huxleyi* was in accordance with regularities of population growth in bath culture (for example see the experiment N 2 in the Table 1, and also the Figures **1**, **2**).

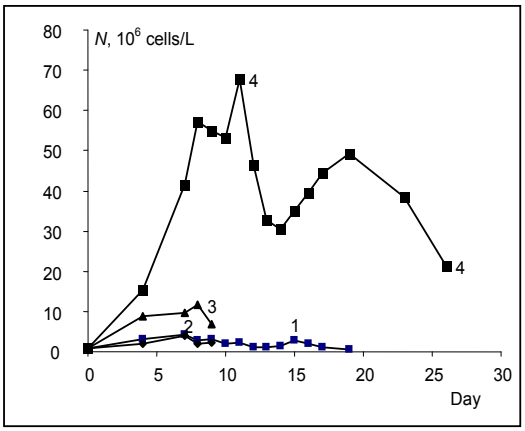

Figure **1**. *Emiliania huxleyi*'s cell number dynamics in bath culture in experiment N 2 (*1-4* – are variants of experiment according to Table 1)

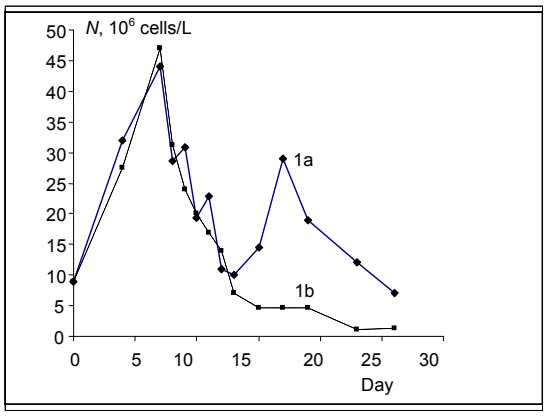

Figure **2**. *Emiliania huxleyi*'s cell number dynamics in bath culture without nutrient supply (var. 1 of experiment N 2, two replications)

In the cultures free of nutrient supply in all experiments cell number differed from the data recorded in the initial culture (Table 1). For example, in 2005 maximal (5 times) rise in coccolitophorids number was registered in the experiment N 2. In 2006 10-times increase of this parameter was fixed in the experiment N 5.

In other variants of the experiments the number of *E. huxleyi* cells in stationary phase depended on added nutrients (Table 1, Figure **1**). So, nitrate supplement (var. 2) did not cause considerable rise of *E. huxleyi* number. At the same time, adding of phosphate (var. 3) resulted in the sharp (from 2 to 10 times) increase of coccolithophorids cells in all experiments. In var. 4 where nitrates and phosphates were added simultaneously, values of maximal cell number were different depending on the experiment. In experiments N 2 and N 3 the number of coccolithophorids cells increased considerably; in other experiments

values of the cell number did not differ substantially from the variant where only phosphorus was added. In these experiments diatoms grow intensively.

The values of regression equation coefficients describing the level of accumulation of cells (N_{st}) in the experiments N 3-5 also testify that concentration of phosphate was the most significant factor of *E. huxleyi* growth in the bath culture (Table 3).

TABLE 3. The regression equation for cells number (10^6 cells/L) of *Emiliania huxleyi* in stationary phase of bath culture (N_{st}) for experiments N 2-5

Experiment 2 (23.05.2005)
$N_{st} = 20.76+12.34X_1+16.34X_2+13.2X_1X_2$ (7.93)
Experiment 3 (22.05.2005)
$N_{st} = 4.28+1.5X_1+3.99X_2+1.32X_1X_2$ (1.56)
Experiment 4 (19.05.2006)
$N_{st} = 15.56+1.64X_1+6.84X_2-0.76X_1X_2$ (4.75)
Experiment 5 (6.06.2006)
$N_{st} = 15.9-3.53X_1+5.15X_2-2.53X_1X_2$ (3.31)

Note. X_1 – nitrogen; X_2 – phosphorus. In brackets – the value of confidence interval for 5% significance level.

In the experiment N 2 simultaneous adding of nitrogen and phosphorus brought considerable effect.

Obtained results allow us to evaluate the growth rate of *E. huxleyi* and the rate of cells degradation in the phase of decay. So, the growth rate in the experiment N 2 (var. 4) under limitation-free conditions in terms of nitrogen and phosphorus can reach 0.53 day^{-1}, i.e. the time of doubling will be close to 1 day. The growth rate of cells decay in var. 1-3 varies from 0.12 to 0.2 day^{-1} (R^2 = 0.48-0.76) meaning in average 0.15 day^{-1}. In var. 4 this parameter was 0.08 day^{-1} with coefficient of determination 0.72.

DISCUSSION

For bath culture two main parameters exist, enabling the description of a growth curve: the maximum growth rate (μ_{max}) and cell concentration in stationary phase of bath culture (N_{st})

(Silkin & Khailov, 1988). The first depends on light intensity, water temperature, and the ratio of concentrations of nutrients. The level of cell number in stationary phase of bath culture is determined by the initial concentration of growth-limiting nutrient. Accumulation of *E. huxleyi* biomass corresponds to the bath culture curve making possible implementation of the methods of algae growth analysis (Figure **1**) (Silkin & Khailov, 1988). The analysis of the dependence of *E. huxleyi* cell number on the phosphate supply in the experiments N 1, N 3-5 allowed us to conclude that intensive growth of this species in studied area in early summer of 2005 and 2006 occurred under conditions of relative phosphorus limit (Tables 1, 3). The fact that adding of nitrates by themselves did not change number of *E. huxleyi* cells in comparison to var. 1 in all experiments indicates that concentration of nitrogen was relatively excessive. Simultaneous adding of nitrogen and phosphorus caused various after-effects in different experiments depending on the composition of phytoplankton community and, first of all, on the contribution of coccolithophorids (Table 3).

In the experiment N 2, where, according to equation of regression, the simultaneous adding of nitrates and phosphates caused considerable increase of coccolithophorids number in the stationary phase of growth, the community was formed completely by coccolithophorids (Table 2). The nitrogen and phosphorus ratio obtained in the var. 4 of the same experiment was in the point of transition from one limiting factor to the other (Silkin & Khailov, 1988). Thus, early summer coccolithophorids bloom in the northeastern part of the Black Sea occurs in the area where the nitrogen and phosphorus ratio is in the range of the point of transition from limiting concentrations of nitrogen to limiting concentrations of phosphorus, and where the growth of *E. huxleyi* is limiting only by phosphorus.

In the var. 4 of the experiments N 3-5 the contribution of coccolithophorids was not crucial: the diatoms, having higher specific growth rate, at the end of experiment became dominants (Table 3). In the experiments N 4 and N 5 maximal specific growth rate of diatoms was 1.53 day^{-1}, and for coccolithophorids it makes 0.515 day^{-1}.

In the var. 1 (free of nitrogen and phosphorus supply) in all experiments except of N 3 the growth of coccolithophorids number was recorded. Though the phosphorus concentration in sea water at the moment of the beginning of experiments (Table 4) had limited the growth of phytoplankton (Thomas & Dodson, 1968), its intracellular reserves were enough for several scale divisions (in the experiments N 1 and N 2 four-five divisions were registered and in the experiments N 4 and N 5 about 10). Thus, in 2006 intracellular accumulation of phosphates was more intensive; it testifies that concentration of phosphates in the sea water was higher in comparison to 2005.

Based on the concept of intracellular regulation of algae growth (Droop, 1974), we can get some estimations of *E. huxleyi* growth rate. Specific growth rate μ is determined by intracellular growth-limiting nutrient content *Q* in accordance with the equation:

$$\mu = \mu_{\max}\left(1 - \frac{q}{Q}\right),$$

where *q* is a minimum nutrient content in a cell under specific growth rate equal to 0; μ_{max} – maximum growth rate (day^{-1}).

Due to the fact that in the experiments free of nutrients *E. huxleyi* cell concentration increased in 5-10 times in comparison to the initial one (which is equal to the cell number in the sea), it is possible to suppose that intracellular content of phosphorus *Q* as the growth-limiting factor is about 5-10 minimal cellular contents of this element *q*, i.e. $Q \leq$ (5-10) *q*.

Evidently, intracellular phosphorus content at the beginning of the experiment was much lower of maximum possible value, because *E. huxleyi* cells can accumulate phosphorus for more than thirty divisions (Riegman et al., 2000). Such phosphorus content in the cell was enough to provide the growth rate over the half from maximal value, and to overcome the rate of cell decay. Relatively high phosphorus content in cells against its low concentration in the medium testifies that in natural conditions this nutrient is periodically supplied to the surface layer of water being the basic biotope of *E. huxleyi* in the initial period of its bloom. At the same time the rate of phosphorus supply in 2006 was higher than in 2005.

Simultaneous adding of nitrogen and phosphorus in the experiment N 2 (where community mainly consisted of coccolithophorids, and the maximum increase in cell number was observed) increases the specific growth rate of *E. huxleyi* as far as energy supply allows. Under the similar to our experiments conditions of light:dark period and temperature, but with light flow equal to 160 μM/(m^2·s) FAR, the specific growth rate of this species would be 1.3 day^{-1} (Paasche, 2001). The light intensity in our experiments was more than a third of above mentioned, probably, it caused the growth rate decrease up to 0.515 ± 0.05 day^{-1}.

In the experiments on mesocosms with nitrogen and phosphorus supply carried out near the Norwegian coast in spring-summer period under natural illumination (light intensity 593±213 μM/m^2 FAR) and temperature of 13 °C the specific growth rate was estimated to be 0.5±0.2 day^{-1} (Engel et al., 2005). The specific growth rate at temperatures 8-10 °C was 0.29 ± 0.32 day^{-1} (Egge & Hiemdall, 1994). These data allows us to suppose that in our experiments applied light intensity limited the *E. huxleyi* specific growth rate.

The decay rate of coccolithophorids biomass in bath culture changes in the range from 0.08 (var. 4 with nitrogen and phosphorus supply) to 0.12-0.20 day^{-1} (var. 1-3). Thus, both nitrogen and phosphorus limitation causes the increase of biomass decay rate. In mesocosm experiments the decay rate of *E. huxleyi* was 0.19-0.23 day^{-1} (Egge & Hiemdall, 1994). In the mathematic model, describing the phytoplankton growth in Norwegian water mesocosm, the coefficient of the mortality of *E. huxleyi* was taken to be equal to 0.14 day^{-1} (Aksness et al., 1994). Since in our experiments the influence of predators was absent, high degradation of biomass was caused by breathing or decay (for example, by means of viruses). Really, virus-like particles were fixed in *E. huxleyi* cells in cultures. Maximum rate of virus decay of *E. huxleyi* cells was 0.4 day^{-1} (Bratbak et al., 1993). At present it is impossible to determine the mechanisms determining the rate of *E. huxleyi* decay in our experiments.

TABLE 4. The phosphates and nitrogen concentration and nitrogen/phosphorus ratio in the sea water on the station of sampling-taking for experiments 2, 4 and 5

Number of experiment, date	PO_4	N_{min}	N_{min}/PO_4
N 2, 23.05.2005	0.15	0.48	3.20
N 4, 19.05.2006	0.44	0.61	1.38
N 5, 06.06.2006	-	0.64	-

Note. N_{min} – sum of nitrate, nitrite and ammonium.

The nitrogen/phosphorus ratio in the water of the northwestern Black Sea during the coccolithophorids bloom in 2005-2006 was low (Table 4), but it is impossible to consider this fact as exclusive, the same was observed in other seas (Lessard et al., 2005). For example, *E. huxleyi* bloom in the Dardanelles in June-July 2003 took place at the value of nitrogen/phosphorus ratio below the Redfield's ratio (Turkoglu, 2008). From the common point of view such water considered to be nitrogen-limited. Our experiments have shown that in the northeastern part of the Black Sea in May-June the value of nitrogen/phosphorus ratio was below the Redfield's ratio, however abundance of coccolithophorids was managed by phosphorus supply.

CONCLUSIONS

Experimental studies with use of natural phytoplankton community have been demonstrated that the intensity of coccolithophorid *E. huxleyi* bloom in May-June 2006 in the shelf water of the northeastern Black Sea was limited by phosphorus concentration in the surface water layer. The low nitrogen/phosphorus ratio not necessarily means that growth-limiting factor is nitrogen. The growth of *E. huxleyi* in the medium with low phosphorus concentration occurred at the account of intracellular reserves; in 2006 their accumulation was more intensive. The reaction of phytoplankton community on nitrogen and phosphorus supply depends on taxonomic composition of dominants. The supplement of nutrients to the base culture with phytoplankton community having diatoms among dominants resulted in their absolute dominance due to the higher specific growth rate (maximum growth rate of diatoms was 1.53 day^{-1}, coccolithophorids – 0.53 day^{-1}). Deficiency of nitrogen and phosphorus caused rise of the coccolithophorids cells degradation rate from 0.08 to 0.20 day^{-1}.

ACKNOWLEGMENTS

This work was fulfilled with the financial support of RFFR (projects No 05-05-64234 and 06-05-64844).

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