Chapter 1

Intrapopulation Changes of Algae under Toxic Exposure

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1.1. Introduction

Algae are a highly diverse group of photosynthetic organisms that play a vital role in aquatic ecosystems, e.g. unicellular algae floating in water make up the phytoplankton and macroscopic algae forming kelp beds on rocky shores. Algae are responsible for sustaining aquatic food webs and carry a large fraction of the aquatic biodiversity. Monitoring of the many species of algae is an essential part of water quality surveys. For the same reasons algae are used to evaluate the risk of new chemicals via laboratory research and these organisms are used for bioassays to measure the toxicity of waste water streams. Laboratory populations of algae are widely used as sensitive test object for the evaluation of the phytotoxicity of chemicals.

Water pollution are altering ecosystem, community, population, organism, cell, subcell, molecular – level processes. It are causing structural-functional alteration in populations and communities and decreasing a biodiversity. Heavy metals, one of the most toxic pollutants, often occur in industrial effluents at very high concentrations, thus posing a serious threat to biota and the environment. While many heavy metals require micronutrients for biological systems, they become toxic to most aquatic lifeforms at only slightly higher concentrations than the minimum requirement. The presence of heavy metal ions in surface water continues to be the most pervasive environmental issues of present time. A wide range of pesticides are used to protect agricultural crops. Residuals of pesticides can be detected in aquatic environments.

Herbicides are toxic to microalgae even in the micromolar concentration range [Nyström 1999].

The cultures of freshwater green microalga *Scenedesmus quadricauda* (Turp.) Breb. and marine diatom *Thalassiosira weissflogii* (Grunow) Fryxell et Hastle were studied under the influence of different toxicants (heavy metals chromium and silver as a part of water-dissolved salt, pesticide imazalil sulfate). Population structure was used for quantifying signs on measurements of toxic action.

1.2. Materials and Methods

The culture of green chlorococcal alga *Scenedesmus qudricauda* (Turp.) Breb. (strain S-3) was grown in Uspenskii medium N1 (composition, g/l: 0.025 KNO_3, 0.025 $MgSO_4$, 0.1 KH_2PO_4, 0.025 $Ca(NO_3)_2$, 0.0345 K_2CO_3, 0.002 $Fe_2(SO_4)_3$; pH 7.0-7.3) in conical flasks in luminostat under periodic illumination. The culture of diatom alga *Thalassiosira weissflogii* (Grunow) Fryxell et Hastle was grown in Goldberg-Kabanova medium (composition, g/l: 0.2024 KNO_3, 0.007105 Na_2HPO_4; mg/l: 0.1979 $MnCl_2$, 0.2379 $CoCl_2$, 0.2703 $FeCl_3$). Cells were counted with a Goryaev's hemocytometer under a light microscope. Cell width was measured with a calibrated ocular micrometer with an accuracy of 0.1μm. Number of dead cells was counted with luminescent microscopes MLD-1 (LOMO, Russia) and Axioskop 2FS (Carl Zeiss, Germany). The functional state of the photosynthetic apparatus of the alga was characterized by *in vivo* measuring of delayed fluorescence (DF) of chlorophyll *a*. The amplitude of the DF decay phase during photosynthetic induction in dark pre-adapted samples was used to characterize the photosynthetic efficiency (PE, η). We investigated the toxic action of potassium dichromate ($K_2Cr_2O_7$, PD), fungicide imazalil sulfate (1-[2-(2,4-dichlorophenyl)-2(2-propenyloxi)ethyl-1H-imidazole sulfate, IS) and silver sulfate (Ag_2SO_4) in the long-term experiments up to 30-36 days in three assays.

1.3. Results and Discussion

Figure 1 shows that, in the culture exposed to the toxicants for 4-7 days, cell number changed in a complicated pattern. At low and high concentrations of IS and PD the number of cells was less than in the control culture, whereas at moderate concentrations of the toxicants had no effect. Such concentration-response dependence we could observe during long-term experiment (up to 30 days). This type of the population number changes (so called “paradoxical reaction”) is a usual behavior of biological systems in increasing of damaging factors intensity. We have shown earlier that nonlinear concentration response curve of cell survival reflects of hierarchy of cell responses to increasing concentration of IS: cell division inhibition in low doses, stress and adaptive tolerance increasing in moderate doses and immature cell division and cell death in high doses [Prokhotskaya 2003]. The number of dead cells in the culture increased only at high toxicant concentration (fig. 1, curve 2). Therefore, the change in the relative cell number at low IS and PD concentrations cannot be explained by the summing of the process of cell division and death.

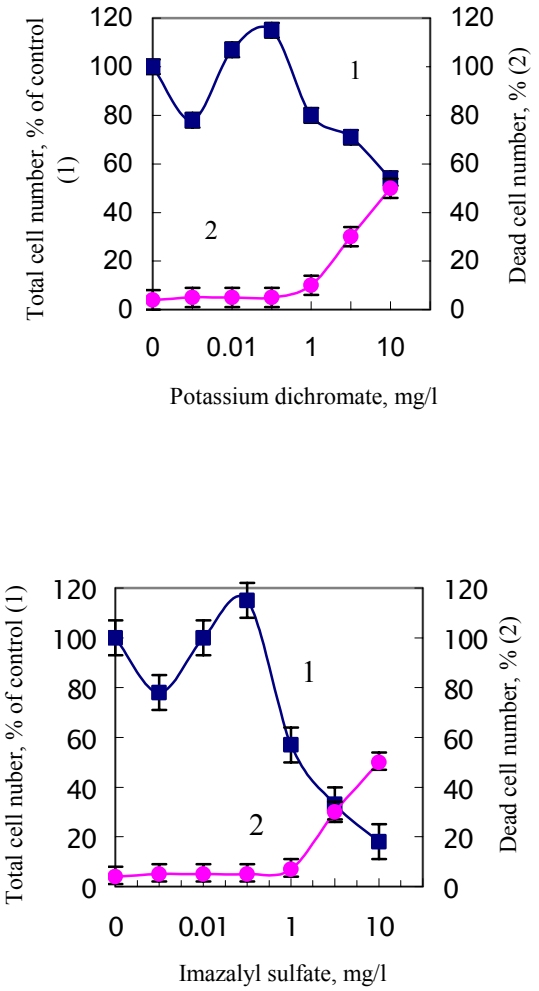

Figure. 1. Changes of the total cell number (1) and dead cell number (2) in the *S. quadricauda* culture as a function of PD and IS concentrations on the 4th – 7th days of treatment.

It was supposed to be existence of certain principles of intrapopulational responses to the toxic exposure, which does not depend on chemical nature of acting factor. These principles reflect the changes of structural and functional characteristics of algal population. We investigated the changes of population structure and average functional characteristics of cells of *S. quadricauda* in the control cultures and in the presence of various concentrations of the toxicants.

1.3.1. Size-age distribution, coenobial composition and functional characteristics of the control culture *S. quadricauda*

The growth curve of the control culture had a stepwise shape apparently due to a partly synchronization of cell division under continuous light-dark periods. We can observe the simultaneous presence of two cell groups differing in size (large and small cells). That fact agrees completely with model previously described for population structure of chlorococcal alga *Chlorella* and *Scenedesmus* [Tamiya 1966, Senger 1986].

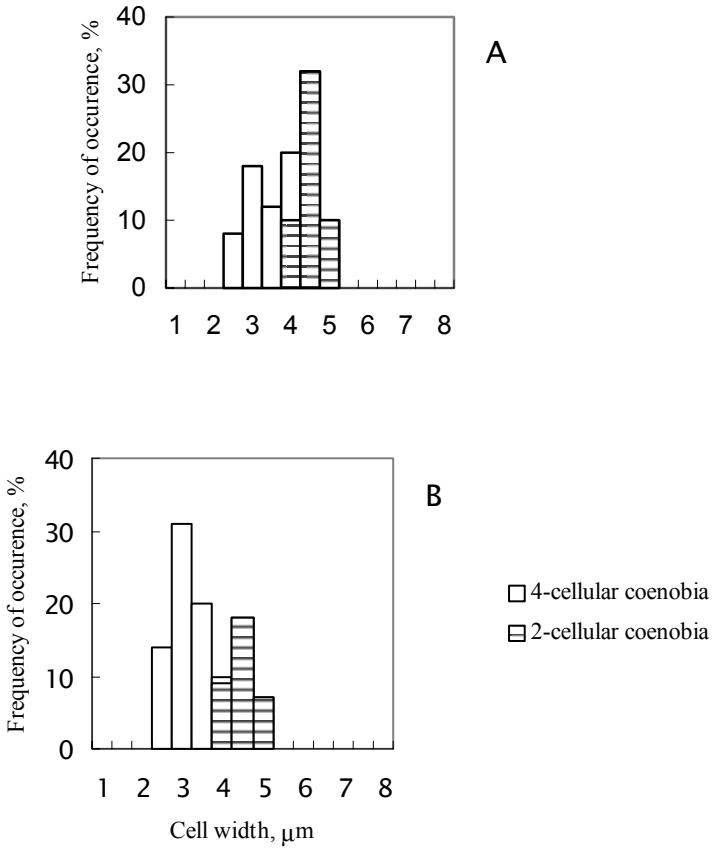

Figure. 2. Cell width distribution in the control culture of. *S. qudricauda*. A – before and B – after increasing of cell number.

Figures 2 shows changes in the cell size distribution during growth of the control culture. Large cells (4.5 μm in width) composing 2-cellular coenobia dominated before the cell number increasing; the share of small cells in 4-cellular coenobia (3.0-3.5 μm) was less, than the share of large cells. The increase of the cell number was accompanied by mirror changes in bimodal distribution with “large” maximum for small-sized cells

and "lower" maximum for large-sized cells. The volumes of large and small cells differed by a factor of two. Hence, it seems likely that large cells are ready for division and small cells are daughter young cells.

The sedimented isolation of young cells from the various-aged culture revealed the functional differences between mature and young cells. The PE was slightly higher in small cells ($\eta = 0.86\pm0.02$) than in large cells ($\eta = 0.80\pm0.02$). The thermal stability of thylakoid membranes in small cells was higher than that of large cells (49.5 ^{0}C and 47.5 ^{0}C, respectively).

1.3.2. Effect of toxicants at low concentrations

The chromium and silver at concentrations of 0.0001, 0.001, 0.01 mg/l (low concentrations) and IS at concentration of 0.001 mg/l were found have an slight influence upon cell number, cell division rates, photosynthetic efficiency and share of alive cells. Any possible changes could be explained on the base of well-known idea of cell population as a complex system with resistant and sensitive cells. Such insignificant differences between control and sample cultures at low concentrations were reversible during the period of experiment. The reason of possible population growth delay under low-level toxic exposure was the arrest of proliferation of some cells rather than deceleration of cell cycle in all cells. In other words, the respond of the algal population to weak toxic effect can be related with cell heterogeneity.

1.3.3. Effect of toxicants at moderate concentrations

In the presence of moderate toxicant concentrations (0.05 mg/l for silver, 0.1 mg/l for chromium and IS), the effect varied from indifferent to toxic according to algal species and season.

During the cell division arrest the PE decreased only slightly ($\eta = 0.70\pm0.02$) as compared to the control culture ($\eta = 0.82\pm0.02$), but it was restored within two days to the control level. The thermal stability of large-sized cells became 1.5 ^{0}C higher than that for the control culture. After cells are being resumed division, they retained the elevated thermal stability. It was suggested that we observed an adaptive increase in cell resistance to the toxicants.

1.3.4. Effect of toxicants at high concentrations

At concentrations of the toxicants over 0.05 mg/l for silver and over 1.0 mg/l for chromium and IS, a total cell number and proportion of living cells decreased. At high PD and IS concentrations (1.0-3.0 mg/l) we can observe long-term cell division inhibition and giant cells forming. Number of dead cells varied from 15 % in the presence of IS (1.0-3.0 mg/l) to 30 % in the presence of PD (1.0-3.0 mg/l). At the concentration 3.0 mg/l of PD the cell number was the same as initial one during the experiment. Analysis of size-age structure and functional characteristics of the cells showed that there were at least two reasons: delay of cell division of one cells and division and death of others. We observed both undividing and proliferating cells.

Sublethal concentrations of IS and PD did not significant inhibit photosynthesis ($\eta = 0.72\pm0.03$, as compared to $\eta = 0.80\pm0.02$ in the control culture). The thermal stability of thylakoid membranes in giant cells exceeded that of the control cells by 1.5 ^{0}C.

At the high silver concentrations 0.1, 0.2 and 0.5 mg/l we observe algostatic effect, e. g. the total number of cells preserved on the constant level close to initial values. We suppose that such effect is related with lysis of the dead cells inhibition. After 1-day incubation at the high concentrations of silver, the photosynthetic activity of the *S. quadricauda* culture was reduced to a double as compared to the control level. It implies that cell damage was irreversible. The number of cells did not attain the control values even after the washing and transferring of silver-treated cells in the toxicant-free medium. It was supposed that the irreversible injuries were caused by silver uptake.

1.3.5. Effect of PD and IS at lethal concentration

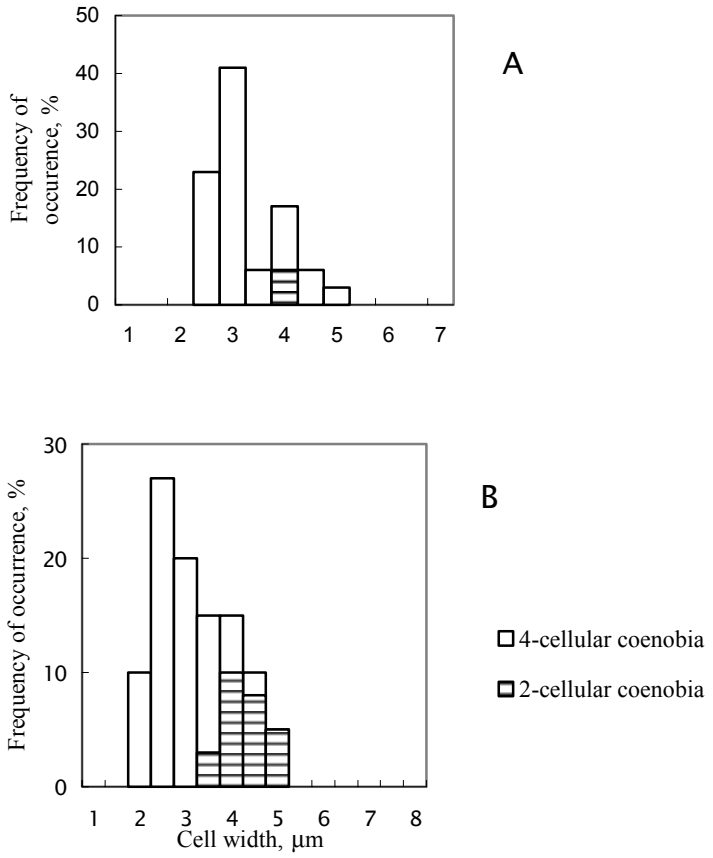

Figure. 3. Size-age and coenobial structure of *S. quadricauda* population after PD and IS 10.0 mg/l incubation. A – PD, 1st day of treatment; B – IS, 1st day of treatment.

At the lethal concentration 10.0 mg/l the cell number decreasing was caused by their death, but during the first day of cultivation the cell number did not change.

The very small cells (width 2.0-2.5 μm) in 2- and 4-cellular coenobia appeared within population (fig. 5, A, B). Therefore, toxicant first initiated cell division in all cells, including those that had not attained the mature cell size. In the normal culture cells divided after attaining about 4.5 μm in diameter, whereas in the presence of toxicant they divided after attaining the size of 3.5 μm. Since the total cell number did not change, it is clear that a certain part of cells died. Therefore, the analysis of size-age population structure can find out the lethal effect earlier than counting of cell number.

Characteristics of DF monotonically changed: the higher the concentrations, the faster they changed. PE was dropped to $\eta = 0.50 \pm 0.05$, as compared to $\eta = 0.80 \pm 0.02$ in the control culture. The thermal stability of thylacoid membranes decreased to 44-45 ^{0}C (against 48.5 ^{0}C in the control culture). These changes accelerated with an increase in IS concentrations within the lethal range (10-20 mg/l) and indicated that cell damage was irreversible.

1.3.6. The number of the toxicant-resistant cells within population

With the aim to estimate the share of resistant cells within the heterogeneous algal population we carried out experiment with triple chromium 10.0 mg/l intoxication during 90 days and double silver 10.0 mg/l intoxication during 60 days. In spite of the long-term exposition with toxicant some algal cells remained alive. Their number was 3-5 % of initial cell number in the presence of silver and 5-6 % in the presence chromium. The cell size spectrum in the presence of silver was rather the same as control one. It indicates that after toxic exposure the normal algal cells remain in population. The photosynthetic activity of these cells was the same as control one, too. The number of these resistant cells corresponds with frequency of mutation for unicellular algae, fungi and bacteria in nature. The presence of resistant cells can be related to their constant presence in population or is the result of selection. The maximal resistance of the algae to the toxicants was revealed in spring-summer, the minimal resistance – in winter.

1.4. Conclusion

The concentration-response curve of cell survival reflects a hierarchy of cell responses to increasing concentration of the toxicants. On the base of structural and functional population characteristics analysis we suggest to appropriate the following types of population reaction to the toxicant action: at low PD and IS concentrations (0.001 mg/l), the decreasing of cell number is the result of cell division arrest; at moderate (0.01-0.1 mg/l), the absence of effect is caused by renewal of cell division after temporary arrest; at high concentrations (1.0-3.0 mg/l), we can observe long-term cell division inhibition and giant cells forming; at lethal concentration (10.0 mg/l), the cell division is stimulated and the small immature cells predominated at the beginning of intoxication. We offer using described types of reaction to the toxic action for risk assessment and biotesting.

Our data demonstrated that the informational value of DF characteristics is most appropriate for recording the responses of algal cultures to lethal concentrations of toxic agents. At low concentrations, DF characteristics are more due to the proportion of various cell types in the population.

There is vast information about chemical waste effects on plants, including algal adaptation to toxicant action [Ahner 1994, Hall 2002, Lasat 2002]. The limits of algal cells resistance to long-term high intensive toxic effects determine survival of population as whole. In the present research we demonstrated the method of proportion of resistant cells estimation in the heterogeneous algal population. Our experiments with algal cultures *Scenedesmus quadricauda* (Turp.) Breb. and *Thalassiosira weissflogii* (Grunow) Fryxell et Hastle grown in the presence of toxicants showed the increasing resistance of pre-adapted cultures by means of the total cell number and share of alive cells growth. The morphological characteristics of the resistant cells were differed from the control ones by the predominance of small cell fraction as a possible result of changes in their growth rates. The population heterogeneity ensured the cell number restoration after the removing of toxic pressure due to the minimal amount of the most resistant cells (3-6 % of the initial cell number). At high silver concentration (0.1 and 0.5 mg/l) the total cell number changed insignificantly, so, we observed the algostatic effect.

Thus, in the long-term intoxication of algal populations experiments we can see the common rules of adaptive and compensation reaction, e. g. elimination of the most sensitive cells and reconstruction the population as a whole system already in the new conditions. Changes of the population structural and functional characteristics can be special way of survival in unfavourable conditions.

Data on the cell number, their photosynthetic characteristics, population structure and share of alive and dead cells will be appropriate for use to predict the most sensitive ecosystem responses and indicate the permissible amount of toxicants in the environment.

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