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# EVALUATION OF THE TOLERANCE THRESHOLD OF MARINE BENTHIC DIATOM *PLEUROSIGMA AESTUARII* (BRÉB. IN KÜTZ.) W. SMITH, 1853 (BACILLARIOPHYTA) UNDER THE IMPACT OF COPPER (II) IONS

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**Abstract.** Copper compounds accumulate in marine bottom sediments as the result of human activity and, being highly toxic, affect microphytobenthos. Evaluation of the tolerance ranges of benthic diatoms to copper pollution is important for biotesting and assessment of coastal marine environment. This work is aimed to reveal the dynamics of growth and cell death of marine benthic diatom *Pleurosigma aestuarii* (Bréb. in Kütz.) W. Smith, 1853 under the impact of a wide range of copper (II) ions concentrations over the course of a 10-day experiment in clonal culture, as well as to identify a tolerance threshold for this species, critical for its survival when exposed to the toxicant. The study has focused on assessment of the changes in the proportion (%) of alive cells, absolute cell number and specific growth rate of the culture at different exposure durations and concentrations of $Cu^{2+}$ ions (32–1024 µg/L) and was meant to evaluate the applicability of this species as a new test object for ecotoxicology. In the control culture and in the cultures exposed to $Cu^{2+}$ in the range of concentrations 32–256 µg/L, the proportion of alive cells did not change over the course of the experiment (95–99 %). At $Cu^{2+}$ concentration 320 µg/L, the proportion of alive cells decreased to 23 % on the 3$^{rd}$ day and to 10 % on the 5$^{th}$ day, which provided a basis to consider this value as a threshold one for *P. aestuarii* survival. At $Cu^{2+}$ concentrations 384 µg/L and higher, up to the maximum one (1024 µg/L), drastic inhibition of the culture was recorded as early as on the 1$^{st}$ day, and on the days 3–5, all the cells died. The increase in the absolute cell number in the concentration range 32–256 µg/L was consistent with the dose–response sigmoid model. Over the timespan of days 1–7, the cell number increased by 3–5 times, reaching its maximum, and then it decreased by 10–12 % by the 10$^{th}$ day. At $Cu^{2+}$ concentrations 320 µg/L and higher, the increase in the cell number was strongly suppressed since the 1$^{st}$ day. Within the 32–256 µg/L range, the test culture is characterized by positive specific cell growth rate for the period up to 7 days; at the threshold concentration 320 µg/L and higher, this test parameter becomes negative. Thus, *P. aestuarii* should be recommended as a new appropriate test object both for toxicological experiments and for monitoring of coastal marine environment affected by technogenic pollution.

**Keywords:** toxicology, biotesting, clonal culture, diatoms, monitoring, pollution, Black Sea

# ОЦЕНКА ПОРОГОВОЙ УСТОЙЧИВОСТИ БЕНТОСНОЙ ДИАТОМОВОЙ ВОДОРОСЛИ *PLEUROSIGMA AESTUARII* (BRÉB. IN KÜTZ.) W. SMITH, 1853 (BACILLARIOPHYTA) К ВОЗДЕЙСТВИЮ ИОНОВ МЕДИ (II)

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**Аннотация.** Соединения меди, будучи высокотоксичными и аккумулируясь в донных отложениях вследствие антропогенной деятельности, оказывают негативное влияние на микрофитобентос. Выявление границ устойчивости бентосных диатомовых к загрязнениям медью — актуальная задача для биотестирования и оценки состояния морской среды. Исследовано воздействие ионов меди (II) в различных концентрациях на новый для токсикологии вид морской диатомовой водоросли *Pleurosigma aestuarii* (Bréb. in Kütz.) W. Smith, 1853 в условиях 10-суточных экспериментов на клоновой культуре. Цель работы состояла в оценке динамики доли живых клеток (%), абсолютной численности и удельной скорости прироста числа клеток при разных сроках экспозиции и различных концентрациях $Cu^{2+}$ (от 32 до 1024 мкг/л), а также установлении критической для выживания вида концентрации токсиканта. В контроле и при концентрациях ионов меди 32–256 мкг/л доля живых клеток *P. aestuarii* на протяжении эксперимента не менялась (95–99 %). При содержании $Cu^{2+}$ 320 мкг/л доля живых клеток в культуре уже на 3-и сутки резко снижалась до 23 %, на 5-е сутки — до 10 %, что определяет данную концентрацию как пороговую для выживания *P. aestuarii*. При повышении концентрации $Cu^{2+}$ до 384 мкг/л и вплоть до максимальной (1024 мкг/л), отмечено резкое угнетение культуры уже в первые сутки, а на 3–5-е сутки доля живых клеток падала до 0 %. Прирост абсолютной численности клеток *P. aestuarii* при концентрациях $Cu^{2+}$ 32–256 мкг/л описывается сигмоидной кривой отклика. В период 1–7-х суток численность возрастала в 3–5 раз, достигая максимума и снижаясь к 10-м суткам на 10–12 %. При концентрации 32–256 мкг/л культура характеризовалась положительным удельным приростом; при концентрации токсиканта 320 мкг/л и выше удельный прирост клеток становился отрицательным. *P. aestuarii* рекомендована как тест-объект для токсикологических экспериментов и мониторинга техногенного загрязнения акваторий.

**Ключевые слова:** токсикология, биотестирование, клоновая культура, мониторинг, диатомовые, загрязнение, Черное море

## INTRODUCTION

Increasing anthropogenic load on the coastal environment resulting from intensification of industrial production and expansion of maritime transport provides relevance to the monitoring of marine water quality. Therefore, adoption of bio-testing and bio-toxicological methods for detection of the response of benthos communities and for identification of the threshold concentrations of toxicants for various taxa is crucial for the protection of coastal ecosystems. Benthic diatoms are considered to be a convenient test object due to their high abundance in microphytobenthos and pronounced sensitivity to pollutants (including heavy metals) accumulated in soft bottom substrates [1, 2]. Identification of various benthic diatom species, their morphophysiological responses and the ranges of tolerance to the impact of heavy metals and other pollutants is among the important tasks of biotesting and biomonitoring. Thus, finding new benthic diatom species that can be used as appropriate indicators for assessment of marine environment quality is a very important task [3]. Copper is a microelement, essential for metabolism in microalgae, electron transfer in respiratory chain of mitochondria, growth, functioning of photosynthetic apparatus, etc. [1, 4, 5–11]. Meanwhile, copper compounds are ecologically significant pollutants with high toxicity that are brought into the marine environment with industrial and municipal effluents and accumulate in bottom sediments [1, 3, 12, 13]. Diatoms are capable of accumulating copper and other heavy metals in their cells in the concentrations $10^3$–$10^4$ times higher than those in the environment [4, 5]. According to Russian State Sanitary Regulations and Norms, the maximum permissible concentration (MPC) of copper in the water column is 5 μg/L, but for marine soft bottom substrates this value is not determined. In silty bottom sediments of the Black Sea nearshore areas, the copper

content ranges from 0.3 to 11.5 μg/g dry weight [12], but in closed and semi-closed industrially polluted bays it can reach 20–37 μg/g dry weight [14, 15]. The importance of copper compounds both for the metabolism of aquatic plants and in biogeochemical cycles in marine environment determined the choice of copper (II) sulfate as a model toxicant for experiments.

Previous studies assessing the toxic effect of copper on marine microalgae have revealed their species-specific resistance to $Cu^{2+}$ ions [5, 7, 9, 16, 17]. Being attached to substrate, benthic diatoms have high sensitivity and great veracity in their response to pollution impact, which substantiates the necessity to expand their study in toxicological experiments and further application for the assessment of marine environmental quality [5, 9, 11, 18–20].

The choice of benthic diatom *Pleurosigma aestuarii* (Bréb. in Kütz.) W. Smith, 1853 for the toxicological experiment was motivated by the following considerations: the large size of its cells, close association with substrate, mobility of cells, relative simplicity of cultivation and high rate of cell division. These properties make it possible to record the vital status and to assess the experimental object *in vitro*. *P. aestuarii* is one of the most abundant and common species that bring a substantial input into the microphytobenthos community of the Black Sea ecosystem in terms of both abundance and biomass. The equability of cell distribution pattern of *P. aestuarii* over the entire bottom area in experimental containers (Petri dishes) was statistically proved before [21]. These results were applied for a reliable comparison of cells development indices based on analysis of a small number of investigated areas (10–15).

This work continues our previous research on evaluation of the response of clonal cultures of some common species of the Black Sea diatoms to the impact of pollutants and determination of their threshold concentrations for survival [11, 21–23]. It should be noted that, in addition to obtaining the new data on the tolerance ranges of various diatom species and optimizing their cultivation conditions, the criteria for detection and accounting of alive cells, as well as assessing the population growth rate at different concentrations of toxicant in culture medium are also being investigated. Moreover, it is important to expand the knowledge of the life cycles of various taxa of Bacillariophyta. This work is focused on studying the dynamics of changes in cell number of *P. aestuarii* culture at different concentrations of copper (II) ions, identification of the tolerance ranges and assessment of the applicability of this species as a new test object for toxicological studies.

## MATERIALS AND METHODS

The investigated strain of benthic diatom *Pleurosigma aestuarii* originated from rocky substrate near Cape Aya (44°28′25″N, 33°37′58″E, Crimea, the Black Sea) collected at the depth of 3 m in July 2018. The clone was established by isolating a single cell and rinsing it with culture medium 7 times [23, 24]. This species is marine, benthic, common in coastal areas; its cells are solitary and mobile, and they easily move along the surface of substrate (Fig. 1: 1–2). Living cells are characterized by two narrow lobed plastids located along valve margin and not reaching the apices (Fig. 1: 3). Valves moderately sigmoid, apices with sharp ends, 135 μm length and 22.5 μm width (Fig. 1: 4–6). Raphe slightly eccentric, raphe sternum narrow, completely straight along the entire length, slightly curved near the apices that bend in opposite directions, central area small, rounded (Fig. 1: 5–6). Valve ornamented by striae (20–21 in 10 μm) consists of small rounded areolae located in oblique and transverse lines (*quincunx*) at angle 60° (Fig. 1: 7–8). In SEM, a slit-like areola in the external view of valve, double areolar foramina in the internal view of the valve (Fig. 1: 9–10). The indicated cell dimensions were recorded at the beginning of cultivation.

The strain was maintained in Petri dishes with 30 ml modified seawater optimized for marine benthic diatoms under diffused natural insolation and temperature 22±2 °C. Seawater for media was taken in a 12-mile zone off the Crimean coast during scientific cruises of the R/V "Professor Vodyanitskiy", filtered through a 0.45 μm filter, then pasteurized three times at a temperature of +75 °C. Nutrients were added in accordance with the protocol [21, 25].

Microphotography of alive cells for counting and assessing their condition was carried out under Carl Zeiss AxioStar Plus light microscope (LM) with an Achroplan ×10 objective lens and Canon PowerShot A640 camera. Identification of cleaned valves was carried out using Carl Zeiss PrimoStar Plus LM with a Plan-Achroplan ×100 objective lens and integrated camera. Ultrastructure of valves was examined using Hitachi SU3500 scanning electron microscope (SEM).

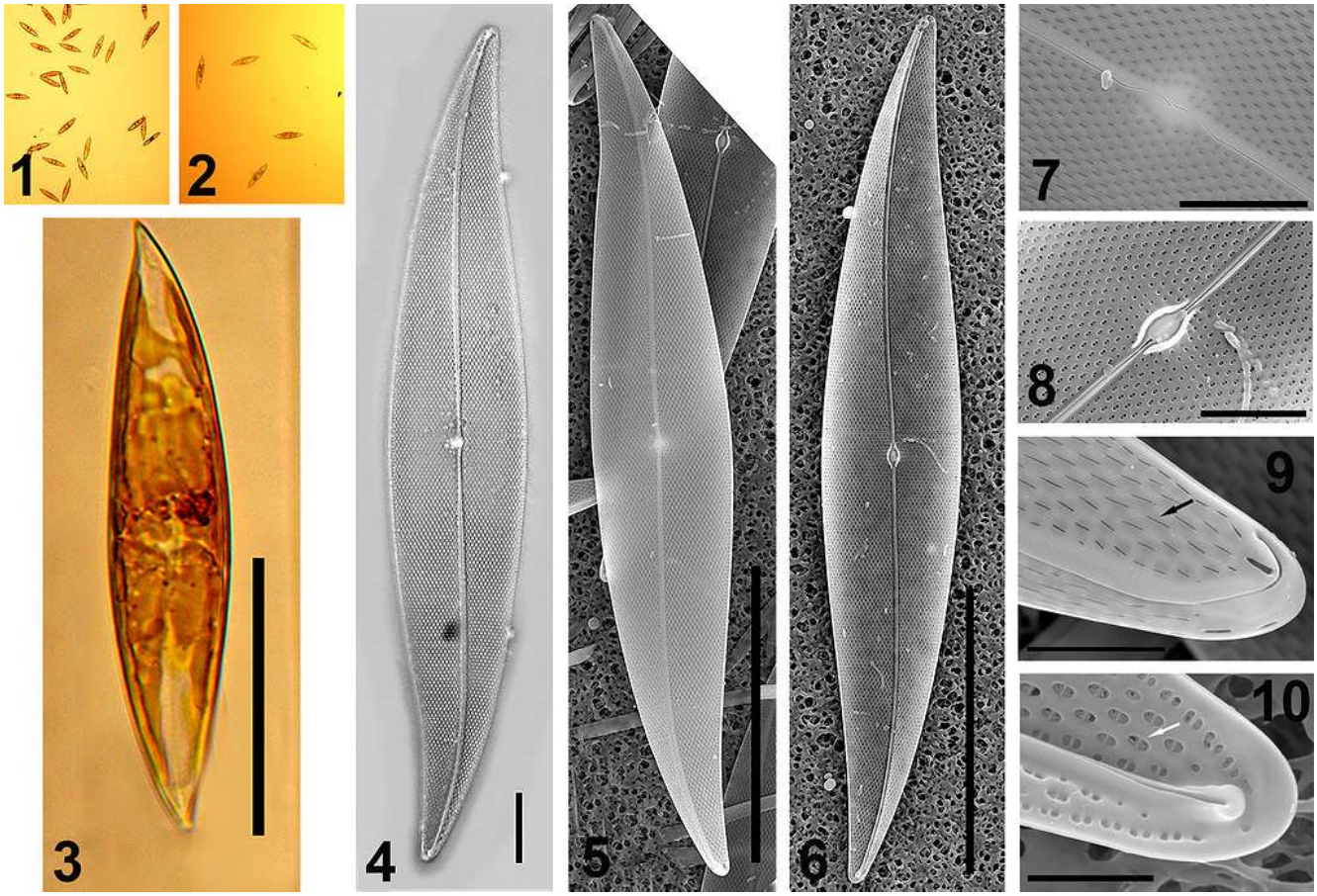


**Fig. 1.** Marine benthic diatom *P. aestuarii*: 1 — alive cells (LM×100); 2 — dead cells (LM×100); 3 — alive cell (LM×630, scale bar 50 μm); 4 — valve internal view (LM×1000, scale bar 10 μm); 5 — valve external view (SEM×1200, scale bar 40 μm); 6 — valve internal view (SEM×1200, scale bar 40 μm); 7 — central node of the raphe, valve external view (SEM×11000, scale bar 5 μm); 8 — central node of the raphe valve, internal view (SEM×9000, scale bar 5 μm); 9 — terminal apices, external view (SEM×18000, scale bar 3 μm); 10 — terminal apices, internal view (SEM×25000, scale bar 2 μm). Black arrow (9) points at the slit-like areola in the external view of the valve; white arrow (10) points at the double areolar foramina in the internal view of the valve. Photo 1–4 (LM) by S. Trofimov, E. Nevrova; photo 5–10 (SEM) by V. Lishaev, E. Nevrova

**Рис. 1.** Морская бентосная диатомовая водоросль *Pleurosigma aestuarii*: 1 — живые клетки (СМ×100); 2 — мертвые клетки (СМ×100); 3 — живая клетка (СМ×630, масштаб 50 мкм); 4 — створка, вид изнутри (СМ×1000, масштаб 10 мкм); 5 — створка, вид снаружи (СЭМ×1200, масштаб 40 мкм); 6 — створка, вид изнутри (СЭМ×1200, масштаб 40 мкм); 7 — центральный узелок шва, вид створки снаружи (СЭМ×11000, масштаб 5 мкм); 8 — центральный узелок шва, вид створки изнутри (СЭМ×9000, масштаб 5 мкм); 9 — апикальный конец створки, вид снаружи (СЭМ×18000, масштаб 3 мкм); 10 — апикальный конец створки, вид изнутри (СЭМ×25000, масштаб 2 мкм). Черная стрелка (9) указывает на щелевидную ареолу на внешней стороне створки; белая стрелка (10) указывает на двойную ареолу на внутренней стороне створки. Фото 1–4 (СМ) С. Трофимова, Е. Невровой; фото 5–10 (СЭМ) В. Лишаева, Е. Невровой

A certain amount of culture media, stock solution of $CuSO_4 \times 5H_2O$ and 1 ml of *P. aestuarii* clone culture inoculum were added in every Petri dish (90 mm in diameter) to make the total volume of 30 ml, in accordance with the protocol [22]. Since the tolerance ranges of the species to copper (II) ions impact were unknown, toxicity screening was performed for sequentially increasing concentrations of the toxicant: 32, 64, 128, 256, 320, 384, 448, 512 and 1024 μg/L (converted to ions $Cu^{2+}$). The control culture (media without toxicant) and each concentration were tested in three replicates. To exclude evaporation and contamination of the solution by external microorganisms, the Petri dishes were sealed with Parafilm®. The duration of all experiments was 10 days, and the exposure check-up periods were 1, 3, 5, 7 and 10 days.

Response of *P. aestuarii* to the impact of $Cu^{2+}$ was assessed by calculating the proportion of alive cells (%). The intravital condition of cells was assessed according to shape and integrity of the cell structure, homogeneity and color of chloroplasts, and regular divergence of frustules after vegetative division. Diatoms with drastic darkening of their plastids, opened valves, and/or lysis of cell contents were considered to be dead (Fig. 1: 2). The number of alive and dead cells was determined based on the averaged data obtained by photographing 10–15 random viewing areas in each dish. Along with that, dynamics of the increase in the absolute number of alive cells for the tested culture was calculated.

Assessment of the specific growth rate in the cell number (as one of the principal parameters of the biotest) was carried out based on the number of cell divisions per day at different stages of the experiment [20]. Parameters of the direct relationship between an increase in toxicant concentration and a decrease in intensity of the specific growth rate in the tested diatom species were also determined. The specific increase in the cell number (V, the average number of divisions per day) was calculated using the equation [17]:

$$V = \frac{N_{(t+\Delta t)} - N_t}{\Delta t \times N_t},$$

where $N_t$ — the average number of cells in culture at the time $t$ (1st day of the experiment); $N_{(t+\Delta t)}$ — the average number of cells at the time $t+\Delta t$ (on 3rd, 5th, 7th and 10th days); $\Delta t$ — exposure period (day).

Obtained results were statistically processed according to the algorithms of parametric and rank analyzes [26]. Comparison of variances of three independent samples (replicates) for each concentration of the toxicant was carried out using the Fisher test (ANOVA, p=0.05), or with the Kruskal–Wallis test (in the absence of normality in the distribution of the data set). Comparison of the reliability of differences in the mean values of test parameters was performed using the Student's t-test. To compare samples in which the variance distribution deviated from normality, the Mann–Whitney rank test (for samples with an equal volume of data) and Dunn's test (for different-sized samples) were applied [26]. The average values of parameters and standard errors (SE) for the samples are given in all plots.

## RESULTS AND DISCUSSION

In the control culture and at $Cu^{2+}$ concentration of 32–256 μg/L, the proportion of *P. aestuarii* alive cells was virtually unchanged throughout the experiment, remaining at 95–99 %. At the copper content of 320 μg/L, the proportion of alive cells drastically decreased to 23 % as early as on the 3rd day, and on the 5th day, it went down to 10 % from the initial 100 % level, which determines this concentration as a threshold one for survival of the species. At the concentration of 384 μg/L and up to the highest one (1024 μg/L), drastic inhibition (down to 10–40 % of the control culture) of the culture was observed starting from the 1st day of the experiment, and on the days 3–5, the proportion of alive cells considerably decreased almost down to 0 % (Fig. 2A).

Change in the absolute number of *P. aestuarii* alive cells was calculated for different exposure durations (Fig. 2B). Our previous experiments showed the statistical insignificance of possible differences in the average number of cells between three replicates, i. e. the variability of this parameter did not exceed the limits of statistical error in different replicates [21]. It resulted from all the replicates (random samples of cells) belonging to the same initial population with a similar pattern of variability. This fact is important for correct comparison of differences in the number of cells in test dishes at different exposure durations and toxicant concentrations.

In the control culture and at $Cu^{2+}$ ion concentrations of 32–256 μg/L, the pattern of the increase in the cell number is described by the "dose–response" sigmoid curve. Over the period spanning from the 1st to 7th day, the absolute number of cells increased by 3–5 times, reaching its maximum, and decreased by about 10 % in the subsequent period. At the copper concentration of 320 μg/L and higher, the increase in cell number is strongly inhibited after the 1st day, and during the further exposure (days 3–10), the alive cell number of *P. aestuarii* in the test dishes decreased almost to zero (Fig. 2B).

Another type of *P. aestuarii* response to the effect of $Cu^{2+}$ ions was investigated, i. e. the change in the number of cell divisions per day (V) and the specific growth rate (SGR) in the cell number (Fig. 3). During the days 1–7, a positive SGR of the culture was observed within the concentrations range 32 to 256 μg/L; the highest SGR (0.7–0.9 divisions per day) was recorded during the days 3–5. At the threshold concentration (320 μg/L) of the toxicant and higher, a negative SGR in the cell number was revealed as soon as after the 1st day of the experiment (0.3–0.5 divisions per day) (Fig. 3). After 5 days of exposure,

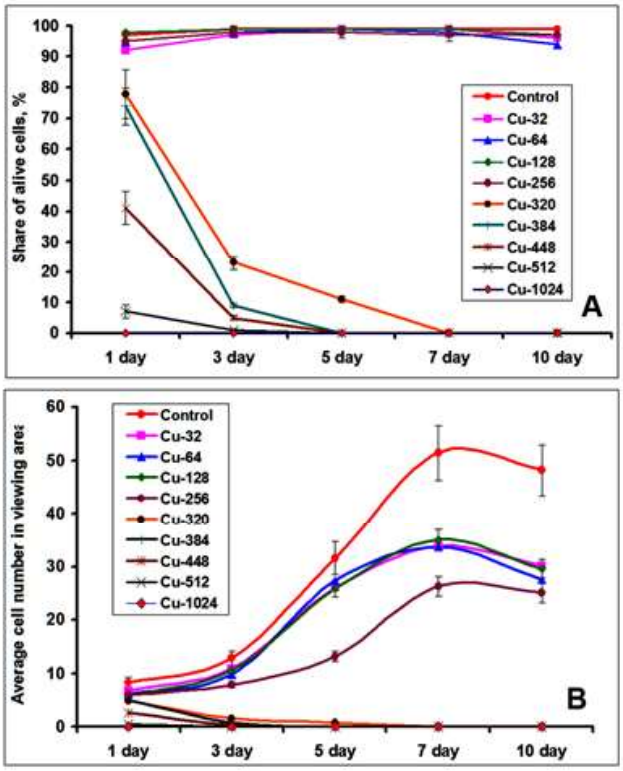


**Fig. 2.** Change in the proportion (%) of *P. aestuarii* alive cells (A) and in the absolute number of cells (mean±SE) in the viewing area (B) at different concentrations of $Cu^{2+}$ over the course of the experiment

**Рис. 2.** Изменение доли живых клеток *P. aestuarii* (%) (A) и среднего числа клеток в поле зрения (B) (среднее±SE) в ходе эксперимента при разных концентрациях $Cu^{2+}$

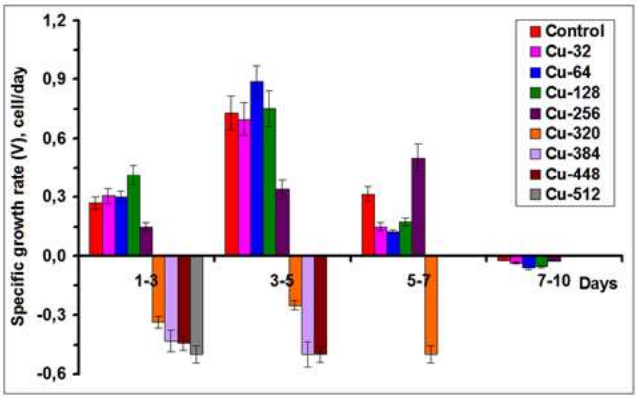


**Fig. 3.** Changes of the specific growth rate in the number of *P. aestuarii* cells (V, cells/day) at different stages of the experiment and at various concentrations of the toxicant

**Рис. 3.** Изменение удельного прироста численности клеток *P. aestuarii* (V, кл./сут) в разные периоды эксперимента и при различных концентрациях токсиканта

the SGR decreased for all tested concentrations of the toxicant, and by the 10th day, the values of this parameter became negative, apparently due to depletion of nutrients in the test medium and aging of the culture.

Thus, in our experiments, a higher tolerance of the benthic diatom *P. aestuarii* to copper impact in comparison with other (mainly planktonic) species of microalgae was revealed [9, 17, 18, 20]. For instance, a pronounced inhibitory effect of $Cu^{2+}$ on the growth rate and cell division, activity of the pigment systems, the size and shape characteristics of *Phaeodactylum tricornutum* Bohlin, 1898 cells manifested as early as on the 4th day of the experiment at copper sulfate concentration of 0.13–0.25 mg/L (or 32–63 μg/L, converted to the concentration of $Cu^{2+}$ ions) [9]. Influence of copper (II) chloride on the survival and reproduction of the green microalga *Scenedesmus quadricaudata* Kiss, 1939 led to a noticeable decrease in the total number and proportion of alive cells at copper (II) concentrations of 10–100 μg/L on the days 10–14 of exposure in the exponential phase of culture growth [17]. It was also noted that even at $Cu^{2+}$ ion concentrations of 1–10 μg/L, the proportion of actively proliferating cells did not exceed 10 %, and the remaining part of the test culture was in dormant state, without affecting the total cell growth rate [17].

Photosynthetic activity of *S. quadricaudata* decreased even at the concentration of copper (II) and other metals (zinc, cobalt, nickel) ions below the MPC (0.0001–0.01 mg/L) [27]. According to the results of testing the effect of $Cu^{2+}$ ions on planktonic microalga *Porphyridium purpureum* (Bory) Drew & Ross, 1965, a noticeable decrease in the concentration of photosynthetic pigments in cells and inhibition of population growth as early as on the exposure days 3–4 at the toxicant concentrations of 50–100 μg/L was found [20]. By the results of benthic diatom *Amphora coffeaeformis* (C. Agardh) Kütz., 1844 biotesting, it was shown that the toxic effect of metals (copper, lead, cadmium) inhibits cell growth, destroys cell membranes and reduces the content of chlorophyll even at the concentration of 0.02 to 10 μg/L [18].

Inhibition of physiological state of diatom cells and growth rate can be caused by the negative effect of copper (II) ions on photosynthetic apparatus and damage to chloroplast membranes, which are involved in a synthesis of amino acids that affect population growth [5, 7, 9, 19], as well as suppression of vegetative reproduction of cells [11, 17].

It is known that the tolerance of various microalgae is conditional on species-specific adaptive abilities; in particular, small-celled species have larger surface of cell, which facilitates sorption of substances from the environment, and, consequently, are more sensitive to the influence of different toxicants [23, 28]. Revealed higher tolerance of large-cell benthic species such as *P. aestuarii* to the toxic effect of copper ions can also result from its heavily silicified frustules, embedded with a raphe and complex areola system at several levels, ensuring the exchange of substances with the aquatic environment but excluding the direct entry of soil particles inside cells (see Fig. 1: 7–10). High concentration of copper ions in soft bottom substrates or in water column can cause in a cell the production of polysaccharides as a protective mucous cover, or glutathione and phytochelatin, which can be one of the universal adaptations of diatoms (protection and detoxification) to the impact of heavy metals [1, 10, 16, 18, 29]. Such adaptations ensure the wide spreading of benthic diatoms on nearshore muddy substrates, in which accumulation levels of copper and other heavy metals are considerably higher than in water column.

The identified morphological adaptations in diatoms belonging to different taxonomic classes may indicate functional elaboration of the structure of valves, areolae and raphe systems over the course of evolution. The revealed regularity could be substantiated by the extended experiments with various representatives of Bacillariophyta.

## CONCLUSION

The obtained results of 10-day toxicological experiments with clone culture of benthic diatom *P. aestuarii* allow for the following conclusions:

At the copper concentration of 32–256 μg/L, the absolute number of cells was increasing by 3–5 times over the days 1–7 and decreasing by 7–10 % during the further exposure. Proportion of alive cells in the culture at the given concentrations of $Cu^{2+}$ ions was virtually unchanged throughout the experiment, remaining at the level of 95–99 %.

At the concentrations of copper ions 320 μg/L and higher, the increase in the absolute number of cells was strongly inhibited starting from the first day; over the course of the continued exposure, the absolute cell number as well as the proportion of alive cells decreased to zero.

A positive specific growth rate in the number of cells was recorded at the concentration range 32 to 256 μg/L, whereas a negative specific growth rate was observed after the first days of the experiment at the toxicant concentration of 320 μg/L. This concentration of copper ions was determined to be species-specific and a threshold one for the survival of *P. aestuarii.*

The threshold is 5–30 times higher than the values of $Cu^{2+}$ ion concentrations critical for survival and growth of some other species of microalgae that are reported in the literature. This fact can be explained by the highly silicified frustules of *P. aestuarii*, equipped with a complex system of areolas and foramines, which may provide reliable protection against the pollution impact, as well as facilitate its successful propagation in the shelf zone.

Thus, diatom *P. aestuarii* can be recommended for use as a promising new test object for toxicological experiments and environmental monitoring of the coastal marine areas exposed to anthropogenic pollution.

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