Evol Ecol (2007) 21:535–547
DOI 10.1007/s10682-006-9134-8

RESEARCH ARTICLE

Resistance to glyphosate in the cyanobacterium *Microcystis aeruginosa* as result of pre-selective mutations

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Received: 8 March 2006 / Accepted: 4 October 2006 / Published online: 30 November 2006

Abstract Adaptation of *Microcystis aeruginosa* (Cyanobacteria) to resist the herbicide glyphosate was analysed by using an experimental model. Growth of wild-type, glyphosate-sensitive (G^s) cells was inhibited when they were cultured with 120 ppm glyphosate, but after further incubation for several weeks, occasionally the growth of rare cells resistant (G^r) to the herbicide was found. A fluctuation analysis was carried out to distinguish between resistant cells arising from rare spontaneous mutations and resistant cells arising from other mechanisms of adaptation. Resistant cells arose by rare spontaneous mutations prior to the addition of glyphosate, with a rate ranging from 3.1×10^{-7} to 3.6×10^{-7} mutants per cell per generation in two strains of *M. aeruginosa*; the frequency of the G^r allele ranged from 6.14×10^{-4} to 6.54×10^{-4}. The G^r mutants are slightly elliptical in outline, whereas the G^s cells are spherical. Since G^r mutants have a diminished growth rate, they may be maintained in uncontaminated waters as the result of a balance between new resistants arising from spontaneous mutation and resistants eliminated by natural selection. Thus, rare spontaneous pre-selective mutations may allow the survival of *M. aeruginosa* in glyphosate-polluted waters via G^r clone selection.

Keywords Cell morphology · Glyphosate · *Microcystis* · Mutation rate · Natural selection

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Abbreviations and symbols

CF	Coefficient of form
G^r	Glyphosate-resistant cells
G^s	Glyphosate-sensitive cells
m_{G^r}	Malthusian fitness parameter from glyphosate-resistant cells
m_{G^s}	Malthusian fitness parameter from glyphosate-sensitive cells
N_0	No. of cells at the start of the experiment
N_t	No. of cells at the end of the experiment
P_0	Proportion of cultures without G^r cells in the set 1 fluctuation analysis experiment
q	Frequency of G^r allele in natural, non-exposed to glyphosate populations
s	Coefficient of selection
μ	Mutation rate

Introduction

Nowadays we are living in a geological instant in which global extinction rates are 50–500 times background and are increasing due to human activities that are altering biosphere-level processes. It has been estimated that several million populations and 300–30,000 species go extinct annually from a total of >10 million species (Woodruff 2001). Distinctive features of the future biosphere could include homogenization of biotas, proliferation of opportunistic species, a pest-and-weed ecology, and unpredictable emergent novelties (Myers and Knoll 2001). The biodiversity crisis is reasonably understood for terrestrial vertebrates and a few other groups, but little is known about organisms as abundant and important as microbes. Studies of bacteria and protists are clearly needed, because crucially important nutrient cycles may become less predictable as essential microbes succumb to anthropogenic toxins (Woodruff 2001). In particular, since microalgae and cyanobacteria are the principal primary producers of aquatic ecosystems (Kirk 1994; Falkowski and Raven 1997), the tolerance of these organisms to contaminated environments is very relevant from an ecological point of view. Herbicides are among the most significant human-synthesized pollutants in aquatic ecosystems (Koenig 2001). Unrelenting application of herbicides during recent decades has resulted in water pollution, with serious environmental implications and evolutionary consequences due to strong selection pressure on numerous species (Belfiore and Anderson 2001; Palumbi 2001). This is the case for the broad-spectrum herbicide glyphosate, which was introduced in 1974 and now constitutes a potent anthropogenic source of selection (Baucom and Mauricio 2004).

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 and Anderson 2001). The neo-Darwinian view that evolutionary adaptation occurs by selection of pre-existing genetic variation was early accepted for multicellular organisms (Huxley 1942; Lewontin 1974; reviewed by Sniegowski and Lenski 1995). However, recent evolutionary studies in bacteria have suggested that hypothetical ''adaptive mutation'' could be a process resembling Lamarckism which, in the absence of lethal

selection, produces mutations that relieve selective pressure (Cairns et al. 1998; Foster 2000). The key to resolving this debate is to know the pre-adaptive or post-adaptive origin of new mutations. Surprisingly, there are almost no studies that have made a direct connection between the rates of origin of favoured mutants and the process of adaptation (Sniegowski 2005). The main reason for this lack of studies is the difficulty in measuring the rate of favoured mutants directly in diploid, multi-celled, sexual organisms living in well-defined populations. In contrast, most microbes (including cyanobacteria and many microalgae) are haploid, single-celled, asexual organisms, and their populations are composed of countless cells (Margulis and Schwartz 1982). Therefore, the study of genetic adaptation of cyanobacteria to extreme environmental changes derived from anthropogenic pollution is an adequate aproximation to the problem of the origin of favoured mutants and the process of adaptation.

The aim of this work was to evaluate, from an evolutionary point of view, the effect of glyphosate on the freshwater cyanobacterium *Microcystis aeruginosa* (Kützing) Lemmermann. For this purpose, we performed a fluctuation analysis (Luria and Delbrück 1943) using glyphosate as selective agent. This experimental model is particularly well-suited to discriminate between cells that become resistant from acquired specific adaptation in response to glyphosate (including both physiological adaptation or acclimation, and possible mutations following glyphosate exposure; the first case is not an evolutionary event) and resistant cells arising from rare spontaneous mutations that occur randomly during propagation of cyanobacteria prior to the glyphosate exposure. Consequently, we have assessed the mechanisms (fitness and mutation-selection balance) that allow cyanobacteria to withstand increasing exposure to glyphosate. We demonstrate the existence of very rapid evolution in populations of *M. aeruginosa* as result of pre-selective mutations from sensitive (G^s) to the glyphosate resistant (G^r) cells.

Materials and methods

Experimental organism and culture conditions

Two strains of *Microcystis aeruginosa* (Kützing) Lemmermann (MaD3 and MaD7) from the Algal Culture Collection of the Universidad Complutense (Madrid), were grown axenically in 100 ml cell culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 ml of BG-11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany), at 20°C under continuous light of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm. Both strains were isolated from pristine ponds in Doñana National Park (SW Spain), where herbicides have never been used. Strains were maintained in mid-log exponential growth by serial transfers of a cell inoculum to fresh medium (details in Carrillo et al. 2003). Prior to the experiments, the cultures were re-cloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the cultures. Cultures were maintained as axenic as possible, and only cultures without detectable bacteria were used in the experiments.

Fig. 1 Schematic diagram of the experiment modified from the classic Luria-Delbrück fluctuation analysis, and the possible results. In the set 1 experiment, different cultures (each started from a small inoculum, $N_0 = 10^2$ cells) were propagated under non-selective conditions until a very high cell density ($N_t = 2.3 \times 10^5$ cells) was reached, and then supplemented with a lethal dose of the selective agent (120 ppm glyphosate). Set 1A: physiological adaptation (i.e., acclimation) or possible adaptive mutations. In this case, the number of resistant cells in all the cultures must be similar. Set 1B: adaptation by mutations occurring in the period of the propagation of cultures, i.e., before exposure to the selective agent. One mutational event occurred late in the propagation of culture 1 (therefore, the density of glyphosate-resistant cells found is low) and early in the propagation of culture 3 (thus, density of glyphosate-resistant cells found is higher than in culture 1); no mutational events occurred in culture 2. Therefore, the variance/mean ratio of the number of resistant cells per replicate must be >1. Set 2 samples the variance of parental populations (the distribution should be Poisson, with a variance ≈ mean) ▶

Toxicity test: effect of glyphosate on growth rate

The toxic effect of glyphosate on growth rate of the two wild-type strains was assessed as follows: a stock solution of glyphosate acid, N-(phosphonomethyl) glycine (Sigma-Aldrich Chemie, Taufkirchen, Germany) was prepared in BG–11 medium to obtain serial dilutions of 0, 10, 30, 60, and 110 ppm. Each experimental culture was inoculated with 5×10^6 cells from mid-log exponentially growing cultures. Four replicates of each concentration of glyphosate, as well as four unexposed controls, were prepared. The effect of the herbicide was estimated by calculating acclimated maximal growth rate (m) in mid-log exponentially growing cells, derived from the equation:

$$N_t = N_0 e^{mt}, \tag{1}$$

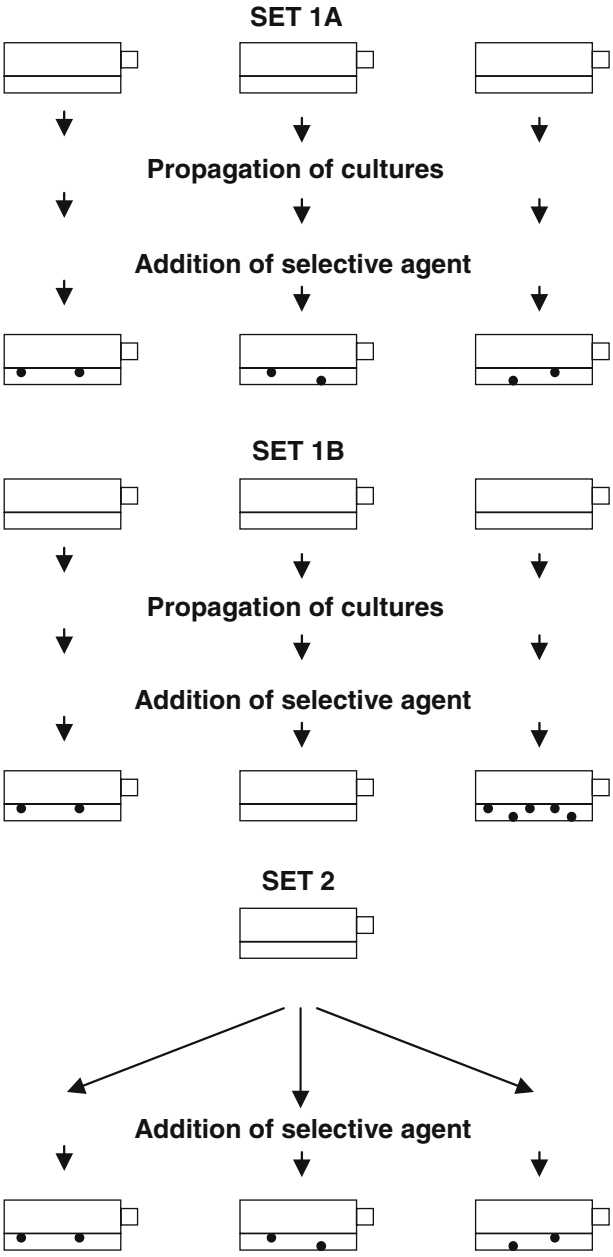
where $t = 7$ days, and N_t and N_0 are the cell numbers at the end and at the start of the experiment, respectively. Therefore, m was calculated as:

$$m = \mathrm{Log_e}\,(N_t/N_0)/7 \tag{2}$$

Acclimated maximal growth rate is the Malthusian parameter of fitness under conditions of r selection (Crow and Kimura 1970; Spiess 1989). Experimentals and controls were counted blind (i.e., the person counting the test did not know the identity of the tested sample), using a haemocytometer and an inverted microscope (Axiovert 35, Zeiss, Oberkóchen, Germany). The number of samples in each case was determined using the progressive mean procedure (Williams 1977), which assured a counting error <5%.

Fluctuation analysis of $G^s \rightarrow G^r$ transformation

A modified Luria–Delbrück analysis was performed as previously described (López-Rodas et al. 2001) to distinguish resistant cells that had their origin in random spontaneous pre-selective mutations (prior to glyphosate exposure) from those arising through acquired post-selective adaptation (during the exposure to glyphosate) (Fig. 1). The modification of the analysis involves the use of liquid medium containing the selective agent rather than plating on a solid medium, as was done by Luria and Delbrück (1943) with bacterial cultures. In short, two different sets of experimental cultures were prepared with both strains of *M. aeruginosa*. In the set 1 experiment, 100 culture flasks were inoculated with $N_0 = 10^2$ cells (a number small



enough to reasonably ensure the absence of pre-existing mutants in the strain). Cultures were allowed to grow until $N_t = 2.3 \times 10^5$ cells and then were supplemented with 120 ppm glyphosate acid. For the set 2 control, 45 aliquots of 2.3×10^5 cells from the same parental population were separately transferred to culture flasks containing fresh liquid medium with 120 ppm glyphosate acid. Cultures were observed for 60 days (thereby insuring that one mutant cell could generate enough

progeny to be detected), 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.

According to Luria and Delbrück (1943), two different results can be found in the set 1 experiment when conducting a fluctuation analysis, each of them being interpreted as the independent consequence of two different phenomena of adaptation. In the first case (Fig. 1, set 1A), the variance in the number of cells per culture could be found to be low if resistant cells arose by physiological adaptation or specific post-selective mutations. Because every cell is likely to had the same chance of developing resistance, interculture (flask-to-flask) variation would be consistent with the Poisson model. By contrast, if high variation in the interculture number of resistant cells is found (i.e., variance/mean > 1), it means that resistant cells appeared by random pre-selective mutations occurring before selection, and the flask-to-flask variation would not be consistent with the Poisson model. That is to say, they occurred during the time in which the cultures reached N_t from N_0 cells, before the exposure to glyphosate (Fig. 1, set 1B).

In the set 2 cultures (Fig. 1), if resistants arose by pre-selective mutations, variance is expected to be low, because set 2 samples the variance of the parental population. Thus, despite the way resistants appear, interculture variance of resistants in set 2 should be similar to the average of resistants in set 2 cultures. Because this set is the experimental control of the fluctuation analysis, if a similar variance/mean ratio between set 1 and set 2 is found, it confirms that resistant cells appeared by acclimation or post-selective mutations, rather than by pre-selective mutations.

In addition, the fluctuation analysis allows estimation of the rate of appearance of resistant cells. There are different approaches for accomplishing this estimation (Rosche and Foster 2000). Due to the methodological limitations imposed by a fluctuation analysis using liquid cultures, the proportion of set 1 cultures showing no mutant cells after glyphosate exposure (P_0 estimator) was the parameter used to calculate the mutation rate (μ). The P_0 estimator (Luria and Delbrück 1943) is defined as follows:

$$P_0 = e^{-\mu(N_t - N_0)}, \tag{3}$$

where P_0 is the proportion of cultures showing no resistant cells. Therefore, μ was calculated as:

$$\mu = -\mathrm{Log}_e P_0/(N_t - N_0) \tag{4}$$

Reliability, reproducibility and precision of the procedure for estimating μ were determined later (British Standards Institute 1979; Thrusfield 1995). Reliability was determined based on the agreement among three iterations of the experiments; reproducibility was determined as the agreement among three sets of observations made on the same experiment by three different observers; finally, precision was calculated as the minimum variation in μ that can be detected using the procedure.

Mutation-selection equilibrium

If the $G^s \rightarrow G^r$ mutation from a normal wild-type, glyphosate-sensitive allele to a glyphosate-resistant allele is recurrent, and the glyphosate-resistant allele is

detrimental in fitness in the absence of the herbicide, then 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; Spiess 1989). At any one time there will be a certain number of cells that are not yet eliminated. The average number of such mutants will be determined by the balance between μ and the rate of selective elimination, in accordance with the equation:

$$q = (\mu/s)^{1/2}, \tag{5}$$

where q is the frequency of the glyphosate-resistant allele and s is the coefficient of selection (Ayala and Kiger 1980), calculated as follows:

$$s = 1 - (m_{G^r}/m_{G^s}), \tag{6}$$

where m_{G^r} and m_{G^s} are the Malthusian fitness of G^r and G^s cells measured in non-selective conditions, respectively (Crow and Kimura 1970).

Analysis of cell morphology of G^s and G^r variants

Maximum and minimum diameters, area, and perimeter of 100 cells of both G^s and G^r variants, from the MaD3 and MaD7 strains of *M. aeruginosa*, were measured directly using an image analysis system (Motic Digital Imaging 3.5, Motic, Xiamen, PRC). As a measure of the shape of the cells, the coefficient of form (CF) proposed by Renau-Piqueras et al. (1985) was calculated:

$$\mathrm{CF} = (4\pi \mathrm{A})/\mathrm{P}^2, \tag{7}$$

where A is the area and P the perimeter of the outline of the cell. According to the formulae of the area of a circle and its circumference, a CF = 1 is derived; identically, the area and the perimeter of an ellipse with semi-axes of 1 and 0.5, respectively, yield a CF = 0.8. More details are given in Goyanes et al. (1990) and Rico et al. (2006).

Results

Glyphosate-sensitive cells from both strains MaD3 and MaD7 showed similar growth rates ($m \approx 1$ doubling d^{-1}) (Fig. 2). The exposure to glyphosate had an analogous toxic effect in both clones: concentrations from 10 to 60 ppm induced a drastic decrease of fitness, and at a concentration of 110 ppm growth was totally inhibited (Fig. 2).

A high fluctuation in set 1 experiments (from 0 to more than 10^4 resistant cells per culture flask) was found in both strains of *M. aeruginosa* (Table 1). The fluctuation observed is not a consequence of experimental error in sampling G^r cells because the analyses of set 2 showed that in all cultures the number of G^r cells per flask was less than 10^3 in both clones (Table 1).

Mutation rates for $G^s \rightarrow G^r$ (estimated with high standards of reliability, reproducibility, and precision, see Table 2) were 3.6×10^{-7} and 3.1×10^{-7} in strains Ma3D and Ma7D, respectively (Table 1).

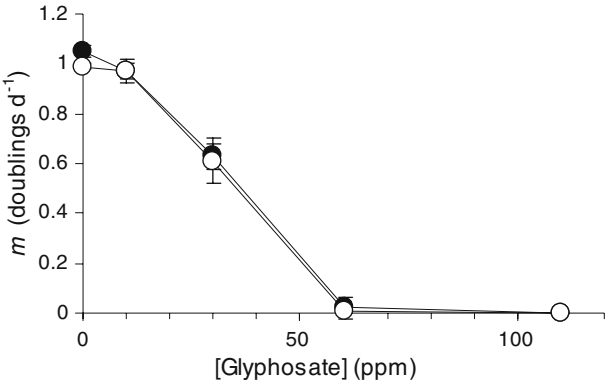


Fig. 2 Effect of glyphosate on acclimated growth rate (m; mean ± SD, $n = 4$) of *Microcystis aeruginosa* wild-type, glyphosate-sensitive Ma3D (open circles) and Ma7D (filled circles) strains

Table 1 Fluctuation analysis of G^r variants in *Microcystis aeruginosa* wild-type strains Ma3D and Ma7D

	Strain Ma3D		Strain Ma7D	
	Set 1	Set 2	Set 1	Set 2
No. of replicate cultures	100	45	100	45
No. of cultures containing the following no. of G^r cells:				
0	92	0	93	0
1–10^3	4	45	3	45
10^3–10^4	3	0	2	0
>10^4	1	0	2	0
Variance/mean (of the no. of G^r cells per replicate)	84.1	0.9	87.2	1.1
μ (mutants per cell per generation)	3.6×10^{-7}		3.1×10^{-7}	

Table 2 Reliability, reproducibility and precision of the procedure to estimate mutation rate (μ) of $G^s \rightarrow G^r$ in two strains of *Microcystis aeruginosa*

	Strain Ma3D	Strain Ma7D
Reliability of μ (%)	90	92
Reproducibility of P_0 (%)	99	97
Precision of μ (mutants per cell per generation)	0.5×10^{-7}	0.5×10^{-7}

Isolated G^r mutants growing in the absence of the selective agent, i.e., without glyphosate in the culture medium, showed growth rates only one-sixth of those found in G^s cells (Fig. 3).

The m_{G^s} and m_{G^r} values were used to compute the coefficient of selection of G^r mutants ($s = 0.84$ in strain Ma3D, and 0.82 in strain Ma7D, respectively). By using the previous values of μ and s, the frequency of glyphosate-resistant alleles was calculated; the value of q ranged from 6.14×10^{-4} in strain Ma3D, to 6.54×10^{-4} in strain Ma7D.

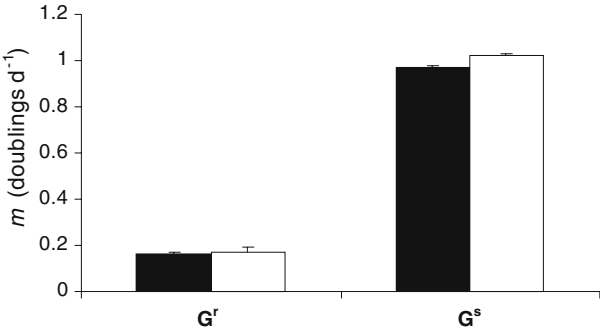


Fig. 3 Acclimated growth rate (m) of the Ma3D (open bars) and Ma7D (filled bars) strains of *Microcystis aeruginosa* wild-type, glyphosate-sensitive (G^s) and glyphosate resistant (G^r) variants. Vertical lines on bars shown SD ($n = 4$)

Table 3 Diameters and coefficient of form (CF) of G^s and G^r cells in two strains of *Microcystis aeruginosa*. Comparisons between G^s and G^r variants were carried out by Student's t-test: in all the comparisons, differences were significant at $P < 0.001$. Values are represented as overall mean ± SD ($n = 100$)

	Strain Ma3D		Strain Ma7D	
	G^s	G^r	G^s	G^r
Maximum diameter (μm)	2.40 ± 0.32	2.01 ± 0.40	2.73 ± 0.41	2.32 ± 0.36
Minimum diameter (μm)	2.35 ± 0.32	1.80 ± 0.36	2.42 ± 0.35	2.01 ± 0.41
CF (dimensionless)	1.00 ± 0.01	0.96 ± 0.01	0.99 ± 0.01	0.90 ± 0.02

Glyphosate-sensitive cells from both strains exhibited significantly greater size (t-test, $P < 0.001$, $n = 100$) than the G^r cells (Table 3). The outline of *M. aeruginosa* G^s cells is circular, with an index of roundness CF ≈ 1 in both strains, corresponding to spherical cells (Table 3). However, the G^r variants showed a significantly (t-test, $P < 0.001$, $n = 100$) lower CF value, corresponding to slightly elliptical rather than spherical cells (Table 3).

Discussion

When *M. aeruginosa* cultures were treated with a lethal dose of glyphosate, they became clear after some days due to the destruction of the sensitive cells by the toxic effect of the herbicide. However, after further incubation, some cultures became colored again, due to the growth of cells that were resistant to the effect of glyphosate. The key to understanding adaptation of cyanobacteria to survive in a glyphosate-contaminated environment seems likely to lie in characterizing the resistant cells that appear after the massive destruction of the sensitive cells. Fluctuation analysis is the appropriate procedure to discriminate between glyphosate-resistant cells arising by rare spontaneous mutations occurring randomly during propagation of organisms under nonselective conditions (i.e., prior to exposure to glyphosate) and glyphosate-resistant cells arising through acclimation or adaptive mutation in response to selection (Luria and Delbrück 1943; reviewed by Sniegowski 2005). The large fluctuation in number of

glyphosate-resistant cells observed in set 1 experiments, in contrast with the scant variation in set 2 controls, unequivocally demonstrates that resistant cells arose by rare spontaneous mutation and not through direct and specific arousal of adaptive mutations in response to glyphosate exposure. The herbicide did not stimulate the appearance of resistant cells at all. The rapid lethal effect of glyphosate seems unlikely to allow the appearance of adaptive mutations. Adaptation of cyanobacteria and algae, which are the principal primary producers of aquatic ecosystems, to environmental changes resulting from anthropogenic contamination (or even to extreme natural environments) seems to be the result of a rare event: the spontaneous mutation from sensitivity to resistance that occurs randomly prior to the cells coming into contact with the selective agent (Costas et al. 2001; López-Rodas et al. 2001; Baos et al. 2002; García-Villada et al. 2002, 2004; Flores-Moya et al. 2005).

The rate of mutation from $G^s \rightarrow G^r$ (from 3.1×10^{-7} to 3.6×10^{-7} mutants per cell per generation) was one to two orders of magnitude lower than the mutation rates we have described (from 2.12×10^{-5} to 1.76×10^{-6} mutants per cell per generation) for the resistance to several biocides in other cyanobacterial and microalgal species (Costas et al. 2001; López-Rodas et al. 2001; Baos et al. 2002; García-Villada et al., 2002, 2004), but of the same order of magnitude found for the resistance to sulphureous waters in the chlorophycean *Spirogyra insignis* (2.7×10^{-7} mutants per cell per generation) (Flores-Moya et al. 2005). Nevertheless, the pre-selective $G^s \rightarrow G^r$ mutations are sufficiently frequent in *M. aeruginosa* populations to allow them to adapt to the presence of glyphosate in culture. The presence of G^r cells in the populations of *M. aeruginosa* is regulated by the recurrent appearance of mutants and their elimination by selection, yielding an equilibrium frequency of 6–7 G^r cells per 10^4 cell divisions. This fraction of resistant mutants is presumably enough to assure the adaptation of cyanobacterial populations to catastrophic water contamination, since the natural populations of cyanobacteria are composed of countless cells. Nevertheless, mutations usually imply an energetic cost that may affect the survival of adapting populations (Coustau et al. 2000), as was demonstrated by a growth rate in G^r cells only one-sixth of that in G^s ones, in the absence of the herbicide. Thus, under a scenario of global change caused by human activies (including the appearance of biocides in ecosystems), it could be hypothesized that G^r cells from *M. aeruginosa* could develop in freshwater ecosystems polluted with glyphosate, but their contribution to primary production will be significantly lower than that occurring in pristine ecosystems with G^s cells.

Finally, in a glyphosate-contamined environment the populations could be formed of cells of smaller size and a slightly different morphology from the typical spherical cells of *M. aeruginosa* (Whitton 2002) since G^r cells showed a CF 5% lower than that of a circular outline associated with a spherical volume. It can be supposed that the mechanism linked to resistance to glyphosate is a pleiotropic gene that is also implicated in the arrangement of microtubular-protein analogues in the cells, but this point remains to be investigated. In freshwater systems located in urban or agricultural areas, cyanobacteria and microalgae are exposed to a multitude of toxicologically different biocides (Junghans et al. 2006). Therefore, it could be hypothesized that the appearance of resistant mutants can originate, simultaneously, the rise of new morphological populations driven by algicide-resistant clones.

However, this aspect remains to be investigated by using more biocides and cyanobacteria and algal species.

The origin of favoured mutants and the process of adaptation can be only achieved if appropiate genetic variability is available (Bradshaw and Hardwick 1989). In a series of preliminary studies analysing the genetic variability in populations of *M. aeruginosa*, significant variability was found for morphological and physiological (fitness and photosynthetic performance) traits, and genetic factors contributed 50–90% of the observed phenotypic variability (Bañares-España et al. 2006; López-Rodas et al. 2006; Rico et al. 2006). This kind of complementary approach can also cast some light on the ability of cyanobacteria to adapt to environmental changes, such as water pollution by herbicides, in recent years. Since *M. aeruginosa* is known to be the most important cause of toxic blooms in inland water systems (reviewed in Skulberg et al. 1993), the occurrence of herbicide-resistant cells could also be of interest to water management.

In conclusion, spontaneous pre-selective mutants, like 'hopeful monsters', are enough to assure the adaptation of cyanobacterial populations to catastrophic environmental changes.

Acknowledgements This work was financially supported by REN 2000-0771 HID, REN 2001-1211 HID, Parques Nacionales 093/2003, P05-RNM-00935 and DOÑANA-2005 grants. Dr. Eric C. Henry (Herbarium, Department of Botany and Plant Pathology, Oregon State University, USA) kindly revised the English style and usage.

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