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ESTIMATION OF CELL DISTRIBUTION HETEROGENEITY AT TOXICOLOGICAL EXPERIMENTS WITH CLONAL CULTURES OF BENTHIC DIATOMS

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An increase in anthropogenic pressure on coastal water areas requires regular monitoring of marine ecosystems. The appropriate bioindicators for indirect assessment of the quality of the near-shore environment are benthic diatom algae, which are a key element of coastal communities and are highly sensitive to environmental impact. Changes in the development of diatoms under the influence of various toxicants may be used as relevant tool for monitoring of marine environment quality. However, scientific and methodological approaches to application of benthic diatom algae as test objects remain unstudied. One of the important methodological problems is the assessment of the significance of the samples in experimental vessels when counting cells abundance at different stages of toxicological test. The study is focused on assessment of the statistical significance of the equality of the initial mean number of cells of clonal culture inoculum placed into each of the replicates, as well as the statistical uniformity of cell distribution over the entire bottom area of Petri dishes. We used clonal cultures of three benthic diatom species belonging to different classes of Bacillariophyta: *Thalassiosira excentrica* Cleve, 1903 (Coscinodiscophyceae), *Ardissonea crystallina* (C. Agardh) Grunow, 1880 (Fragilariophyceae), and *Pleurosigma aestuarii* (Bréb. in Kütz.) W. Smith, 1853 (Bacillariophyceae). They significantly differ in valve morphology and life history (floating in water mass, attached to substrate, and motile). The results of statistical comparison of cell number variability in the experiment for all studied species confirmed the absence of significant differences between the mean values of the tested parameter at a standard significance level (0.05). It was shown that despite specific differences in cell growth rate during the experiment, the variability in cell number in the microscope viewing fields varies irregularly. The highest value of the variability coefficient was observed on the 5th day for the small-sized species *T. excentrica* ($C_v = 42...55\%$), and the lowest variability – for the large-cell species *A. crystallina* ($C_v = 27...31\%$). The absence of significant differences in cell number between three replicates (for each species) was established both during the initial placing of inoculum into the dishes and on the following days of the experiment. The conclusion is applicable for each of diatom species studied, which allows to consider all replicates as subsamples of the replicate sample and to average the results obtained at different stages of the toxicological experiment. The uniformity of cell distribution throughout experimental dishes bottom, which does not depend on species and absolute cell number, was statistically proven. The results obtained allow to statistically reliably estimate the changes in cell number at different stages of toxicological experiment according to replicate sampling, based on cell counting in a limited number of viewing fields.

Keywords: toxicological experiment, methodology, statistical estimation, Bacillariophyta, *Thalassiosira excentrica*, *Ardissonea crystallina*, *Pleurosigma aestuarii*, Black Sea

Due to the significant anthropogenic pressure on the Black Sea, which is especially evident in coastal waters off the coast of Crimea, it is necessary to monitor changes in the state of planktonic and benthic communities. As one of the most appropriate objects for bioassay and bioindication, planktonic microalgae are widely used, and there are numerous methodological developments for their applying [3 ; 8 ; 12 ; 19].

Extensive literature on the effect of different toxicants on microalgae is mainly devoted to Chlorophyta [10] and Cryptophyta [17 ; 25], while the contribution of these phyla (they include 23 and 2 species, respectively) to Black Sea benthic microalgae cenoses is quite insignificant, in contrast to the contribution of the phylum Bacillariophyta (1094 species and intraspecific taxa) [13], representatives of which bring up 99 % of the abundance and biomass in the World Ocean microphytobenthos [29]. The fact mentioned above indicates an insufficient level of knowledge for obtaining a comprehensive data of the effect of toxicants on microphytobenthos communities.

Many mass benthic diatom species (Bacillariophyta), being important structural components of marine ecosystems, are characterized by development only within the certain microhabitats and high sensitivity to the influence of adverse environmental factors [14]. This allows to apply benthic diatoms as appropriate test objects, and the changes in their physiological parameters (specific growth rate, mortality, and chloroplast state) under the impact of different pollutants can be a convenient tool for indirect assessment of environmental quality [3 ; 10 ; 12 ; 18 ; 24 ; 25 ; 27].

Within the tasks of hydrobiological monitoring of coastal water areas, the use of benthic diatoms as test objects is a poorly developed scientific and methodological problem [9 ; 23]. Its solution allows to obtain new experimental data on the tolerance ranges of various marine diatom species when exposed to model toxicants (copper sulfate, synthetic surfactants, pesticides, etc.) during subacute and chronic experiments [1 ; 10 ; 11 ; 24 ; 25]. In addition, it becomes possible to solve a number of issues of a methodological nature, which affects the reliability of conclusions based on the results of toxicological tests [19].

One of the main methodological problems is to check the significance of sample assessment when counting cell number in experimental vessels (in our case, Petri dishes) at different stages of a multi-day toxicological experiment. The accuracy of results may be affected by the uneven number of cells initially contained in the inoculum aliquot (1 ml) input in Petri dishes at the beginning of each experiment, as well as by possible uneven cell distribution on dishes bottom in the following days of exposure.

Due to small size and high abundance of diatom algae, direct total cell counting in each dish at different stages of experiment is hardly possible. Therefore, cell number is counted under a microscope in a certain number of discrete viewing fields of known area, and then the numerical data obtained are recounted for the entire bottom area of experimental vessel. In case of such indirect counting of the total cell number in dishes, the final results can vary greatly, which can lead to distortion of conclusion about the degree of toxicant impact on cell number changes. These difficulties determined the need for special methodological study, the results of which can be used to optimize the carrying out of toxicological experiments on benthic diatoms and to provide reliable conclusion when interpreting the quantitative data obtained.

The purpose of the work carried out using clonal cultures of three benthic diatom species is to verify the validity of the following methodological hypotheses based on an assessment of the statistical significance of the results obtained:

- 1) the mean number of clonal culture inoculum cells, input into each Petri dish at the beginning of the experiment, should be approximately equal, *i. e.* the initial cell numbers in each of three replicates, that are put in each line, should not statistically differ from each other;

- 2) cell distribution in each dish, controlled during repeated counting in viewing fields over the entire bottom area, is relatively uniform, *i. e.* there is no statistically significant spatial heterogeneity in cell numbers *in vitro*.

The possible aggregation of cell distribution when counting a certain number of viewing fields (their sum area is not more than 4–5 % of Petri dish total bottom area), when recounting on experimental vessel entire bottom area, can lead to a significant overestimation (or underestimation) of the total cell number. Thus, such results may distort the conclusion concerning the degree of impact of different toxicant concentrations as well as the time of exposure on the total changes in cell numbers.

MATERIAL AND METHODS

Research objects. To assess microalgae distribution in experimental vessels, three benthic diatom species were used: *Thalassiosira excentrica* Cleve, 1903 (Coscinodiscophyceae), *Ardissonaea crystallina* (C. Agardh) Grunow, 1880 (Fragilariophyceae), and *Pleurosigma aestuarii* (Bréb. in Kütz.) W. Smith, 1853 (Bacillariophyceae). The choice of species was based on the following reasons: 1) significant differences in valve morphology (diskoid, linear, and sigmoid); 2) different life history (planktonic – floating in water mass, benthic – attached to substrate, and motile – moving on substrate); 3) ability to form colonies; 4) species-specific rate of reproduction, and therefore, the presumably different nature of cell distribution on dishes bottom, as well as growth rate of cell numbers during a long-term experiment; 5) affiliation with three different taxonomic classes of Bacillariophyta (according to taxonomic system [28]). A comparative statistical assessment of cell distribution features, having cardinal differences in life history, allows us to verify the reliability of results when conducting further toxicological experiments using representatives of different classes of Bacillariophyta.

According to the results of molecular genetic studies and experiments on sexual reproduction, the systematic position of *A. crystallina*, previously transferred from the class Fragilariophyceae to Coscinodiscophyceae, is questioned. It is assumed that *Ardissonaea* (and other representatives of Toxariales) can represent a unique evolutionary group isolated from the pennate diatoms [22]. Taking into account the fact that this species is more similar to Fragilariophyceae in terms of its valve outline and ability to form bundle-shaped colonies attached to substrate, we consider *A. crystallina* as belonging to this class for the purposes of our experiment.

Selected diatom algae species were isolated into clone lines by micropipetting and 7-fold single cell washing under MBS-10 binocular at magnification $\times 40$ [2 ; 5 ; 18].

Thalassiosira excentrica strain was isolated from microphytobenthos of soft substrate, sampled in Laspi Bay in September 2017 at a 9-meter depth. The species is marine, bento-planktonic, and able to float in water column or dip to bottom. It is characterized by high abundance in Black Sea sublittoral zone. Cells form chain-shaped colonies of 4–6 individuals connected by a thin transparent filament [13 ; 15]. The valves are flat-cylindrical; disk diameter is of $25 \mu\text{m}^*$; height is of $3 \mu\text{m}$ (Fig. 1A, D).

Ardissonaea crystallina strain was isolated from phytoperiphyton of artificial substrate, sampled in Kazach'ya Bay in April 2018 at a 5-meter depth. The species is marine, benthic, often found in coastal areas. Cells attach to substrate, forming bundle-shaped colonies of 4–30 individuals [4 ; 13]. Valves are narrowly linear; length is of $410 \mu\text{m}$; width is of $18 \mu\text{m}$ (Fig. 1B, E).

*Hereinafter, cell sizes are indicated as they were at the beginning of the experiment.

Pleurosigma aestuarii strain was isolated from microphytobenthos of rocky substrate, sampled from Cape Aya in July 2018 at a 3-meter depth. The species is marine, benthic, often found in the Black Sea sublittoral zone. Cells are single, motile; they quickly move along substrate surface [13 ; 16]. Valves are narrow-lanceolate, sigmoid curved at the ends; length is of 135 μm ; width is of 22 μm (Fig. 1C, F).

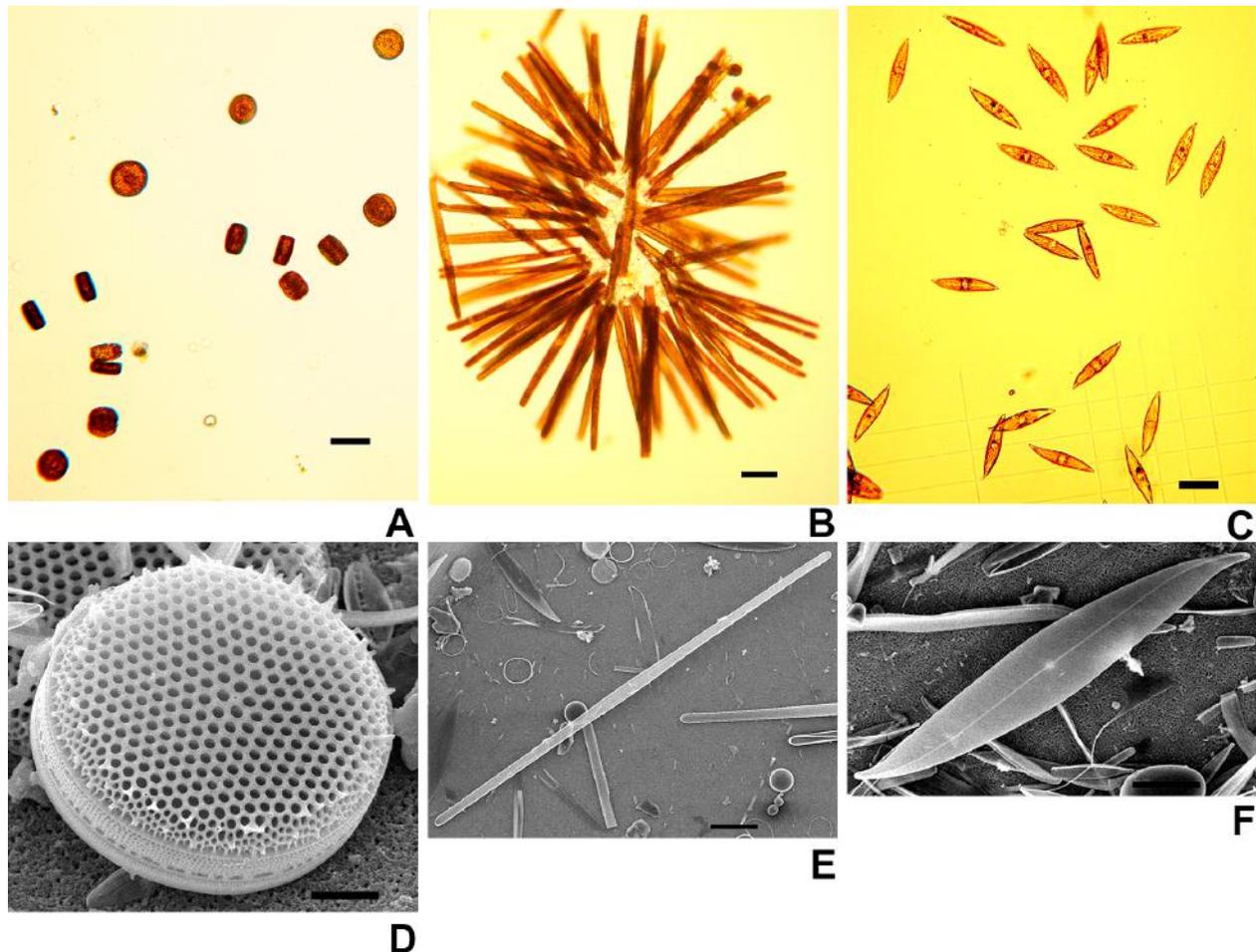


Fig. 1. Benthic diatoms used in experiment: A, D – *Thalassiosira excentrica*; B, E – *Ardissonaea crystallina*; C, F – *Pleurosigma aestuarii*. Light microscope – A, B, C (scale bar = 10 μm); scanning electronic microscope – D (scale bar = 5 μm), E (scale bar = 50 μm), F (scale bar = 20 μm)

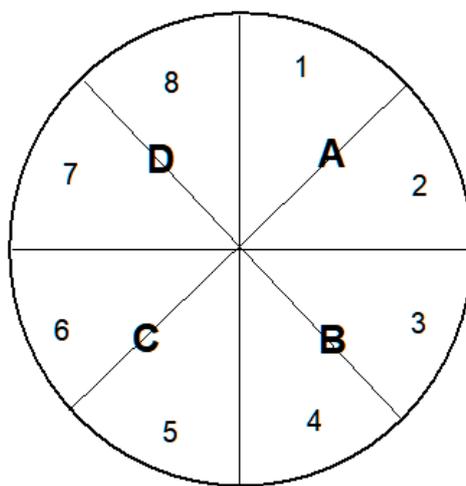
Cultural media maintenance. Clonal cultures were maintained in natural seawater media by Goldberg [7 ; 21] modified for the cultivation of marine benthic diatoms, at a constant temperature of $(22 \pm 2)^\circ\text{C}$ in ambient light on the north window of IBSS laboratory. To prepare the medium, seawater was taken in a 12-mile off-coast zone and filtered through a 0.45- μm filter, then pasteurized three times at a temperature of $+75^\circ\text{C}$ and enriched with nutrients according to the protocol (Table 1).

Experiment design. Possible discrepancies between three replicates in mean cell number in each Petri dish after 1 day and 5 days of exposure were assessed in an experiment with clonal cultures of three diatom species. For each species, the experiment was carried out using modified natural seawater media by Goldberg, without adding a toxicant. Totally, 30 ml of natural medium and 1 ml of clonal culture inoculum were added to each dish (inner diameter of 85 mm; bottom area about 5700 mm^2); then the contents were thoroughly mixed, and the dish was sealed with Parafilm film to prevent medium evaporation.

Table 1. Recipes for modified natural seawater media by Goldberg

No. of solution	Substance	Amount of substance per 100 ml of dH ₂ O	Quantity of solution used per 1 L of seawater, ml
1	KNO ₃	10.1 g	2.0
2	NaH ₂ PO ₄ ×2H ₂ O	1.421 g	0.5
3	MnCl ₂ ×4H ₂ O CoCl ₂ ×6H ₂ O	0.01979 g 0.02379 g	1.0
4	Na ₂ EDTA×2H ₂ O FeCl ₃ ×6H ₂ O	0.244 g 0.144 g	1.0
5	FeNH ₄ -citrate	0.072 g	0.5
6	Na ₂ SiO ₃ ×5H ₂ O	1.5 g	2.0
7	Thiamine (vitamin B ₁)	0.05 mg	0.5
8	Cyanocobalamin (vitamin B ₁₂)	0.5 mg	5.0

To control distribution uniformity of random viewing fields over the entire area, dish bottom was divided by lines into 8 equal parts (Fig. 2). Within the boundaries of each sector, photographs of 8–9 viewing fields were carried out. Viewing fields were selected randomly over the whole bottom area; so, 64–72 fields were taken into account in each dish. Microphotography was carried out under Carl Zeiss Axiostar Plus light microscope with an Achroplan ×10 lens using Canon PowerShot A640 digital camera (IBSS RAS, Sevastopol) and under JEOL JSM-6390LA scanning electron microscope (Komarov Botanical Institute RAS core facility, Saint Petersburg). Taxonomic identification was carried out according to species guides [6 ; 15 ; 16 ; 26].

**Fig. 2.** Estimation of distribution heterogeneity of random viewing fields over the entire Petri dish bottom area in the experiment

Cell counting was performed using photographs of each sector. The area of one viewing field was about 4.0 mm², *i. e.* during the viewing, 4.5–4.9 % of bottom area of each dish was counted. Further, when assessing distribution uniformity of diatom cells over dish bottom at different stages of the experiment, the mean values of cell number in viewing fields in 4 sectors of dish bottom (A, B, C, and D) were compared, and the previously obtained calculation data for viewing fields from 8 adjacent parts were combined in pairs: 1 + 2, 3 + 4, 5 + 6, and 7 + 8. These methodical features were related to the fact that the absolute

variation in cell number, when comparing individual viewing fields even within one of 8 sectors, could be significant, especially on the 5th day of exposure. For example, for *A. crystallina* the range of variation was 16÷41 cells, for *T. excentrica* – 11÷48, for *P. aestuarii* – 36÷91. The coefficient of variation, when counting cell numbers in individual sectors separated by lines, could also reach 70–78 %. When making comparative estimation for 4 sectors (A–D) with combined data from adjacent parts of dishes bottom, the variability indexes of cell number (variance and standard error) were significantly lower due to taking into account the doubled number of measurements for each replicate. To assess discrepancy degree between the replicates (dishes), the statistical significance of pairwise differences in mean cell number was counted both between 4 sectors of the same dish (*i. e.* the degree of cell aggregation within the bottom of one dish was assessed) and between sectors belonging to different dishes.

Since these diatom species are characterized by different growth rates of cell number during the experiment, for the correct assessment of statistical differences by the mean number, the relative cell growth rate (V) for all studied species was calculated using the formula [19 ; 20]:

$$V = (N_{(t+\Delta t)} - N_t) / (\Delta t \cdot N_t),$$

where N_t is the mean cell number in a culture in a Petri dish at time t (the 1st day of the experiment);

$N_{(t+\Delta t)}$ is the mean cell number in a culture at time $t+\Delta t$ (the 5th day of the experiment);

Δt is the period of exposure (four days).

Statistical data processing. Statistical processing of the experimental results was carried out using standard routines of parametric and rank analysis included in the statistical analysis platform “SigmaPlot 12.5” [30].

Normality of cell distribution mode in the sample (cell number in 64–72 viewing fields in each Petri dish) was estimated according to Shapiro – Wilk or Kolmogorov – Smirnov criterion with preliminary data testing and exclusion from calculations of sharply differed values in each sample estimated by quintile method. Such aggregations with an abnormally high cell abundance are not the result of a natural increase in their number during the experiment, but occur sometimes either as a result of the initial placing into the Petri dish of inoculum, which already contains cell aggregation linked by polysaccharides, or as a result of fluctuations of dish contents while photographing.

Comparison of variances for 3 and more independent subsamples was carried out using the Fisher criterion (ANOVA), as well as the Kruskal – Wallis test by ranks (in case of the non-normal distribution mode) for significance level $P = 0.05$. The following comparison of the statistical significance of differences in mean feature values (mean cell number in random viewing fields in 4 sectors of dishes taking into account various exposure stages) was performed using Student’s t -test (in case of normality of the distribution mode in samples and the equality of variances). To compare independent samples, in which the distribution mode differed from the normal one, non-parametric Holm – Šidák test (for samples equal in amount) and Dunn’s test (when different-sized samples were compared) were applied [30].

RESULTS AND DISCUSSION

The results of the analysis showed that the variability of data, when counting cell number in viewing fields, can significantly differ both when comparing different species and in one species but at different exposure stages (Table 2).

Table 2. Variation characteristics for three benthic diatom species reflecting the changes in mean cell number in Petri dishes (3 replicates) on the 1st and 5th days of the experiment

Species	Replicate	The 1 st day			The 5 th day		
		n	$N \pm SE$	$Cv, \%$	n	$N \pm SE$	$Cv, \%$
<i>Ardissonea crystallina</i>	I	65	6.16 ± 0.41	51.3	63	22.70 ± 0.82	31.4
	II	63	6.82 ± 0.41	44.8	62	24.71 ± 0.90	32.2
	III	62	6.79 ± 0.42	46.2	68	24.65 ± 0.76	26.7
<i>Thalassiosira excentrica</i>	I	63	9.19 ± 0.39	34.1	63	20.68 ± 1.42	54.6
	II	64	9.67 ± 0.44	37.3	63	20.91 ± 1.21	46.1
	III	62	11.45 ± 0.41	28.3	62	24.00 ± 1.26	41.6
<i>Pleurosigma aestuarii</i>	I	65	17.37 ± 0.38	29.6	72	63.44 ± 1.37	22.3
	II	67	18.55 ± 0.54	24.0	72	60.78 ± 1.71	23.9
	III	72	19.79 ± 0.52	22.8	72	61.83 ± 1.80	24.7

Note: n – total number of examined viewing fields in each Petri dish minus the sharply distinguished values (statistical outliers); $N \pm SE$ – mean cells number \pm sampling standard error; Cv – coefficient of variation.

Possible statistical differences in distribution pattern of diatom cells on dish bottom at different stages of the experiment could be caused by the fact that on the 1st day cell distribution was mainly determined by thorough mechanical mixing of the inoculum before and after placing it into a dish, which theoretically caused a more uniform cell distribution in viewing fields. On the 5th day of exposure, distribution pattern of diatom cells on dish bottom could be mainly determined by individual motility of cells and by tendency to attach to substrate and to form aggregations or to soar passively in the cultural medium. The factors mentioned above could influence the non-uniformity of values when counting cell numbers in random viewing fields.

The highest coefficient of variation was observed in *A. crystallina* samples on the 1st day of the experiment (45–51 %), as well as in *T. excentrica* samples on the 5th day (42–54 %), although the mean cell number in viewing fields of these species differed by 3.5 times. The high variability of the data could be caused by distribution heterogeneity of these species, when along with single cells in viewing fields there are aggregations, in which cells are bound by polysaccharide secretions (*T. excentrica*) or form bundle-shaped colonies (*A. crystallina*) attached to dish bottom at single point. Thereby, *P. aestuarii* samples in viewing field are characterized by minimal variability (23–29 %) in terms of abundance, regardless of exposure stage, which was explained by cell motility of this species that freely move throughout dish bottom during the experiment and do not concentrate in one point.

The results of analysis for studied diatom species showed that the variances between the samples did not differ statistically, when comparing three replicates: the probability (P) of the accepting the null-hypothesis is much higher than the critical one (0.05) and ranges 0.27–0.49 on the 1st day and 0.16–0.47 on the 5th day. The results of a pairwise comparison of the mean values of cell number in each Petri dish (comparison between replicates) at different stages of the experiment are shown in Table 3.

For *A. crystallina*, all pairwise differences in the mean cell number between replicates on both the 1st and 5th days of exposure were insignificant ($P_{exp} \gg 0.05$).

For *T. excentrica*, on the 1st day, significant differences in the mean cell number in viewing fields were identified between pairs of replicates I – III and II – III ($P_{exp} \leq 0.003$). The differences were unreliable when comparing the pair I – II ($P_{exp} = 0.416$). On the 5th day, there were no significant differences in the mean cell number values between all pairs of replicates.

Table 3. Results of testing the differences between the mean cell numbers in Petri dishes under pairwise comparison of three replicates for diatom species at different stages of experiment

Species	Pair of replicates	Mean value (the 1 st day)		<i>P</i>	Mean value (the 5 th day)		<i>P</i>
<i>Ardissonea crystallina</i>	I – II	6.16	6.82	0.252	22.70	24.71	0.090
	I – III	6.16	6.79	0.283	22.70	24.65	0.092
	II – III	6.82	6.79	0.951	24.71	24.65	0.747
<i>Thalassiosira excentrica</i>	I – II	9.19	9.67	0.416	20.68	20.91	0.906
	I – III	9.19	11.45	0.000	20.68	24.00	0.084
	II – III	9.67	11.45	0.003	20.91	24.00	0.080
<i>Pleurosigma aestuarii</i>	I – II	17.37	18.55	0.163	63.44	60.78	0.077
	I – III	17.37	19.79	0.001	63.44	61.83	0.200
	II – III	18.55	19.79	0.135	60.78	61.83	0.671

Note: *P* – probability of acceptance of the null-hypothesis that there are no differences between the mean values of cell number in samples compared ($P_{\alpha} = 0.05$). Statistically significantly different results are indicated in bold.

For *P. aestuarii*, on the 1st day, there were no reliable mean differences under the pairwise comparison between replicates I – II and II – III. Only when comparing the pair I – III, the differences in mean cell number values were reliable ($P_{exp} \leq 0.001$). On the 5th day, there were no significant differences in mean cell number values when comparing all replicates ($P_{exp} > 0.05$).

The rate of relative increase in cell number was in average higher in *P. aestuarii* (0.59) and *T. excentrica* (0.40), than in *A. crystallina* (0.37), which could affect the variability indexes, although the resulting differences in cell number between replicates of the experiment turned out to be not significant for both species (see Table 3).

Thus, it can be assumed that the variability range of the mean cell number for each species in different replicates in most cases does not exceed the statistical error. This fact gives reason to consider all replicates (random subsamples of cells) as corresponding to one initially taken sample (inoculum of each species cells) with a similar degree of variability.

The results of assessment of cell number distribution uniformity in 4 sectors of Petri dishes bottom showed the following.

The 1st day. For all diatom species studied, there were no statistically significant differences ($P \gg 0.05$) between the mean values of parameter (cell number in 16–18 viewing fields) at pairwise comparison of 12 sectors, *i. e.* three replicates (4 sectors in each dish – A, B, C, and D). Consequently, at the initial stage of experiment, cell distribution over dishes bottom was statistically uniform, and there were no noticeable differences between dishes in the results of counting the total cell number by the mean values from selected viewing fields.

The 5th day. Considering that the distribution of cell number in samples in many sectors differs from the normal one (Kolmogorov – Smirnov test: 0.125–0.210) and has significantly different variances, the testing of significance of possible differences between dish sectors was carried out using rank criteria (Kruskal – Wallis test).

Ardissonea crystallina. The results of 66 pairwise rank comparisons of the mean cell number values in each sector of Petri dish bottom showed the absence of statistically significant differences ($P_{exp} = 0.067$) both between sectors of the bottom of one dish and between dishes (replicates).

Thalassiosira excentrica. The results of 66 pairwise rank comparisons of the mean cell number values in each sector indicate the absence of statistically significant differences ($P_{exp} = 0.071$) both between sectors of the bottom of one dish and between dishes.

Pleurosigma aestuarii. In all 66 pairwise rank comparisons of 12 sectors from three replicates, only in pairs of sectors where the data from 1D sector were considered (1D vs 2A; 1D vs 2B; 1D vs 3B), the differences in the mean cell number values were significant (P_{exp} was of 0.001, 0.003, and 0.008, respectively). For the other pairwise rank comparisons of the mean values, performed by the Dunn's test, differences between sectors were not significant ($P_{exp} > 0.05$). In case of exclusion of sector 1D (the only one with abnormally high cell number in the viewing fields) from the analysis, no statistically significant differences were identified both between sectors of the bottom of one dish and between dishes ($P_{exp} = 0.272$).

The data obtained confirm statistical uniformity of cell distribution pattern of benthic diatoms through bottom of experimental vessels even in case when not more than 5 % of dish bottom area is considered by direct visual counting. The results remain valid regardless of microalgae species used, their morphological structure, and life history, as well as differences in the values of their absolute number in dishes at different stages of experiment.

Conclusion. The results of statistical comparison of degree of variation in cell number in experimental dishes of three marine benthic diatom species belonging to three different classes of Bacillariophyta (*Thalassiosira excentrica*, *Ardissonea crystallina*, and *Pleurosigma aestuarii*) confirmed that overwhelmingly there were no statistically significant differences between the mean values of the studied parameter at standard significance level (0.05). It was shown that despite species-specific differences in cell number growth rate during the experiment, the variability of parameter varied irregularly. After a 5-day exposure, the highest variability coefficient in cell number in viewing fields ($Cv = 42...55$ %) was observed for the benthoplanktonic small-sized species *T. excentrica*, mainly soaring in water mass, and the lowest ($Cv = 27...31$ %) was noted for large-cell species *A. crystallina*, that attaches to dish bottom.

It was found that the counted mean diatom cell number did not differ significantly between three replicates both on the 1st day, after the initial placing of inoculum into the dishes, and at the final stage of experiment. The conclusion is valid for all studied diatom species used as test objects, and this allows us to consider all replicates as subsamples of one sample and to average the results obtained on them at different stages of toxicological experiments.

Cell distribution uniformity within experimental dishes bottom was statistically proven (even when counting not more than 5 % of bottom area). The uniformity of distribution pattern is not species-specific and does not depend on the absolute number of diatom cells in the dishes. The results obtained make it possible to statistically reliably assess the changes in cell number of studied species at different stages of toxicological experiment according to replicate samples, obtained on the base of cell counting in a limited number of viewing fields.

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REFERENCES

1. Aizdaicher N. A., Reunova Yu. A. The effect of detergents on growth of the diatom *Thalassiosira pseudonana* in culture. *Biologiya morya*, 2002, vol. 28, no. 5, pp. 362–365. (in Russ.)
2. Gaisina L. A., Fazlutdinova A. I., Kabirov R. R. *Sovremennye metody vydeleniya i kultivirovaniya vodoroslei* : uchebnoe posobie. Ufa : Izd-vo BGPU, 2008, 152 p. (in Russ.)
3. Gelashvili D. B., Bezel' V. S., Romanova E. B., Bezrukov M. E., Silkin A. A., Nizhegorodtsev A. A. *Printsipy i metody ekologicheskoi toksikologii*. Nizhny Novgorod : Nizhegorodskii gosuniversitet, 2015, 742 p. (in Russ.)
4. Guslyakov N. E., Zakordonets O. A., Gerasimyuk V. P. *Atlas diatomovykh vodoroslei bentosa severo-zapadnoi chasti Chernogo morya i prilegayushchikh vodoemov*. Kiev : Naukova dumka, 1992, 115 p. (in Russ.)
5. Davidovich N. A., Davidovich O. I., Podunay Yu. A. Diatom culture collection of the Karadag scientific station (Crimea). *Morskoy biologicheskij zhurnal*, 2017, vol. 2, no. 1, pp. 18–28. (in Russ.). <https://doi.org/10.21072/mbj.2017.02.1.03>
6. *Diatomovy analiz*. Kn. 3. *Opredelitel' iskopaemykh i sovremennykh diatomovykh vodoroslei*. *Poryadok Pennales* / A. I. Proshkina-Lavrenko (Ed.). Moscow ; Leningrad : Gosgeolitizdat, 1950, 398 p. (in Russ.)
7. Kabanova Yu. G. *Organicheskii fosfor kak istochnik pitaniya fitoplanktona* : avtoref. dis. ... kand. biol. nauk : 03.00.18. Moscow, 1958, 13 p. (in Russ.)
8. Krainyukova A. N. Biotestirovanie i okhrana vod ot zagryazneniya. In: *Metody biotestirovaniya vod*. Chernogolovka : GK OP SSSR, 1988, pp. 4–21. (in Russ.)
9. Markina Zh. V. *Primenenie mikrovodoroslei dlya otsenki kachestva morskoi vody i deistviya detergentov* : avtoref. dis. ... kand. biol. nauk : 03.00.18 ; 03.00.16. Vladivostok, 2008, 21 p. (in Russ.)
10. Markina Zh. V. Influence of detergents and surface-active substances on unicellular algae growth, physiological and biochemical parameters (review). *Izvestiya TINRO*, 2009, vol. 156, pp. 125–134. (in Russ.)
11. Markina Zh. V., Aizdaicher N. A. The influence of detergents on the abundance dynamics and physiological state of the benthic microalgae *Attheya ussurensis* (Bacillariophyta) in laboratory culture. *Biologiya morya*, 2007, vol. 33, no. 6, pp. 432–439. (in Russ.)
12. Markina Zh. V., Aizdaicher N. A. *Phaeodactylum tricornutum* Bohlin bioassay of water quality of Amur Bay (the Sea of Japan). *Sibirskii ekologicheskii zhurnal*, 2011, vol. 18, no. 1, pp. 99–105. (in Russ.)
13. Nevrova E. L. *Bentosnye diatomovye vodorosli (Bacillariophyta) Chernogo morya: raznoobrazie i struktura taksotsenov razlichnykh biotopov*. [dissertation]. Moscow, 2015, 445 p. (in Russ.). <https://dlib.rsl.ru/01005555099>
14. Nevrova E. L., Snigireva A. A., Petrov A. N., Kovaleva G. V. *Guidelines From Quality Control of the Black Sea. Microphytobenthos* / A. V. Gaevskaya (Ed.). Sevastopol ; Simferopol : N. Orianda, 2015, 176 p. (in Russ.). <https://doi.org/10.21072/978-5-9907290-2-5>
15. Proshkina-Lavrenko A. I. *Diatomovye vodorosli planktona Chernogo morya*. Moscow ; Leningrad : Izd-vo AN SSSR, 1955. 222 p. (in Russ.). <https://repository.marine-research.org/handle/299011/6623>
16. Proshkina-Lavrenko A. I. *Diatomovye vodorosli bentosa Chernogo morya*. Moscow ; Leningrad : Izd-vo AN SSSR, 1963, 243 p. (in Russ.)
17. Reunova Yu. A., Aizdaicher N. A. Vliyanie detergenta na sodержanie khlorofilla *a* i dinamiku chislennosti u mikrovodorosli *Chroomonas salina* (Wils.) Butch. (Cryptophyta). *Al'gologiya*, 2004, vol. 14, no. 1, pp. 32–38. (in Russ.)
18. Romanova D. Yu., Petrov A. N., Nevrova E. L. Copper sulphate impact on growth and cell morphology of clonal strains of four benthic diatom species (Bacillariophyta) from the Black Sea. *Morskoy biologicheskij zhurnal*, 2017, vol. 2, no. 3, pp. 53–67. (in Russ.). <https://doi.org/10.21072/mbj.2017.02.3.05>
19. Spirikina N. E. *Issledovanie kul'tury zelenoi mikrovodorosli Monoraphidium arcuatum kak novogo*

- test-ob"ekta dlya otsenki kachestva vodnoi sredy*. [dissertation]. Moscow, 2016, 172 p. (in Russ.)
20. Schlegel H. G. *Allgemeine Mikrobiologie*. Moscow : Mir, 1987, 567 p. (in Russ.)
 21. Andersen R. A., Berges J. A., Harrison P. J., Watanabe M. M. Recipes for freshwater and seawater media. In: *Algal culturing techniques* / R. A. Andersen (Ed.). San Diego : Elsevier Academic Press, 2005, pp. 429–538.
 22. Davidovich N. A., Davidovich O. I., Podunay Yu. A., Gastineau R., Kaczmarek I., Poulíčková A., Witkowski A. *Ardissonea crystallina* has a type of sexual reproduction that is unusual for centric diatoms. *Scientific Reports*, 2017, vol. 7, article 14670 (16 p.). <http://www.nature.com/articles/s41598-017-15301-z>
 23. Kim J. W., Price N. M. The influence of light on copper-limited growth of an oceanic diatom, *Thalassiosira oceanica* (Coscinodiscophyceae). *Journal of Phycology*, 2017, vol. 53, iss. 5, pp. 938–950. <https://doi.org/10.1111/jpy.12563>
 24. Markina Z. V., Aizdaicher N. A. Content of photosynthetic pigments, growth, and cell size of microalga *Phaeodactylum tricornerutum* in the copper-polluted environment. *Russian Journal of Plant Physiology*, 2006, vol. 53, no. 3, pp. 305–309. <https://doi.org/10.1134/S1021443706030034>
 25. Markina Zh. V., Aizdaicher N. A. Influence of the ariel detergent on the growth and physiological state of the unicellular algae *Dunaliella salina* (Chlorophyta) and *Plagioselmis protonga* (Cryptophyta). *Hydrobiological Journal*, 2010, vol. 46, iss. 2, pp. 49–56. <https://doi.org/10.1615/HydrobJ.v46.i2.60>
 26. Reid G. *A revision of the family Pleurosigmales / A. Witkowski (Ed.). Ruggell : A. R. G. Gantner Verlag K. G.*, 2012, 163 p. (Diatom Monographs ; vol. 14).
 27. Rijstenbil J. W., Gerringa L. J. A. Interactions of algal ligands, metal complexation and availability, and cell responses of the diatom *Ditylum brightwellii* with a gradual increase in copper. *Aquatic Toxicology*, 2002, vol. 56, iss. 2, pp. 115–131.
 28. Round F. E., Crawford R. M., Mann D. G. *The Diatoms. Biology and Morphology of the Genera*. Cambridge : Cambridge University press, 1990, 747 p.
 29. Seckbach J., Kociolek J. P. (Eds). *The Diatom World*. Berlin ; Heidelberg ; New York : Springer Verlag, 2011, 533 p. (Book series : Cellular Origin, Life in Extreme Habitats and Astrobiology ; vol. 19). <https://doi.org/10.1007/978-94-007-1327-7>
 30. *SigmaPlot 12.5 User's Guide*. USA : Systat Software Inc., 2013, 455 p.

ОЦЕНКА НЕОДНОРОДНОСТИ РАСПРЕДЕЛЕНИЯ КЛЕТОК ПРИ ТОКСИКОЛОГИЧЕСКИХ ЭКСПЕРИМЕНТАХ С КЛОНОВЫМИ КУЛЬТУРАМИ БЕНТОСНЫХ ДИАТОМОВЫХ ВОДОРΟΣЛЕЙ

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Увеличение антропогенной нагрузки на прибрежные акватории требует постоянного отслеживания состояния их экосистем. Удобными биоиндикаторами для опосредованной оценки качества морской среды служат донные диатомовые водоросли, являющиеся ключевым звеном морских прибрежных сообществ и обладающие высокой чувствительностью к влиянию экологических факторов. Изменение показателей развития микроводорослей под воздействием различных токсиантов может быть подходящим инструментом при мониторинге качества морской среды, однако научно-методические подходы использования бентосных диатомовых как тест-объектов остаются недостаточно разработанными. Одной из важных проблем является оценка достоверности выборок при подсчёте обилия клеток в сосудах на разных этапах токсикологического эксперимента. Цель работы — провести статистическую оценку достоверности равенства среднего

исходного числа клеток инокулята клоновой культуры, вносимого в каждую из повторностей, а также достоверности равномерного распределения клеток по всей площади дна чашек Петри. Используются клоновые культуры трёх видов бентосных диатомовых водорослей — *Thalassiosira excentrica* Cleve, 1903 (Coscinodiscophyceae), *Ardissonea crystallina* (C. Agardh) Grunow, 1880 (Fragilariophyceae) и *Pleurosigma aestuarii* (Bréb. in Kütz.) W. Smith, 1853 (Bacillariophyceae). Эти виды относятся к разным классам Bacillariophyta и значительно различаются по морфологии панциря и образу жизни (парящие в водной массе, прикрепленные, подвижные). Статистическое сравнение вариативности числа клеток в эксперименте подтвердило отсутствие достоверных различий между средними значениями исследуемого параметра у всех видов при стандартном уровне значимости (0,05). Показано, что, несмотря на видоспецифические отличия в темпе приращения числа клеток, вариативность числа клеток в полях зрения микроскопа в ходе эксперимента меняется незакономерно. Наибольшая вариативность отмечена на 5-е сутки у мелкоразмерного вида *T. excentrica* ($Cv = 42...55\%$), а наименьшая — у крупноклеточного вида *A. crystallina* ($Cv = 27...31\%$). Установлено отсутствие достоверных различий в численности клеток между тремя повторностями (для каждого из видов) как при исходном внесении инокулята в чашки, так и в последующие дни опыта. Вывод справедлив для каждого из изученных видов диатомовых, что позволяет рассматривать все повторности как выборки одной совокупности и осреднять результаты, полученные на разных стадиях токсикологического эксперимента. Статистически доказана равномерность распределения клеток по дну экспериментальных чашек, которая не зависит от видовой принадлежности клеток и их абсолютной численности. Результаты позволяют надёжно оценивать изменения численности клеток тестируемых видов диатомовых водорослей на разных этапах эксперимента по выборкам, полученным на основе подсчёта клеток в ограниченном числе полей зрения.

Ключевые слова: токсикологический эксперимент, методика, статистическая оценка, Bacillariophyta, *Thalassiosira excentrica*, *Ardissonea crystallina*, *Pleurosigma aestuarii*, Чёрное море