Chapter

Structural Changes and Adaptation of Algal Population under Different Regimens of Toxic Exposure

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1.1. Introduction

Algae are the principal primary producers of aquatic ecosystems. Modern chemical residues from water pollution (such as pesticides in surface and ground waters, antibiotics, chemical substances of military use, heavy metals, oil, oil products, etc.) are a challenge to survival of microagal populations. Growth of many species was restricted even by micromolar concentrations of such xenobiotics.

Laboratory populations of microalgae are widely used as sensitive test object for the evaluation of the phytotoxicity of chemicals and wastewater streams. Cell populations of microalgae are complex systems with resistant and sensitive cells. When pollutants are added to a dense microalgal culture, the cell density will be reduced after a few days due to the death of sensitive cells. However, after further incubations, the culture will sometimes increase in density again due to the growth of cell variant, which is resistant to the contaminants. Numerous studies have shown that heavy metals are extremely toxic to microalgae in both laboratory cultures and natural populations. It has also been reported that microalgae from contaminated sites appear to have adapted to high metal concentrations whereas algae from unpolluted sites remain sensitive [Knauer 1999].

Rapid adaptation of microalgae to environmental changes resulting from water pollution has been demonstrated recently [Costas 2001, López-Rodas 2001]. Unfortunately, the evolution of microalgae subsequent to a catastrophic environmental change is insufficiently understood. Little is known about the mechanisms allowing algal adaptation to such extreme conditions. Within limits, organisms may survive in chemically-stressed environments as a result of two different processes: physiological adaptation (acclimation), usually resulting from modifications of gene expression; and, adaptation by natural selection if mutations provide the appropriate genetic variability [Belfiore 2001]. Because physiological adaptation is bounded by the types of conditions commonly encountered by organisms, it remains for genetic adaptation to overcome extreme environmental conditions [Hoffmann 1991].

The changes of population structure of freshwater green alga *Scenedesmus quadricauda* and marine diatom alga *Thalassiosira weissflogii* were studied under different regimens of heavy metal (chromium) exposure. Adaptation of the algae to growth and survival in an extreme environment was analysed by using an experimental model. The main aims of this work were: (1) to estimate the effect of chromium contamination on microalgal populations under different regimens of chromium addition; (2) to determine the nature and origin of chromium-resistant cells that arise; (3) to estimate the mutation rate from chromium sensitivity to chromium resistance.

1.2. Materials and Methods

The culture of green chlorococcal alga *Scenedesmus qudricauda* (Turp.) Breb. (strain S-3) was grown non-axenically 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 (12:12 h). The culture of diatom alga *Thalassiosira weissflogii* (Grunow) Fryxell et Hastle was grown non-axenically 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 $FeCl_3$).

Toxicity test: effect of chromium on population growth. We investigated the toxic action of potassium dichromate ($K_2Cr_2O_7$, PD), well known as standart toxicant [Wang 1997], in view of maintenance of a constant dose of chromium per one cell during experiments in order to pass from concentration dependence to dose dependence. The laboratory algal cultures were exposed to increasing concentrations of the toxicant in the long-term experiments in three replicates. The experiments were performed both with single chromium addition at the start of experiment and with multiple additions during exposure time. The periods between toxicant additions approximately corresponded to doubling time for algae so that the dose of the toxicant per one cell was particularly the same as that at the initial day of experiment. The effect of chromium on *S. qudricauda* and *T. weissflogii* was estimated by calculating total cell number, a share of alive, dead and dying cells during exposure time (28 and 21 days, respectively). Cells were counted with a Goryaev's hemocytometer and Nazhotta cytometer under a light microscope. Number of alive, dead and dying dead cells was counted with luminescent microscope Axioskop 2FS (Carl Zeiss, Germany).

For experiment with *S. quadricauda* we used concentration of chromium: 0.001; 0.01; 0.1; 1; 5 and 10 mg/L. Concentration of toxicant in a stock solution was 1 mg/mL (counting per chromium). Initial number of cells after inoculation was 50 000 cells/mL. After that cells grew within 5 days on reaching of a logarithmic growth phase by culture. Number of cells at this moment was $28\text{-}30 \cdot 10^4$ cells/mL. Experiment was performed in conic flasks in volume of 100 mL, volume of culture in which was 50 mL. We added toxicant to cultures at 0 day of experiment (single addition) and further at 3, 6, 10 and 17 day until necessary concentrations (multiple additions). Frequency of toxicant addition was defined by growth rate of cultures and rate of cell division.

For experiment with *T. weissflogii* we used concentration of chromium: 0.001; 0.01; 0.1 and 1 mg/L. The initial number of cells taken for experimemt was 5 000 cells/mL. Experiment was performed in small phials with 10 mL of culture. Chromium was introduced into growth medium at 0 day of experiment until necessary concentrations (single addition). Further, in one series of culture we did not add the toxicant (conditionally named by us as "control") and in another series chromium was added at 3, 6, 10 and 13 day (multiple additions).

Average growth rate of both cultures (without chromium) was 0.33 division/day. Toxicant was introduced into the growth mediums proportionally to an increase of cell number of *S. quadricauda* and *T. weissflogii* so that the toxicant quantity per one cell (dose) was kept constant.

Fluctuation test: analysis of transformation from chromium sensitivity to chromium resistance. A modified Luria–Delbrück fluctuation analysis was performed as previously described [López-Rodas 2001] in liquid medium to distinguish resistant cells that had originated as a result of random spontaneous pre-selective mutations (prior to chromium exposure) from those arising through acquired post-selective adaptation (during the exposure to chromium).

Two different sets of experimental cultures were prepared with both species of algae. The first set of experiments was performed in 52 (*S. quadricauda*) and 49 (*T. weissflogii*) parallel culture flasks with cell number $N_0 = 200$ cells and $N_t = 2.8 \cdot 10^4$ (*S. quadricauda*), $N_t = 10^5$ (*T. weissflogii*) cells; and treated with 2.5 (*S. quadricauda*), 1.5 (*T. weissflogii*) mg/L chromium after reaching N_t. For the second set of experiments, 30 aliquots of 10^4 (*S. quadricauda*) and 10^5 (*T. weissflogii*) cells from the same parental populations were separately transferred to flasks containing fresh liquid medium with 2.5 (*S. quadricauda*) and 1.5 (*T. weissflogii*) mg/L chromium. Cultures were observed for approximately 14 days, and the resistant cells in each culture (both in set 1 and set 2) were counted. The cell count was performed by at least two independent observers.

If resistant cells arise by rare spontaneous mutations, each parallel culture in set 1 would have a given probability of generating resistant variants with each cell division. Then, inter-flask variation would not be consistent with the Poisson model. The number of cells from each flask in set 2 would show variation due only to random sampling; variation from flask to flask would be consistent with the Poisson model. If there is rare spontaneous mutation, the variance/mean ratio_{set1} is usually many times higher than the variance/mean ratio_{set2}. The method allows estimation of the rate of spontaneous mutation in algae and the rate of appearance of resistant cells. The proportion of set 1

cultures showing no mutant cells after chromium exposure (P_0 estimator) was the parameter used to calculate the mutation rate (μ). The P_0 estimator [Luria 1943] is defined as follows: $P_0 = e^{-\mu\ (Nt-No)}$, where P_0 is the proportion of cultures showing no resistant cells. Therefore, μ was calculated as: $\mu = -Log_e P_0 / (N_t - N_0)$.

1.3. Results and Discussion

We tried to develope an experimental model of toxic effect using constant toxicant dose per cell during the experiments.

The presented data show (Fig. 1), that at presence of high chromium concentration (1 mg/L and more) the total cell of both species slightly varied or decreased, since the moment of the first chromium addition and down to the end of experiment in comparison with the initial cell number and drastically decreased in comparison wich control without chromium. At toxic influence of such intensity, the dose of chromium per one cell remains practically constant during all term of experiment. Therefore with reference to high concentration of substances it is possible to speak about concurrence of concepts "concentration" and "dose" even if we add the toxicant one time at the beginning of the experiment.

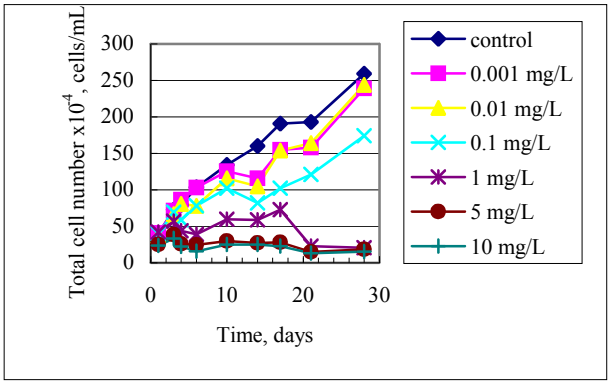

Figure. 1. Changes of the total cell number of *S. quadricauda* under chromium exposure (multiple chromium additions).

At medium chromium concentration of 0.1 mg/L number of cells increased, but growth rate of culture has been slowed down in comparison with control one. At low chromium concentration of 0.001 and 0.01 mg/L growth rate of *S. quadricauda* corresponded to the control parameters down to 10 day of experiment, then growth rate have decreased, however by the end of experiment number of cells at presence of these concentrations of chromium has appeared close to the control. Thus, the most sensitive stage at repeated additions of chromium in medium is, apparently, second half of logarithmic growth phase (10-14 day of experiment). As concentration of chromium of 0.001 and 0.01 mg/L are low enough, it is not likely, that they provoke selection of resistant cells. In this case chromium could cause "synchronization" (full or partial) of cultures seaweed by delay or arrest of cellular division at 7-10 day of experiment. After that there was an acclimation of algal cells, and cellular division also was synchronously restored. Thus cultures have reached "control" levels of number of cells.

Thus, at low chromium concentrations of 0.001 and 0.01 mg/L during the experiments with the periodical additions growth rate of *S. quadricauda* was close to

the control (without chromium) although the total final concentrations were 3.3-3.4 times more than initial ones.

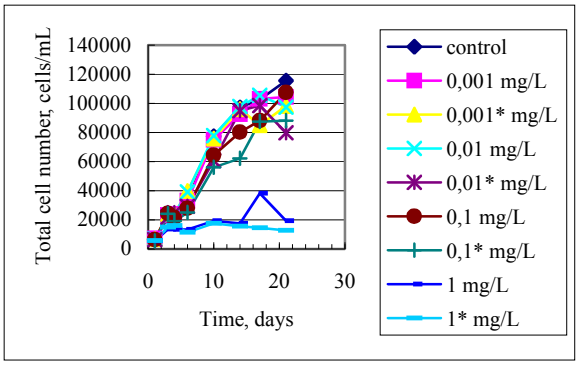

Figure. 2. Changes of the total cell number of *T. weissflogii* under chromium exposure: single addition at the start of experiment and multiple (*) additions at 0, 3, 6, 10 and 13 day of experiment.

The final cell number of *T. weissflogii* was slightly decreased in the presence of 0.001 mg/L chromium and was reliably smaller in the presence of 0.01; 0.1 and 1 mg/L chromium during the multiple intoxication as compared with the single one (Fig. 2).

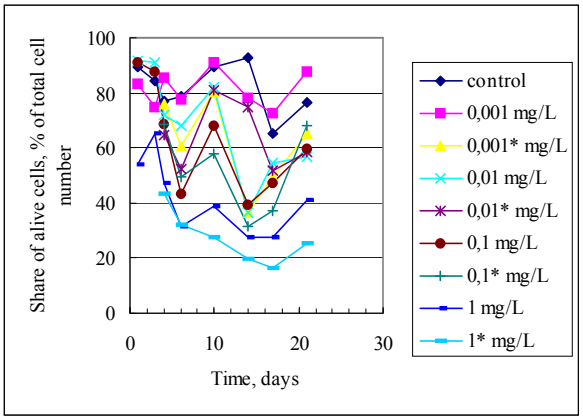

Figure. 3. Changes of share of alive cell of *T. weissflogii* under chromium exposure: single addition at the start of experiment and multiple (*) additions at 0, 3, 6, 10 and 13 day of experiment.

The share of dead and dying cells was slightly higher at the multiple intoxication than at the single one (Fig. 3) during experiments with both species (data for *S. quadricauda* are not presented).

We have determined earlier [Prokhotskaya 2006] the number of resistant cells within the heterogeneous *S. quadricauda* population under triple chromium 3.5 mg/L intoxication during 90 days. In spite of the long-term exposition with the toxicant some algal cells remained alive. Their number was 5-6 % of initial population density.

In the present study we have analysed the spontaneous occurrence of chromium-resistant cells in cultures of chromium-sensitive (wild-tipe) cells of *S. quadricauda* and *T. weissflogii*. Modified Luria-Delbrück fluctuation analysis with algae as experimental organisms [Luria 1943; López-Rodas 2001] was used to distinguish between resistant cells arising by rare spontaneous pre-adaptive mutations occurring randomly during replication of organisms prior to the incorporation of chromium and chromium resistant cells arising through post-selective adaptation in response to chromium and, subsequently, to estimate the rate of occurrence of resistant cells.

On the base of hypothesis that adaptation to chromium occurs by selection on spontaneous mutations, the controls should have had a low variance-to-mean ratio consistent with the error in sampling resistants from one large culture, whereas the fluctuation test cultures should have had a high variance-to-mean ratio. Spontaneous mutation thus predicts a high variance-to-mean ratio in the number of resistant cells among cultures, whereas resistance acquired in response to exposure predicts a variance-to-mean ratio that is approximately 1, as expected from the Poisson distribution.

When algal cultures were exposed to 2.5 mg/L (*S. quadricauda*) and 1.5 mg/L chromium (*T. weissflogii*), growth of the algae was inhibited. Chromium killed the wild-type sensitive cells but allowed the growth of resistant cells. Every experimental culture of both sets 1 and 2 apparently collapsed following chromium exposure. In set 1, only some cultures recovered after 14 day of chromium exposure, apparently due to the growth of chromium resistant cells (recovered cultures increased their cell number compared to the control level). A high fluctuation in set 1 (in contrast with the scant variation in set 2) was found in both species (Table 1, 2), which indicated that the high variance found in set 1 cultures should be due to processes other than sampling error.

Table. 1. Fluctuation analysis of resistant variants in *Scenedesmus quadricauda*

	Set 1	Set 2
No. of replicate cultures	52	30
No. of cultures containing the following no. of resistant cells/mL:		
0	45	0
$0\text{-}2\times10^4$	2	0
$2\times10^4\text{-}10^5$	5	30
$>10^5$	0	0
Variance/mean (of the no. of resistant cells per replicate)	61.5	3.2
μ (mutants per cell division)	5.2×10^{-6}	

Table. 2. Fluctuation analysis of resistant variants in *Thalassiosira weissflogii*

	Set 1	Set 2
No. of replicate cultures	49	30
No. of cultures containing the following no. of resistant cells/mL:		
0	36	0
1-1300	4	0
1300-5000	9	30
>5000	0	0
Variance/mean (of the no. of resistant cells per replicate)	16.8	0.95
μ (mutants per cell division)	3.1×10^{-6}	

The data from a fluctuation test were used to calculate a spontaneous mutation rate per cell division using the proportion of cell cultures that exhibit no mutants at all [Luria 1943]. The estimated mutation rates (μ) using the P_0 estimator were $5.2 \cdot 10^{-6}$ and $3.1 \cdot 10^{-6}$ mutants per cell division in *S. quadricauda* and *T. weissflogii*, respectively.

The data of this study correspond to the results of other work carried out on understanding algal adaptation to anthropogenic chemical water pollutants [Costas 2001; López-Rodas 2001; Baos 2002; García-Villada 2002; Flores-Moya 2005]. The mutation rate from $3.1 \cdot 10^{-6}$ to $5.2 \cdot 10^{-6}$ mutants per cell per generation was the same order (or one order lower and higher) of magnitude found for the resistance to several pollutants in other cyanobacterial and microalgal species. The presence of resistant cells in the populations of algae is regulated by the recurrent appearance of mutants and their elimination by selection, yielding an equilibrium frequency of 3-5 resistant cells per 10^6 cell divisions. This fraction of resistant mutants is presumably enough to assure the adaptation of algal populations to catastrophic water contamination, since the algal natural populations are composed of countless cells. Nevertheless, mutations usually imply an energetic cost that may affect the survival of adapting populations [Coustau 2000], as it has been demonstrated by a decreased growth rate in resistant cells compared to growth rate in sensitive ones [Flores-Moya 2005; López-Rodas 2007]. Thus, resistant cells could develop in freshwater ecosystems polluted with the toxicants, but their contribution to primary production will be significantly lower than that occurring in pristine ecosystems with sensitive cells.

1.4. Conclusion

The present study is a simple model of algal adaptation to stressful environments. Our results suggest that rare preselective mutants can be sufficient to ensure the adaptation of eukaryotic algae to extreme natural habitats. These values are low ($\sim 10^{-6}$ mutants per cell division). Such mutation rate coupled with rapid growth rates, are presumably enough to ensure the adaptation of microalgae to water contamination. The resistant cells arise randomly by rare spontaneous mutation during replication of cells prior to the addition of the contaminant. Resistant mutants are maintained in the absence of contaminants as the result of balance between new resistant cells arising from spontaneous mutation and resistant cells eliminated by natural selection, so that about 3-5 chromium-resistant mutants per million cells are present in the absence of chromium. Within limits microalgal species should survive in polluted environments as a result of physiological adaptation. With increasing concentrations of contaminants, however, physiological adaptation is not enough, but the genetic variability of natural populations could assure the survival of at least some genotypes [Mettler 1988]. Genetic variability in natural populations is the most important guarantee of surviving most environmental changes [Lewontin 1974; Mettler 1988]. Some populations are being exposed to new xenobiotics for the first time. Sudden toxic spills of residual materials can be lethal to microalgae. Rare spontaneous pre-adaptive mutation is enough to ensure the survival of microalgal populations in contaminated environments when the population size is large enough. Adaptation of algal populations to modern

pollution-derived environmental hazards seems to be the result of a rare instantaneous events and the result of resistant cells selection within heterogeneous population.

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