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Adaptation of Phytoplankton to Novel Residual Materials of Water Pollution: An Experimental Model Analysing the Evolution of an Experimental Microalgal Population Under Formaldehyde Contamination

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Abstract The adaptation mechanisms of microalgae to grow in contaminated waters were analysed using a *chlorophyta* species under formaldehyde exposure as experimental model. Cultures initially collapsed after exposure to 16 ppm formaldehyde, but occasionally resistant cells were able to grow after further incubation. Resistant cells arose by rare spontaneous mutations that appeared before the exposure to formaldehyde (mutation rate = 3.61×10^{-6}), and not as result of physiological mechanisms. Although mutations may be the mechanisms that should allow the survival of microalgae in polluted waters in a world under rapid global change, mutants have a diminished growth rate.

Keywords Adaptation · Formaldehyde · Mutation · Water pollution

Water pollution by anthropogenic substances is a problem of great magnitude that urgently needs more basic research to facilitate predictions about the future and to determine actions to mitigate this environmental crisis. In this sense, studies focused on knowing if essential microbes succumb to anthropogenic toxins are of great importance. Particularly, the tolerance of microalgae to contaminated environments is very relevant from an ecological point of view, as these organisms are the principal primary producers of aquatic ecosystems.

Among these toxics, formaldehyde has become widely used as a chemical intermediate, analytical reagent, in concrete and plaster additives, wood preservation, in agriculture, disinfectants and fumigants (EPA 1988; WHO 1989). Formaldehyde has a half-life of 24–168 h in surface waters and 48–336 h in deeper waters (Howards et al. 1991), causing acute toxicity in phytoplankton (Chiayvareesajja and Boyd 1993; Burridge et al. 1995).

In order to study adaptation of microalgae to grow and survive in formaldehyde-polluted environments, a fluctuation analysis (Luria and Delbrük 1943) was performed. Usually, formaldehyde treatment produces massive destruction of microalgae (Chiayvareesajja and Boyd 1993; Burridge et al. 1995), but some cell variants could survive in formaldehyde-contaminated environments. The fluctuation test (Luria and Delbrück 1943) provides the appropriate procedure to discriminate between adaptation by selection of rare spontaneous mutations and other procedures. Recently, fluctuation test has been conducted entirely in liquid media, growing microalgae cultures first in a benign medium and then exposing them to contaminants (López-Rodas et al. 2001, 2007; Costas et al. 2001, 2007; Baos et al. 2002; García-Villada et al. 2002; Flores-Moya et al. 2005).

Materials and Methods

Experiments were performed with a wild-type strain of *Dictyosphaerium chlorelloides* (Naumann) Komárek and Perman (Chlorophyta) isolated from a pristine lagoon (without previous formaldehyde contamination) in Sierra

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Nevada (SE Spain). This strain was isolated from a single cell to assure no genetic variability within it. Before the experiments, cells were grown axenically in cell-culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 mL of BG-11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany) at 22°C under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm. Cultures were axenically maintained in mid-log exponential growth by serial transfers of subcultures to fresh medium.

To determine formaldehyde toxicity, the effects of increasing doses of formaldehyde on growth and photosynthetic performance of *Dictyosphaerium chlorelloides* were measured. Experimental cultures were seeded each with 1.3×10^6 cells from mid-log exponentially growing cultures. A stock solution of about 38% formaldehyde (Sigma, Aldrich Chemie, Taufkirchen, Germany) was prepared in BG-11 medium to obtain serial dilutions of 1.60×10^{-3} % w/w (16 μg/mL), 9.94×10^{-4} % w/w (10.6 μg/mL), 6.14×10^{-4} % w/w (6.4 μg/mL), and 3.80×10^{-4} % w/w (4 μg/mL) to be used for algal exposure. Three replicate cultures of each formaldehyde concentration as well as three unexposed controls were prepared. In these cultures and controls, growth rate (m) was calculated using the equation:

$$m = \frac{Log_e \frac{N_t}{N_0}}{t}, \quad \text{(Crow and Kimura 1970)}$$

where $t = 7$ d, and N_0 and N_t are the cell numbers at the start and at the end of the experiment, respectively. Experiments and controls were counted using a spectrofluorimeter (Schimadzu RF-551S, Durisburg, Germany) relating the chlorophyll *a* fluorescence with cell density within the lineal range.

The effective quantum yield (Φ_{PSII}) was also measured in experiments and controls using a ToxY-PAM fluorimeter (Walz, Effeltrich, Germany) 24 h after formaldehyde exposure. Effective quantum yield was calculated as follows:

$$\Phi_{PSII} = \frac{F'_m - F_t}{F'_m}$$

where F'_m and F_t are the maximum and the steady-state fluorescence of light-adapted cells, respectively (Schreiber et al. 1986).

The fluctuation analysis (Fig. 1) was performed at 22°C and under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm, and consisted of two Sets of culture flasks. Set 1 included 105 parallel cell-culture flasks, each one initially seeded with $N_0 = 125$ cells (i.e. a small number enough to assure the absence of pre-existing mutants). These cultures were allowed to grow (as previously detailed) until they reached approximately $N_t = 4.2 \times 10^5$ cells per flask, and then BG-11 medium containing formaldehyde (final concentration 1.6×10^{-3} (16 μg/ml) % w/w) was added. Control (Set 2) consisted on 25 parallel cell-culture flasks containing each 4.2×10^5 cells from the same parental population and with the same concentration of formaldehyde in BG-11 medium as Set 1. Both Sets were inoculated simultaneously. Cultures were grown for 50 days and then resistant cells in each culture were detected using a spectrofluorimeter (Schimadzu RF-551S, Durisburg, Germany). If resistant cells arose only from spontaneous mutations before selection (formaldehyde addition), then a high variance in their presence per culture (fluctuation) should be found as the chance of mutation would occur earlier in some cultures, later or even not occur in others. On the opposite, if resistant cells arose only in response to the selective medium, physiological mechanisms or post-adaptive mutation, every cell should present the same (and low) probability to adapt to the new medium. Thus, their distribution per culture should not exhibit any fluctuation at all. The control (Set 2) estimates the error in sampling resistant cells. Since this Set 2 is the experimental control of the analysis of fluctuation, a similar variance/mean ratio between Sets 1 and 2, would confirm that resistant cells arose in response to the selective medium.

The mutation rate from formaldehyde sensitive to formaldehyde-resistant cells was estimated by fluctuation analysis. The proportion of cultures from Set 1 showing no resistant cells after formaldehyde exposure was the parameter (P_0 estimator) used to calculate the mutation rate (μ). The P_0 estimator (Luria and Delbrück 1943) is defined as follows:

$$P_0 = e^{-\mu(N_t - N_0)}$$

where P_0 is the proportion of cultures showing no resistant cells, and N_0 and N_t are the initial and the final population size, respectively.

Therefore, μ (mutation rate) was calculated as:

$$\mu = \frac{-\mathrm{Log}_e P_0}{N_t - N_0}$$

The mutation from a normal wild-type formaldehyde-sensitive allele to a formaldehyde-resistant allele is recurrent. In addition, the formaldehyde-resistant allele is detrimental to fitness in the absence of formaldehyde. As a result, new resistant mutants arise in each generation, but most of these mutants are eliminated sooner or later by natural selection, if not by chance (Crow and Kimura 1970). At each time there will be a certain number of resistant cells that are not yet eliminated. The average number of such mutants will be determined by the balance between mutation rate and selective elimination rate, in accordance with the equation:

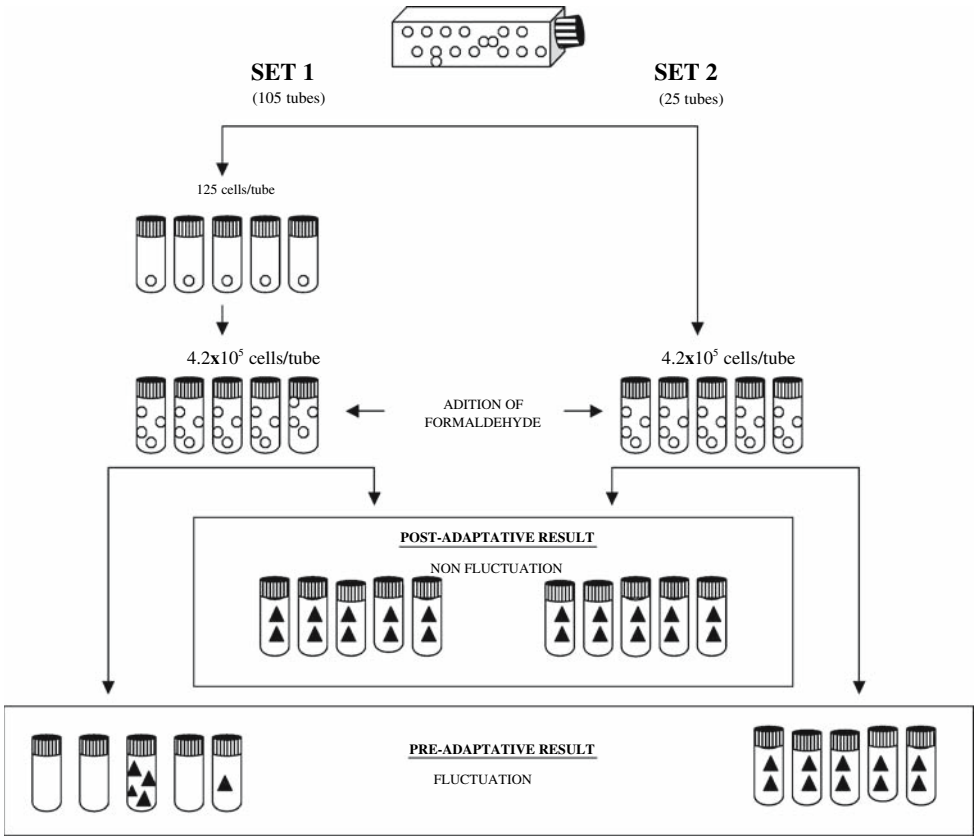

Fig. 1 Schematic diagram of the experiment modified from the classic Luria and Delbrück(1943) fluctuation analysis. Set 1 consists on 105 cultures each one containing 125 cells. They were allowed to grow, before adding the substance in study, till they reached the number of 4.2×10^5 cells. Set 2 consists on 25 tubes control with 4.2×10^5 cells that directly incorporated the studied product. If the adaptation to medium is due to the uncommon pre-selective mutations, between both Sets the existence of a huge fluctuation should be evident, as mutation appears by chance. In Set 1, some tubes would contain some mutants that had appeared early during cell division, in other tubes mutants would have appeared later, and, in the rest of them, there would be no mutants at all. On the other hand, if the resistance needs specific adaptation in response to formaldehyde exposure, both Sets 1 and 2, would be very similar, as every cell would have the same small probability to survive in that medium

$$q = \sqrt{\frac{\mu}{s+\mu}} \quad \text{(Kimura and Maruyama 1966)}$$

where q is the frequency of the formaldehyde-resistant allele, μ is the mutation rate and s is the coefficient of selection calculated as follows:

$$s = 1 - \frac{m_f^r}{m_f^s}$$

where m_f^r and m_f^s are the fitness of formaldehyde-resistant and formaldehyde-sensitive cells, respectively (Crow and Kimura 1970).

Results and Discussion

Low concentrations of formaldehyde have significant toxic effects on wild-type *D. chlorelloides* cells (Table 1). Growth rate and photosynthesis performance were severely reduced even by 6.4 μg/mL, whereas concentrations of 16 μg/mL inhibited completely growth and photosynthesis performance.

When microalgae were treated with 16 μg/mL formaldehyde in Set 1, all cultures initially collapsed due to the destruction of sensitive cells by the toxicant. But some cells were able to grow in some culture flask after 50 days, suggesting that rare formaldehyde-resistant cells occur (Table 2). A high fluctuation in the number of resistant cells per culture was observed in Set 1 (from 0 to more than 2.6×10^8 resistant cells per culture flasks). In contrast in Set 2 all the cell cultures contain formaldehyde-resistant cells showing low fluctuation (Table 2).

The mutation rate from formaldehyde susceptibility to formaldehyde resistance in *D. chlorelloides* (3.61×10^{-6} divisions) was found to be on the same order of magnitude than mutation rates we have described for resistance to many other biocides in chlorophyta (Costas et al. 2001; López-Rodas et al. 2001; Baos et al. 2002; García-Villada et al. 2002), significantly higher than mutation rates for

Table 1 Inhibition of growth and photosynthetic performance (effective quantum yield) of *Dictyosphaerium chlorelloides* by increasing doses of formaldehyde, calculated as percentage of untreated controls (dose-effect)

Formaldehyde concentration yield ($\mu g\ mL^{-1}$) (mean ± SE)	Growth rate inhibition (%) (mean ± SE)	Effective quantum inhibition (%)
0.0	0 ± 0	0 ± 0
4.0	17 ± 6	5 ± 0
6.4	61 ± 5	13 ± 1
10.6	100 ± 0	98 ± 2
16.0	100 ± 0	100 ± 0

Table 2 Fluctuation analysis of *Dictyosphaerium chlorelloides* exposed to formaldehyde (16 μg/mL)

	Set 1	Set 2
No. of culture replicates	105	25
N_0 (cells)	125	–
N_t (cells)	4.2×10^5	4.2×10^5
No. of cultures containing the following no. of formaldehyde-resistant cells:		
0	23	0
$1–10^7$	32	0
$10^7–2 \times 10^7$	11	0
$2 \times 10^7–3 \times 10^7$	8	0
$>3 \times 10^7$	31	25
Mutation rate (mutants per cell division) 3.61×10^{-6}		

sulphurous water of La Hedionda, 2.7×10^{-7} (Flores-Moya et al. 2005) and on the same order of magnitude than mutation rates for sulphurous water of Spain's Tinto River (Costas et al. 2007). Some stressful environments support populations of algal species at the extreme limits of their physiological tolerance (Fogg 2001). Algae survive in such hostile environments as a result of physiological acclimation by modifications of gene expression (Belfiore and Anderson 2001). Beyond physiological limits, adaptive evolution depends on the occurrence of new mutations that confer resistance (Belfiore and Anderson 2001).

On the opposite of formaldehyde-sensitive wild-type algae, the formaldehyde-resistant mutants isolated from Set 1 were able to grow under 16 μg/mL of formaldehyde. Furthermore, such a high formaldehyde concentration just inhibited 75% of their quantum yield. Isolated formaldehyde-resistant mutants growing in absence of formaldehyde showed a coefficient of selection of $s = 0.06$ respect to the wild-type formaldehyde-sensitive cells. Since mutation is recurrent, but mutant is usually detrimental in fitness, in each generation new mutants arise, but most of them are finally eliminated by natural selection (Crow and Kimura 1970). The frequency (q) of formaldehyde-resistant alleles in non-extreme environment was estimated, by using the values of μ and s, in 7.68 formaldehyde-resistant mutants per 10^3 cells, as the consequence of the balance between mutation and selection. Consequently, the ancestral microalgae population would be predominantly constituted by a clone line of wild-type sensitive genotype and, simultaneously, in a very small fraction, by a clone line of formaldehyde-resistant mutants. Thus, a rare spontaneous mutation from formaldehyde susceptibility to formaldehyde resistance seems to be enough to assure survival of microalgae populations in formaldehyde-contaminated environments.

Synthetic chemicals, like formaldehyde, causing water pollution could exert drastic selective pressures to facilitate rapid fixation of rare pre-adaptive mutations in natural populations of microalgae. Although some phytoplankton species could be able to rapidly adapt to new residual substances, such process usually implies a high cost for the ecosystem, as they reduce growth and photosynthetic performances.

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