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Оригинальная статья

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Evaluation the toxic effect of copper ions on the condition indices of benthic diatom *Actinocyclus subtilis* (W.Gregory) Ralfs 1861 in the experiment

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Introduction. Pollution of marine coastal areas lead to the relevance of environmental monitoring including application of biotesting methods based on- the cultures of unicellular algae. Microalgae have different species-specific resistance to pollutants that expands application of different species as bioindicators of marine pollution.

The aim of the study was to determine the threshold concentration of copper ions (Cu^{2+}) for the survival and increase in the cells number of benthic diatom *Actinocyclus subtilis* (W.Gregory) Ralfs 1861 (Bacillariophyta) under the wide range of toxicant concentrations during 10-day toxicological experiments.

Material and methods. The response of strain culture of the benthic diatom *A. subtilis* to various concentrations of copper sulfate (ranged from 16 to 1024 μg/l in terms of Cu^{2+} ions) was studied. In accordance with the previously developed protocol, the following indices were evaluated: alterations in the absolute number and proportion (%) of alive cells in the test-culture, as well as the specific growth rate in the number of *A. subtilis* cells at different concentrations of toxicant. Counting of alive and dead cells was carried out by micrographs taken for 12–15 random viewing fields under Nikon Eclipse inverted light microscope.

Results. It was found that in the control and at concentration of copper ions 16 μg/l, the increase in the absolute number of cells in culture is described by sigmoid response curve. At the control ehe exponential growth phase occurs on days 5–7 and at concentration of 16 μg/l on days 3–5 of the experiment. The threshold concentration of copper ions (32 μg/l) which is critical for the survival of *A. subtilis* was determined, which is 3–7 times lower than threshold level for other benthic diatom species. At concentration of 32 μg/l, the phases of acceleration and exponential growth on the abundance curve are absent. The proportion of living cells in the culture decreases to 80% of the control level on day 3 and to 39% by day 10. At Cu^{2+} concentrations of 64 μg/l and above, sharp inhibition and death of culture is observed as early as 1–3 days. A positive specific growth rate of *A. subtilis* culture was revealed in the period of 1–5 days at copper concentration of 16 and 32 μg/l, and at concentration of 64 μg/l and higher the culture dies off. Negative values of the specific growth rate for all concentrations of the toxicant within the period of 5–10 days were obtained.

Limitations. By the results of 10-day experiments the effect of 8 concentrations of copper sulfate on the culture of marine benthic diatom *A. subtilis* was studied. Three replicates in each concentration and exposure time were measured (1350 measurements in total), which is sufficient sampling for statistically reliable determination of the threshold values of copper ion toxicity for given test object.

Conclusion. Considering the results obtained, the benthic diatom *A. subtilis* is highly sensitive to copper ions impact and can be recommended as new test-object for toxicology, as well as for application in monitoring of marine water areas subject to technogenic pollution.

Keywords: *biotesting; microalgae; clonal strain; copper; threshold concentration; cell number; Black Sea*

Compliance with ethical standards. The study does not require the decision of biomedical ethics committee or other documents, since all experiments were carried out on common unicellular non-toxic algae, which does not violate any prohibitions associated with damage to the ecological environment, the living space of bio-communities, and also does not lead to irreversible changes in the biological (genetic) nature and human health.

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Оценка токсического воздействия ионов меди на показатели состояния бентосной диатомовой водоросли *Actinocyclus subtilis* (W.Gregory) Ralfs 1861 в эксперименте

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Введение. Загрязнение морских прибрежных акваторий обусловливает актуальность экомониторинга на основе биотестирования с использованием микроводорослей с различной видоспецифической устойчивостью к действию поллютантов, что расширяет их применение в качестве биоиндикаторов.
Цель работы — определение пороговой концентрации ионов меди (Cu^{2+}) для выживания и прироста численности клеток бентосной диатомовой водоросли *Actinocyclus subtilis* Ralfs 1861 под воздействием широкого диапазона концентрации токсиканта в ходе 10-суточных экспериментов.
Материал и методы. Изучена реакция клоновой культуры *A. subtilis* на воздействие возрастающих концентраций сульфата меди (от 16 до 1024 мкг/л в пересчёте на ионы Cu^{2+}). В соответствии с разработанным протоколом, оценены следующие показатели: абсолютная численность и доля (%) живых клеток в тест-культуре, а также удельный прирост численности клеток при разных концентрациях токсиканта. Подсчет живых и мертвых клеток проведен по микрофотографиям для 12—15 случайных полей зрения под световым микроскопом Nikon Eclipse.
Результаты. В контроле и при концентрации ионов меди 16 мкг/л прирост численности клеток в культуре описывается сигмоидной кривой отклика. В контроле фаза экспоненциального роста приходится на 5—7-е сутки, а при концентрации 16 мкг/л — на 3—5-е сутки. Определена пороговая концентрация ионов меди (32 мкг/л) для выживания *A. subtilis*, что в 3—7 раз ниже, чем для других видов бентосных диатомовых. При концентрации 32 мкг/л на кривой численности отсутствуют фазы ускорения и экспоненциального роста, а доля живых клеток снижается до 80% от контроля уже на 3-и сутки и до 39% — к 10-м суткам. При концентрациях Cu^{2+} 64 мкг/л и выше наблюдается угнетение и гибель культуры уже в 1—3-и сутки. В период 1—5-х суток отмечен положительный удельный прирост культуры *A. subtilis* при концентрации 16 и 32 мкг/л, при концентрации 64 мкг/л и выше наблюдается отмирание культуры. Для периода 5—10-х суток получены отрицательные значения удельного прироста культуры при всех концентрациях токсиканта.
Ограничения исследования. По результатам 10-суточных экспериментов с культурой морской бентосной диатомовой *A. subtilis* изучено влияние 8 концентраций сульфата меди. Для каждой концентрации учитывалось по 3 повторности, всего выполнено более 1350 измерений, что представляет достаточную выборку для статистически надежного определения видоспецифичных пороговых значений токсичности ионов меди.
Заключение. Полученные результаты позволяют рекомендовать *A. subtilis* в качестве нового высокочувствительного объекта для токсикологических экспериментов, а также при экологическом мониторинге акваторий, подверженных техногенному загрязнению.

Ключевые слова: *биотестирование; микроводоросли; клоновая культура; сульфат меди; пороговая концентрация; численность клеток; Чёрное море*

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Соблюдение этических стандартов. Исследование не требует представления заключения комитета по биомедицинской этике или иных документов, так как все эксперименты проводились на массовых одноклеточных нетоксичных водорослях, что не нарушает запреты, связанные с ущербом для экологической среды, жизненного пространства биологических сообществ, а также не приводит к необратимым изменениям в биологической (генетической) природе и здоровье человека.

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Introduction

The relevance of monitoring of the marine environment, including the use of biotesting methods based on cultures of unicellular algae is increased due to enhancement the anthropogenic impact on the coastal zones of the sea. Microalgae are characterized by different species-specific resistance to many pollutants and can be applied as bioindicators of the marine environment pollution (water mass and bottom sediments) [1, 2].

One of the groups of the most dangerous pollutants are heavy metals and, in particular, copper compounds, which accumulate in bottom sediments, especially in technogenically polluted water areas [3–7]. The content of copper ions in marine waters nearby ports and industrial zones can reach 50–100 µg/l, mainly due to input with sewage and emission from biocidal paints covering hydrotechnical constructions and ship hulls [9, 10]. In silty-sandy sediments of the Crimean Black Sea coasts, the copper content can be 8–11 µg/g [11], and in closed and semi-closed technogenically polluted bays may reach up to 22–37 µg/g [12, 13]. Such high levels of copper ions accumulation pose potential threats to hydrobionts involved in the trophic chains of the coastal marine ecosystem, in which benthic diatoms are a key primary level.

Microamounts of copper may incorporate into the active centers of many enzymes and play an important role in the microalgae cells physiology [6, 14–19], however at higher concentrations copper ions are acutely toxic for most organisms [14, 16, 20, 21]. The relevance of copper compounds in the metabolism of hydrobionts and in biogeochemical cycles in the marine environment determined the choice of copper sulfate as a tested model toxicant. Planktonic microalgae most often are used in the toxicological experiments assessing the impact of copper compounds [2, 9, 17, 22, 23], while benthic microalgae are more objectively capable of responding to environmental pollution due to their low mobility and closed association with the substrate. The level of copper content in bottom sediments determines the spatial distribution of benthic diatom, their taxocene structure, and the cell health and development [10, 12]. These peculiarities stipulate the relevance of expanding toxicological research on benthic Bacillariophyta to search for suitable species for use as bioindicators in assessing the copper impact of marine environment taking into account the species-specific thresholds resistance to the Cu^{2+} ions [2, 8, 16, 23–26].

The tested microalgae species should be eurybiont, widely represented in the coastal biotopes, easily cultivated on standard medium, ensuring a high rate of vegetative reproduction in culture. The choice of *Actinocyclus subtilis* (W.Gregory) Ralfs 1861 as a test object was made taking into account the above criteria. Besides, this species is large-celled, which makes it easier counting the cell number and recognition the vital condition and morphological features of diatom cells during photoregistering.

Aim of the study – to evaluate the growth dynamics of benthic diatom *A. subtilis* clonal strain under the influence of a wide range of toxicant concentrations (Cu^{2+}), to determine the threshold concentration of copper ions for cell survival, to assess the suitability of this species as a promising test object for the tasks of marine ecotoxicology.

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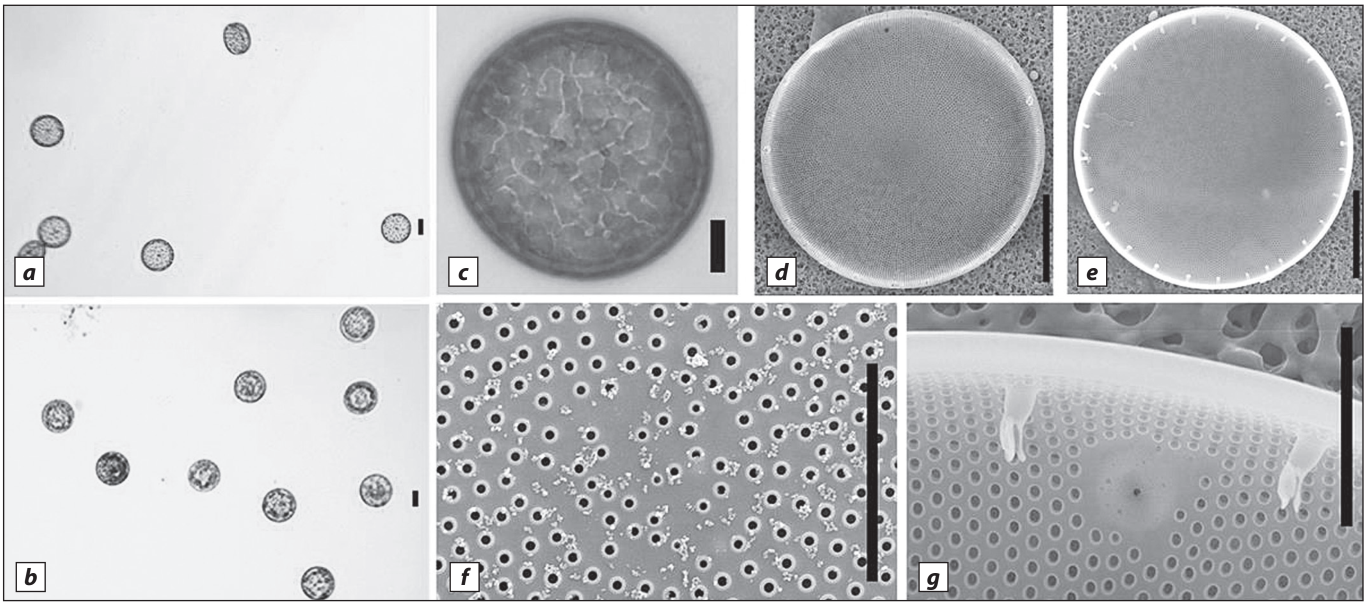

Fig. 1. *A. subtilis*, used in experiment: *a* – alive cells (LM ×100); *b* – dead cells (LM×100); *c* – alive cell with chloroplasts (LM ×630); *d* – valve external view (SEM ×1300); *e* – valve inside (SEM ×1300); *f* – areolae, external view (СЭМ×10000); *g* – labiate process and pseudonodulus, internal view (СЭМ×7000). Scale bar: *a–д* – 20 μm; *е*, *ж* – 5 μm.

Рис. 1. *A. subtilis*, использованный в эксперименте: *a* – культура живых клеток (СМ×100); *b* – погибшие клетки (СМ×100); *c* – живая клетка с хлоропластами (СМ ×630); *d* – створка, вид снаружи (СЭМ×1300); *e* – створка, вид изнутри (СЭМ×1300); *f* – локулярные ареолы центра, створка снаружи (СЭМ×10000); *g* – лабиатные выросты и псевдонодулюс, створка изнутри (СЭМ×7000). Размерная шкала: *a–д* – 20 μм; *е*, *ж* – 5 μм.

Material and methods

The clonal strain of benthic diatom *Actinocyclus subtilis* (W. Gregory) Ralfs 1861 was used for the experiments. The species was sampled from the phytoperiphyton of rocky substrate at a depth of 0.5 m in Sevastopol Bay (N 44°28′25″, E 33°37′58″, Crimea, the Black Sea). The clone was obtained by isolating single cell under a binocular lens (magnification ×40) and growing at a temperature of 15±2 °C and natural light on Goldberg medium optimized for marine benthic diatoms [27, 28]. During cultivation, the clonal batch culture was being reseeded every 3 weeks.

Cells of this species are solitary, round, plastids numerous, discoid, and evenly fills the entire volume of the cell (Fig. 1: 1-3). Species is free-living, and often abundant in the marine benthos and phytoplankton. The cell diameter is 60–70 μm, the areolae are 18–20 in 10 μm, one operculate pseudonodulus at the valve edge is present (Fig. 1).

In accordance with the protocol [29], a certain amount of medium, a stock solution of the toxicant $CuSO_4{\cdot}5H_2O$ and 1 ml of clonal culture inoculum containing an average of 6840±350 cells of *A. subtilis* was added to each Petri dish, bringing the total volume of liquid to 30 ml . The duration of each experiment was 10 days. To prevent evaporation of the solution during exposure, the Petri dishes were sealed with Parafilm®. The resistance of *A. subtilis* to the following concentrations of Cu^{2+} ions was assessed: 16, 32, 64, 128, 256, 320, 512 and 1024 μg/l. Each toxicant concentration was tested in three replicates. Quantitative assessment of the cell culture condition was performed after 1, 3, 5, 7 and 10 days.

Photofication of alive and dead cells during the experiment was carried out with a Carl Zeiss Axiostar Plus (Germany) light microscope (LM) equipped with an Achroplan ×10 objective and Canon PowerShot A640 camera. Taxonomical identification was performed under LM Carl Zeiss PrimoStar Plus (Germany) with a Plan-Achroplan ×100 objective lens and integrated camera as well as using a scanning electron microscope (SEM) Hitachi SU3500 (Japan). The percentage (%) of alive and dead cells in each stage of experiment was determined by using an averaged data obtained from photographs of 12–15 random viewing fields. The intravital condition of cells was assessed according to the integrity of the frustules, the invariability of the cell structure, homogeneity, color and location of chloroplasts in the cell volume, and the completeness of cell divergence after vegetative division. Diatom cells with drastic darkening of their plastids, opened valves, and/or lysis of cell contents were considered to be dead (Fig. 1, 2). Previously [28], the cell distribution homogeneity along the bottom of experimental vessels was statistically confirmed ($p<0.05$) for several benthic diatom species and based on the analysis of a limited number (64–72) of viewing

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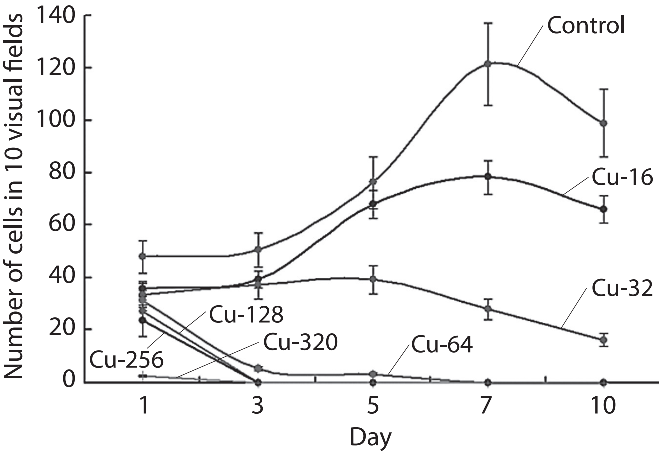

Fig. 2. Change in the total number (mean value ± *SE*) of alive cells of *A. subtilis* in 10 random viewing fields at different concentrations of toxicant.

Рис. 2. Изменение в ходе эксперимента суммарной численности (среднее значение ± *SE*) живых клеток *A. subtilis* в 10 случайных полях просмотра при разных концентрациях токсиканта.

Change in the share of alive cells (mean ± *SE*, %) in strain *A. subtilis* at different concentrations of toxicant

Изменение доли живых клеток (среднее ± *SE*, %) в культуре *A. subtilis* при разных концентрациях токсиканта

Concentration of the toxicant Cu^{2+}, mcg/l	Exposure periods, day				
	1	3	5	7	10
Control	95 ± 2	95 ± 1	93 ± 1	95 ± 2	94 ± 1
16	93 ± 1	91± 3	91 ± 2	93 ± 1	83 ± 2
32	90 ± 1	80 ± 2	77 ± 4	53 ± 8	39 ± 3
64	81 ± 2	13 ± 2	8 ± 1	0	0
128	79 ± 2	0	0	0	0
256	71 ± 12	0	0	0	0
320	8 ± 1	0	0	0	0
512	0	0	0	0	0

fields. These results make it possible to consider all replicates (random cell samples) as a single set, which is important for statistically corrected comparison of cell numbers at different toxicant concentrations.

Comparative assessment of the diatom culture state at various concentrations of copper ions was carried out based on change in the absolute number of alive cells at different stages of the experiment [8, 24]. Cell number growth rate (v, cells/day) was calculated by the formula:

$$v = \frac{\ln N_{(t+\Delta t)} - \ln N_t}{\Delta t \bullet \ln 2},$$

where N_t is the average number of cells in the culture at time t (the first day of the experiment); $N_{(t+\Delta t)}$ – average number of cells in culture at time $t+\Delta t$ (3, 5, 7 and 10 days); Δt – exposure period (days).

Statistical processing of the experimental results was carried out based on standard algorithms for parametric and rank analyzes included in the MS Excel package. The reliability of differences in the condition indices of diatom strain at various copper concentrations and exposure periods, was evaluated for the significance level p = 0.05. The quantitative indices of the culture development are presented as an average values with a standard error of the mean (SE).

Results

According to the results of calculation in the absolute number of *A. subtilis* alive cells, it was found that both in the control and at minimum concentrations of Cu^{2+} ions (16 µg/l), the model of cell culture growth corresponds to the dose-response sigmoidal curve (Fig. 2). In the period of 1–7 days, the absolute number of cells increases 2–2.5 times from the initial values and reached a maximum. In the subsequent period (7–10 days), the cell number decreases from the maximum by 12–20% due to negative impact of the toxicant, accumulation of metabolites and aging of the culture. On the 7th day of experiment, the average cell number at a concentration of 16 µg/l was significantly lower compared to the control due to less intensive growth during the exponential phase.

At copper ions concentration of 32 µg/l, no increase in the number of *A. subtilis* strain was recorded in the period from the 1st to the 5th day. In the subsequent days of experiment the number of alive cells decreases and is no more than 20% of the control level on the 10th day. At concentrations of 64 µg/l and higher, the number of alive cells go down to almost zero on the 3rd day (see Fig. 2). These results evidence that copper ion concentrations within the range of 32–64 µg/l can be considered as critical for the survival of the tested diatom.

Results of calculation the share (%) of alive cells *A. subtilis* in culture at different stages of the experiment have shown that in the control and at minimum concentrations of copper ions (16 µg/l), the proportion of alive cells almost does not change within 1st to 7th day, remaining at the level of 93–95%, and gradually decreases to 83% only by the 10th day (Table).

At copper ions concentration of 32 µg/l and higher, the differences between control and test dishes in the average share (%) of alive cells become statistically highly significant (p<0.01–0.001) after 3 days of exposure.

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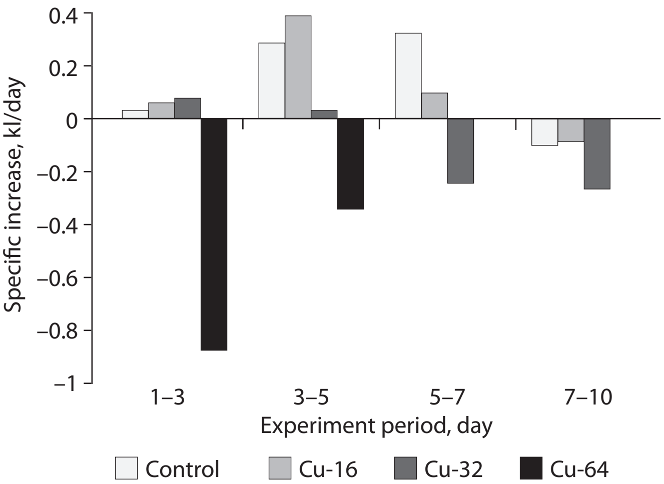

Fig. 3. Change of the specific growth rate in the number of *A. subtilis.* cells (*v*, cells/day) at various concentrations of copper ions in different stages of the experiment.

Рис. 3. Изменение удельного прироста численности клеток *A. subtilis* (*v*, кл/сут) при различных концентрациях ионов меди в разные периоды эксперимента.

At a concentration of 64 μg/l, the cell mortality in the culture is increased, and by days 7–10 the share of alive cells diminished to zero (see table). These results also confirm that toxicant concentration of 64 μg/l can be considered as threshold for survival and development of this diatom species.

The diurnal changes in the number of cell divisions was studied as another response of *A. subtilis* test culture to the copper ions impact (Fig. 3).

Within the period of 1st to 3rd day, in control and at concentrations of 16–32 μg/l, the culture characterized by a slight increasing in the cell number, which may correspond to the lag phase and the growth acceleration phase. At a threshold concentration of 64 μg/l, the test culture intensively dying off since the first day, and the growth rate became negative. At the 3rd to 7th days of exposure, an intensive specific growth, corresponding to the exponential phase of culture development, was observed only for the control (0.30–0.33 divisions/day) and for copper concentration of 16 μg/l (0.11– 0.39 divisions/day). At the concentration 32 μg/l, the growth rate decreased to 0.04 divisions/day on the 5th day. In the period from 5th to 7th day the specific growth rate became negative (the culture is dying out) (see Fig. 3). Within the period 7th – 10th day, the negative values of growth rate in the number of *A. subtilis* cells registered for all tested concentrations of toxicant, including the control. It could be caused not only due to the direct influence of the toxicant, but also by depletion of nutrients in experimental vessels and accumulation of metabolites that inhibit culture growth.

Discussion

Based on the experimental results, it was revealed that the culture of *A. subtilis* is characterized by lower resistance to the copper ions impact in comparison with other species of benthic diatoms (Bacillariophyta). So, in the culture *Pleurosigma aestuarii* W. Smith 1853, the proportion of alive cells did not change within copper ion concentrations range from 16 to 256 μg/l during the 10 days of experiment, but at 320 μg/l the share of alive cells sharply decreased from the initial level to 23–10% by 3th and 5th days. It defines this concentration as the threshold for the survival of this species [26]. For *Thalassiosira excentrica* Cleve 1903, a Cu^{2+} ion concentration of 128 μg/l is critical for the survival and reproduction of the strain [30]. For microalgae species from another taxonomical groups, in particular for *Porphyridium purpureum* (Bory) K.M. Drew et R. Ross 1965, a pronounced inhibition of cell growth and decrease in the content of photosynthetic pigments compared to the control was observed at copper ion concentrations of 100 μg/l [8]. Decrease in the total number and proportion of alive cells in test culture of *Scenedesmus quadricaudata* (Turpin) Brebisson 1835 was registered at copper concentration of 10–100 μg/l, while the share of actively reproducing cells did not exceed 10%, and the rest of the culture remained in an inactive stage [24].

The suppression of cell population growth and physiological condition of diatom *A. subtilis* at relatively low concentrations of copper ions may also be associated with the so-called “paradoxical effect” [18], when microalgae cells may accumulate more considerable amounts of the toxicant at low concentrations in the environment than at high concentrations. Inhibition the processes of cell division at low concentrations of toxicant can serve as mechanism to maintain the population integrity and its ability to survive for a long time under the impact pollution. This effect apparently exhibits species-specific features due to the metabolic activity and effectiveness of the protective mechanisms of microalgae cells under the influence of copper on the photosynthetic apparatus and synthesis of amino acids that determine population growth [2, 17, 19, 24].

Conclusion

The results of 10-day toxicological experiments with the clonal culture of benthic diatom *A. subtilis* under the wide concentration range of copper ions (16 to 1024 μg/l) showed that at concentration of 16 μg/l the share of alive cells significantly decreased on the 10th day of exposure, and at 32 μg/l – on the 3rd day compared to the control. The abundance of cells in the culture reaches a

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maximum level on the 7th day and diminishes in the subsequent period due to influence both the toxicant and accumulation of metabolites in the cultural medium. At toxicant concentrations of 64 μg/l and higher, the number of alive cells in the strain descend to zero already on the 3rd day of the experiment, along with a sharp decreasing in the specific growth rate.

For the first time, the threshold concentration of copper ions (32 μg/l), critical for survival and growth of *A. subtilis* cells, was determined, which is 3–7 times lower than the known Cu^{2+} threshold values for other species of microalgae.

Extending of experiments to determine threshold concentrations of copper and other heavy metals ensures the selection of diatom species with different sensitivity to toxicants for the purpose of their further use as new test objects for toxicological studies. The obtained results allowed to recommend *Actinocyclus subtilis* as suitable indicator for assessing the technogenic pollution impact on the state of microphytobenthos assemblages during ecological monitoring of coastal marine areas.

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