DR. ELENA MARTIN-CLEMENTE (Orcid ID : 0000-0002-0365-6377)

Article type : Regular Article

Adaptation dynamics and evolutionary rescue under sulfide selection in cyanobacteria: a comparative study between *Microcystis aeruginosa* and *Oscillatoria* sp. (cyanobacteria)[1]

Elena Martín-Clemente[2], *Ignacio J. Melero-Jiménez, Elena Bañares-España*

Antonio Flores-Moya and María J. García-Sánchez

Departamento de Botánica y Fisiología Vegetal, Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos s/n, E-29071 Málaga, Spain

[2] Author for correspondence: e-mail; elena.mc@uma.es; phone: +34 952134220

Condensed running title: Cyanobacteria and sulfide

Editorial Responsibility: B. Palenik (Associate Editor)

ABSTRACT

Experimental evolution studies using cyanobacteria as model organisms are scarce despite their importance in the evolution of photosynthetic organisms. For the first time three different experimental evolutionary approaches have been applied to shed light on the sulfide adaptation process, which played a key role in the evolution of this group. With this purpose, we used a *Microcystis aeruginosa* sulfide-sensitive strain, unable to grow above ~0.1 mM, and an *Oscillatoria* sp. strain, isolated from a sulfureous spa (~0.2 mM total sulfide). Firstly, performing a fluctuation analysis design using the spa waters as selective agent, we proved

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jpy.12911
This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

that *M. aeruginosa* was able to adapt to this sulfide level by rare spontaneous mutations. Secondly, applying a ratchet protocol, we tested if the limit of adaptation to sulfide of the two taxa was dependent on their initial sulfide tolerance, finding that *M. aeruginosa* adapted to 0.4 mM sulfide, and *Oscillatoria* sp. to ~2 mM sulfide, twice it highest tolerance level. Thirdly, using an evolutionary rescue approach, we observed that both speed of exposure to increasing sulfide concentrations (deterioration rate) and populations' genetic variation determined the survival of *M. aeruginosa* at lethal sulfide levels, with a higher dependence on genetic diversity. In conclusion, sulfide adaptation of cyanobacteria sensitive strains is possible by rare spontaneous mutations and the adaptation limits depend on the sulfide level present where strains inhabit. The high genetic diversity of a sulfide-sensitive strain, even at fast environmental deterioration rates, could increase its possibility of survival even to a severe sulfide stress.

Key index words: evolutionary rescue; fluctuation analysis; *Microcystis aeruginosa*; *Oscillatoria* sp.; ratchet protocol; sulfide

Abbreviations: A_{750}, absorbance at $\lambda = 750$ nm**;** *m*, acclimated growth rate**;** CD, cell density; ER, evolutionary rescue**;** FM, fresh mass**;** LHW, La Hedionda waters**;** P_0, first term of the Poisson distribution; *q*, frequency of the LHW-resistant allele; *s*, coefficient of selection; μ, mutation rate

This article is protected by copyright. All rights reserved.

Accepted Article

INTRODUCTION

Acclimation is the mechanism allowing tolerance at low levels of selective pressure, but when the limit of acclimation is exceeded, survival is only possible by the selection of new mutations conferring resistance or genetic adaptation (Hughes 1999). Previous studies on the adaptation of photosynthetic microorganisms to extreme environments showed that in most cases survival was due to the selection of new genetic variants originated by mutation (Costas et al. 2007, 2008, López-Rodas et al. 2007, 2008a,b, 2009, 2011, Fernández-Arjona et al. 2013, Melero-Jiménez et al. 2019). Although these studies revealed that the coupled interplay of mutation and selection allowed organisms to surpass a given level of selection pressure, experimental assays evaluating the maximum capacity of an organism to adapt to a selective agent have not been reported until recently. In particular, it was found that the maximum adaptation capacity for the same selective agent varied among different taxonomic and ecological groups of photosynthetic microorganisms, and that this capacity differed depending on the selective agent used (Huertas et al. 2010, 2011, Rouco et al. 2014, Melero-Jiménez et al. 2019). It must be taken into account that all the studies previously cited were performed with strains having a homogeneous eco-evolutionary history (i.e., all the experimental culture cells were founded from inocula having a similar history, and they were exposed to a similar selection pressure). However, it is known that evolutionary processes are contingent, closely dependent on the previous history (Gould 1989, Travisano et al. 1995, Blount et al. 2008, Flores-Moya et al. 2008, 2012). Thus, the possibility that natural populations exposed to lethal conditions may adapt through natural selection, a process known as 'evolutionary rescue' (ER), could depend on the previous eco-evolutionary history (Gonzalez et al. 2013). In fact, in experimental populations of bacteria and yeasts, it has been found that greater genetic variation favors the possibility of ER events and that the rate at which the selective pressure increases is inversely correlated with the capacity to proliferate

This article is protected by copyright. All rights reserved.

under lethal conditions (Bell and Gonzalez 2011, Lindsey et al. 2013). Moreover, it should be noted that, in the recent field of research based on the use microorganisms to study evolution in action, most of them have been performed with viruses, bacteria and yeasts (Elena and Lenski 2003, Garland and Rose 2009). However, the unique characteristics of cyanobacteria make them ideal model organisms in experimental evolution, especially if a selective agent with eco-evolutionary implications is chosen, such as sulfide in cyanobacteria.

Sulfidic conditions played a key role in the history of life and, in particular, in the evolution of the cyanobacteria (Meyer and Kump 2008, Scott et al. 2008, Poulton et al. 2010, Poulton and Canfield 2011), because sulfide inhibits oxygenic photosynthetic and respiratory electron transport (Bagarinao 1992, Stal 1995, 2012). Cyanobacteria developed the ability to tolerate a variety of sulfide environments throughout their evolutionary history (Cohen et al. 1975a,b, Padan 1979, de Wit and van Germeden 1987, García-Pichel and Castenholz 1990, Klatt et al. 2015). It is known that strains isolated from environments where sulfide is not present are extremely sensitive to very low levels (<0.1 mM) of this ion (Cohen et al. 1986) whereas those isolated from sulfureous habitats exhibit one or more different adaptations to overcome the toxic effect of sulfide. These adaptations maintain oxygenic photosynthesis by the resistance of PSII (Castenholz 1977, Cohen et al. 1986, Dodds and Castenholz 1990, Miller and Bebout 2004) or enable PSII-independent, anoxygenic photosynthesis with sulfide as an electron donor to PSI (Cohen et al. 1975a,b, 1986, Hamilton et al. 2016, Leister 2017, Walter et al. 2017). Also, it is known that some cyanobacteria can detoxify sulfide by oxidation to sulfur (Stal 1995), and a channel has been described in the eubacterium *Clostridium* that specifically exports HS^- from the cytosol (Czyzewski and Wang 2012). However, sulfide-tolerance in cyanobacteria seems not to be a taxon-dependent trait, rather it may be gained or lost relatively rapidly in response to variation in sulfide levels and it is not constrained by phylogeny (Miller and Bebout 2004).

This article is protected by copyright. All rights reserved.

Accepted Article

Recently, a sulfide-resistant mutant of the cyanobacterium *Microcystis aeruginosa* that proliferates in sulfureous waters containing 0.24 mM sulfide, was obtained by genetic adaptation from a wild-type, sulfide-sensitive strain isolated from non-sulfureous waters (Fernandez-Arjona et al. 2013). This sulfide-resistant strain of *M. aeruginosa* showed lower growth rates than wild-type cells in a sulfide-free medium, as well as decreased maximum quantum yield and photosynthetic efficiency in comparison to wild-type cells (Fernandez-Arjona et al. 2013, Bañares-España et al. 2016). This finding raises the following question: what is the limit of adaptation that a given taxon of cyanobacteria can achieve under increased sulfide levels? It could be hypothesized that the potential adaptation will correlate with the sulfide levels observed where the isolate was found. For instance, *M. aeruginosa* could be able to adapt (by the mutation-selection process) to levels of sulfide lower than sulfide-tolerant cyanobacterial taxa would, such as some strains of *Oscillatoria* sp. (Cohen et al. 1975a, Castenholz 1977). Moreover, it is also relevant to know how the previous eco-evolutionary history of the sulfide-sensitive cyanobacteria could modulate the ER dynamics allowing the survival to originally lethal sulfide levels.

Therefore, the aim of this work was to determine the limits of the potential adaptation to increased sulfide levels of a *Microcystis aeruginosa* strain, isolated from non-sulfureous waters, and an *Oscillatoria* sp. strain, isolated from the sulfureous La Hedionda spa. Additionally, we studied how the dispersal of original populations (a proxy for genetic diversity) and the rate of increase of sulfide levels modulate the ER dynamics in *M. aeruginosa*. For these purposes, we performed three types of evolutionary experiments. The first one was a fluctuation analysis with *M. aeruginosa,* which let us to disentangle the mechanisms (i.e., acclimation vs. genetic adaptation), allowing the tolerance of this species to the sulfide levels present at La Hedionda spa. On the other hand, a modified ratchet experiment, in which sulfide levels increased at each ratchet cycle, was applied in order to

This article is protected by copyright. All rights reserved.

Accepted Article

detect the maximum adaptation that could be achieved by both cyanobacteria. Finally, we performed an ER experiment by submitting populations of *M. aeruginosa* to different rates of increase of sulfide levels, as well as to different dispersal mechanisms, in order to understand the role of the genetic diversity and the deterioration rate, on the natural selection process allowing the survival of this cyanobacterium under lethal sulfidic conditions.

MATERIAL AND METHODS

Sample collection and sulfide concentration determination at La Hedionda spa.

La Hedionda spa is located on the Manilva River bank (Málaga, Spain) and it has two sectors, one external, which is the river itself, and one internal, walled and roofed for bathing. Samples (microorganisms and water) used in the experiments were collected from the interior area, where microbial mats cover the walls.

Considering the annual mean pH value (7.23 ± 0.06, n = 20) found in La Hedionda waters (LHW) and according to the pK_{a1} of H_2S (close to 7.0), H_2S and HS^- species are at a proportion of around 35% and 65% respectively. To measure total sulfide (H_2S and HS^-) concentration in situ, a DR900 Multiparameter Portable Colorimeter (Hatch Co., Loveland, CO, USA) was used, based on the methylene blue method. Sulfide determinations were conducted in triplicate, with a value of 109 µM (CV<3%) at the time of mat sample collection. Samples of LHW were also collected weekly during June and July 2016 for the fluctuation analysis experiment. Samples were stored in plastic bottles (1.5 L) that were tightly sealed without air bubbles, in the dark at 4 °C before use. Total sulfide concentrations in LHW ranged from 97-207 µM along the six weeks of collection with a mean value of 147 ± 36 µM ($n = 6$).

This article is protected by copyright. All rights reserved.

Accepted Article

Experimental organisms and culture conditions.

Experiments were performed with the wild-type strain Ma1Vc of the cyanobacterium *Microcystis aeruginosa* and an *Oscillatoria* sp. strain isolated from mats from La Hedionda spa. The Ma1Vc strain was provided by the Veterinary School Algal Culture Collection, Complutense University (Madrid, Spain) and was isolated from non-sulfureous waters from the Valmayor reservoir (Madrid, Spain). *Oscillatoria* sp. was isolated following a successive dilution process. For this purpose, samples of microbial mats were collected in June of 2016. The mat sample was diluted and gently homogenized in 900 mL of BG11 medium (Sigma-Aldrich Chemie, Taufkirchen, Germany), diluted at 50% (BG11-50%). The strain isolated was named O1LH.

Both *Microcystis aeruginosa* and *Oscillatoria* sp. cultures were grown axenically in 50 mL ventilated cell-culture flasks (Greiner; Bio-One, Longwood, NJ, USA) with 20 mL of culture medium (or in 250 mL culture flasks, with 100 mL of growth medium). Cultures were axenically maintained in mid-log exponential growth by weekly transferring inoculum to fresh growth medium. Cultures were incubated at 20°C under a continuous photon flux density of 50 μmol · m^{-2} · s^{-1} over the waveband 400–700 nm, provided by cool-white fluorescent lamps (Sylvania GRO-LUX, F36W/GRO, Feilo Sylvania, Spain). The culture medium of *M. aeruginosa* was BG11-50%. The culture medium of *Oscillatoria* sp. was BG11-50% buffered with 5 mM HEPES (pH 7.2), and sulfide was added every day at a final concentration of 200 μM, from a Na_2S aqueous NaOH master stock solution (pH 13, concentrations ranging 21-24 mM). In this way, sulfide concentrations in the culture (see below) were maintained as close as possible to the highest sulfide concentrations observed in LHW.

This article is protected by copyright. All rights reserved.

Accepted Article

Toxicity test: effect of sulfide on growth rate.

In order to test the toxic effect of sulfide on cells of *Microcystis aeruginosa*, changes in acclimated growth rate (m) were measured in mid-log exponentially growing cultures (Crow and Kimura 1970):

$$m = \log_e (N_t/N_0)/\ t \qquad \text{(Eq. 1)}$$

where N_t and N_0 are the cell number at the end (after $t = 5$ d) and at the start of the experiment, respectively. Samples of *M. aeruginosa* with an initial cell density of 2.5×10^5 cells·mL^{-1} were placed in 15 mL tubes (Falcon™, BD Biosciences, San Jose, CA, USA) containing 4 mL of BG11-50% buffered with 20 mM HEPES (pH 7.2), and increasing sulfide concentrations (0, 0.05, 0.1, 0.15, 0.2 and 0.35 mM). Five replicates were prepared for each sulfide concentration and were maintained for 5 d at the same culture conditions as indicated above. Sulfide was added to the medium every day from a Na_2S-aqueous NaOH master stock solution (pH ~13, 21-24 mM) to maintain sulfide concentrations as close as possible to the concentration tested. The sulfide added to the cultures disappeared along the day due to redox reactions and H_2S volatility, being the half-life ($t_{1/2}$) of total sulfide around 1 h when the initial concentration was 0.35 mM. However, it is known that sulfide downstream biological effects can persist for hours (Olson and Straub 2016). On the other hand, Na_2S additions did not change significantly the medium pH after 5 d of incubation due to the buffer concentration used.

The number of cells of *Microcystis aeruginosa* was estimated at the start and the end of the incubation by linear regression fit between cell density (CD; units in 1×10^6 cells · mL^{-1}) and absorbance at $\lambda = 750$ nm (A_{750}):

This article is protected by copyright. All rights reserved.

$$CD = 1.1\times10^7 \times A_{750}\ (R^2 = 0.980,\ n = 18) \qquad \text{(Eq. 2)}$$

It must be highlighted that, under culture conditions, the experimental *M. aeruginosa* strain grew as single cells and did not produce coenobia or colonies, as occurs in nature.

Due to the filamentous nature of *Oscillatoria* sp. absorbance could not be used as an estimator of the number of cells. Instead, the biomass present in the cultures was estimated by chlorophyll *a* (Chl *a*) content. A relationship was established between the fresh mass (FM, mg) of the sample and the amount of Chl *a* (μg):

$$\text{Chl } a = 0.19\ \text{FM} + 1\ (R^2 = 0.982,\ n = 5) \qquad \text{(Eq. 3)}$$

Then, the FM at the start (FM_0) and after t = 5 d (FM_t) values were used in the Eq. 1, instead of N_0 and N_t. For the estimation of the FM, cultures were centrifuged at 8000 rpm for 8 min and the pellet weighed. The Chl *a* concentration was determined according to Wellburn (1994), using N, N-dimethylformamide as a solvent. Samples of *Oscillatoria* sp. were prepared in 50 mL tubes (Falcon™) containing 20 mL of BG11-50% buffered with 20 mM HEPES (pH 7.2) and with increasing sulfide concentrations (0, 0.05, 0.1, 0.15, 0.2, 0.35, 0.5, 0.7, 0.9, 1.2 and 1.5 mM). The initial biomass in each tube (four replicates for treatment) was 0.05 mg FM · mL^{-1}. This value was chosen because its equivalent Chl *a* content was similar to the Chl *a* present in *M. aeruginosa* samples at the initial cell density used in the toxicity test. Sulfide was added to the medium every day from a Na_2S-aqueous NaOH master stock solution (pH ~13, 210-240 mM), as indicated before ($t_{1/2}$ for total sulfide at 1.5 mM initial concentration was around 1.6 h). However, a slight increase on pH could be detected in the last days of incubation at the highest sulfide concentrations tested which could partially decrease sulfide toxicity due to the decrement on the hydrogen sulfide form (Howsley and

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Pearson 1979). Nevertheless, the toxic effect of the $^{-}$HS form has also been described (Olson and Straub 2016). After 5 d of culture in the presence of sulfide, samples were centrifuged at 8000 rpm for 8 min, and the pellet was frozen for Chl *a* determination.

Fluctuation analysis of LHW-sensitivity to LHW-resistance transformation in Microcystis aeruginosa.

In order to distinguish between genetic adaptation (i.e., spontaneous preselective mutations) and acclimation to the selective agent (LHW) of the Ma1Vc *Microcystis aeruginosa* strain, a modified fluctuation analysis initially described by Luria and Delbrück (1943) was performed. The modification allowed carrying out the experiment with liquid cultures instead of solid medium. The running of the experiment and the interpretation of the possible results (Fig. 1), have been extensively detailed in our previous studies (Costas et al. 2001, 2007, López-Rodas et al. 2001, 2007, 2008a,b, 2009, 2011, García-Villada et al. 2002, 2004, Flores-Moya et al. 2005, Marvá et al. 2010, Fernández-Arjona et al.2013, Melero-Jiménez et al. 2019). In short, two different sets of experimental cultures were prepared (Fig. 1). Set 1 consisted of 90 independent cultures inoculated with $N_0 = 1\times10^3$ cells (a number small enough to make it likely that no pre-existing mutants were present). Cultures were prepared in 13 mL reagent and centrifuge tubes, sealed with a cap (Sarstedt AG and Co, Nümbretcht, Germany). Cells were grown in 2 mL BG11-50% (non-selective conditions) until $N_t = 1\times10^6$ cells. Then, cultures were centrifuged, the medium was decanted, and 2 mL of LHW was added to the cells in the pellet (selective conditions). Cultures were grown for 6 weeks, thereby insuring that one mutant cell could generate enough progeny to be detected. Set 2 consisted of 40 aliquots of 3×10^6 cells from the parental population, cultured in 2 mL of LHW for 3 weeks.

This article is protected by copyright. All rights reserved.

According to the daily rate of sulfide loss (which disappeared within one day of culture at the initial low micromolar sulfide concentration in LHW), the LHW in culture tubes was replaced every 3 d in order to maintain its toxicity as much as possible throughout the duration of the experiment. For this purpose, fresh LHW samples were collected from La Hedionda spa each week, as indicated before, and filtered before use with a 0.22 µm mixed cellulose ester filter (Merck Millipore Ltd., Merck KGaA, Darmstadt, Germany).

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 resistance to sulfide. If resistant cells arose by acclimation, the variance in the number of cells per culture would be low (Fig. 1) because every cell is likely to have the same chance of developing resistance. Therefore, the CV (SD×100/mean) of the number of resistant cells per tube must be relatively low. By contrast, if cells appeared by random, pre-selective mutations occurring before the exposure to sulfide, high variation in the inter-culture (tube-to-tube) number of resistant cells should be found (Fig. 1) and, consequently, the CV value should be relatively high. If a similar CV value is found in sets 1 and 2, this confirms that resistant cells appeared by acclimation (i.e., after the exposure to sulfide). By contrast, if the CV from set 1 is significantly higher than the CV in set 2, it means that resistant cells arose by spontaneous mutations prior to exposure to sulfide. Obviously, another result (0 resistant cells in each culture) could also be found, indicating that neither selection on spontaneous mutations that occur prior to sulfide exposure nor acclimation during the exposure to sulfide took place.

In addition, the fluctuation analysis allows the estimation of the mutation rate (μ) of appearance of resistant cells when genetic adaptation is detected. The parameter P_0 (i.e., the first term of the Poisson distribution) was computed as the proportion of set 1 cultures showing no mutant cells after LHW exposure and μ was calculated as:

This article is protected by copyright. All rights reserved.

Accepted Article

$$\mu = -\log_e P_0 / (N_t - N_0) \quad \text{(Eq. 4)}$$

where N_0 and N_t are the number of cells at the start and at the end of the propagation period (i.e., before the addition LHW), respectively (see Fig. 1). Cell counting was performed using a hematocytometer, because the presence of dead cells interfered in the estimation of cell density by absorbance.

Mutation–selection equilibrium.

If the mutation from a normal wild-type LHW-sensitive allele to a LHW-resistant allele is recurrent, new mutants arise in each generation which will be outcompeted by the wild type cells due to their lower fitness in the absence of LHW. The average number of such mutants will be determined by the balance between μ and the rate of selective eliminations (s), in accordance with Kimura and Maruyama (1966):

$$q = \mu / (\mu + s) \quad \text{(Eq. 5)}$$

where q is the frequency of the LHW-resistant allele, and s is the coefficient of selection against this resistant allele, calculated as follows:

$$s = 1 - (m^r / m^s) \quad \text{(Eq. 6)}$$

where m^r and m^s are the acclimated growth rates of the LHW-resistant and the LHW-sensitive cells measured in non-selective culture medium (i.e., BG11-50%), respectively. Four independent isolates of both LHW-resistant and the LHW-sensitive strains were used to

Accepted Article

This article is protected by copyright. All rights reserved.

compute the m^r and m^s values, as indicated in Eq. 1. Isolates of the LHW-sensitive cells were founded from the original strain Ma1Vc. On the other hand, the LHW-resistant inoculum, isolated from one randomly selected culture of the set 1 after running the fluctuation analysis (1–1.5 $\times 10^6$ resistant cells per culture), was used to prepare the LHW-resistant cultures of *M. aeruginosa*.

Ratchet experiment with Microcystis aeruginosa *and* Oscillatoria *sp.*

To study the adaptation of *Microcystis aeruginosa* and *Oscillatoria* sp. to the exposure to increasing concentrations of sulfide and to estimate the maximum sulfide concentration to which each strain was able to adapt, a modified ratchet protocol (Huertas et al. 2010, Rouco et al. 2014, Melero-Jiménez et al. 2019) was carried out. A requisite in this experimental design is to balance a strong selection pressure and a population size large enough to ensure the occurrence of new mutations that confer resistance. With this objective, experimental cultures were inoculated with an elevated cell density and were exposed to three different selection levels (i.e., three sulfide concentrations) in each ratchet cycle (Fig. 2).

Four replicates were prepared for each one of the sulfide doses tested. It must be highlighted that each replicate is an independent population, and they could be ratcheted to the next cycle at different times once they reached the desired cell density. This means that each independent population has a different random chance to experience a mutation conferring sulfide resistance. The choice of the initial dose and the increase in sulfide concentrations for each ratchet cycle was different for each strain due to their difference in sulfide tolerance, as detailed below.

The ratchet experiment with *Microcystis aeruginosa* started with four independent populations, for each three initial sulfide doses (10, 30 and 90 µM sulfide) and four controls without sulfide (Fig. 2). The initial doses were low, because this strain was isolated from non-

This article is protected by copyright. All rights reserved.

sulfureous waters. Each population (replicate) was founded with 6×10^5 cells, which were incubated in 15 mL tubes (Falcon™) containing 4 mL culture medium. All culture media contained BG11-50% buffered with 20 mM HEPES, pH 7.2, and the corresponding sulfide addition. Culture tubes were closed with a cap and maintained under continuous irradiance of 50 μmol · m^{-2} · s^{-1}, at 20°C. Cultures were grown for at least 7 d at each sulfide dose. In order to maintain sulfide concentrations as close as possible to the desired level, sulfide was added every day to the culture medium from a Na_2S-aqueous NaOH master stock solution (pH~13, 21-24 mM) and culture medium was changed weekly to maintain a constant pH during the experiment. After 7 d of culture, cell concentrations in sulfide-enriched cultures were compared to those in control ones. If treated cultures showed a cell concentration similar to that observed in controls, they were transferred to the next ratchet cycle; however, if treated cultures showed a lower cell growth than controls, they remained another week at the same sulfide concentration (Fig. 2). Each ratchet cycle entailed a 3-fold increase in sulfide concentration at the beginning of the experiment and later a 1.5-fold increase. Changes in each ratchet cycle were as follows: from 10 to 30, from 30 to 90, from 90 to 270, from 270 to 405, and from 405 to 607 μM sulfide. At the starting point of each ratchet cycle, both control and treated tubes were inoculated with the same cell density used at the beginning of the ratchet experiment (i.e., 6×10^5 cells).

In the experiment with *Oscillatoria* sp. the first ratchet cycle was started with four populations for each three initial doses of sulfide (300, 400 and 500 μM) plus the control at 200 μM sulfide. Control sulfide concentration was the one used for the cultivation of *Oscillatoria* sp. and the increase in each ratchet cycle was lower than those used in the *Microcystis aeruginosa* ratchet cycles (100 μM each time). Tubes (50 mL volume, Falcon™) were inoculated initially with 5 mg FM in 20 mL BG11-50%, buffered with 20 mM HEPES (pH 7.2) and the corresponding sulfide addition, and were grown for at least 7 d. Changes in

This article is protected by copyright. All rights reserved.

each ratchet cycle were performed increasing by 100 µM the preexisting sulfide concentration. Daily sulfide additions and weekly renewal of culture medium were performed as for the *M. aeruginosa* ratchet experiment. However, at concentrations above 1.5 mM sulfide, at the end of the 7 d of incubation the medium pH showed an increase between 0.5 and 0.8 units (which was restored with the medium renewal) that could slightly mask the toxic effect of sulfide, as indicated before.

In both experiments, if the treated cultures reached a biomass similar to controls, they were transferred to the next ratchet cycle. Cultures with a lower biomass than controls were not transferred, and were analyzed again after the end of the next ratchet cycle period. A population was considered to have reached the maximum capacity of adaptation when no further cell growth was observed after 90 d of exposure to sulfide additions. Results were expressed in terms of days taken by each replicate under selective sulfide conditions to reach the same biomass as the control. Consequently, the number of days was culture-dependent, as growth was a function of their evolutionary potential to adapt to the selective conditions.

Evolutionary rescue in Microcystis aeruginosa *under sulfide selection: effect of dispersal and deterioration rate.*

Evolutionary rescue occurs when a population exposed to selective conditions survives and, consequently, is able to grow as well as before, or at even higher rates. In order to study the effects of the environmental deterioration and dispersal mode on the dynamics of ER under stress by sulfide, an experiment based on the design from Bell and González (2011) was performed with *Microcystis aeruginosa* (Fig. 3).

We imposed nine different conditions, consisting of combinations of three environmental deterioration speeds (control -no deterioration-, slow or fast deterioration rates, Fig. 3a) and three dispersal modes (control -no dispersal-, local or global dispersal; Fig.

This article is protected by copyright. All rights reserved.

Accepted Article

3b). Cultures under each treatment were subjected to four sulfide stress gradients (G), which included four increasing sulfide concentrations per gradient, increasing 30 μM each time: G1 (0, 30, 60, 90 μM), G2 (30, 60, 90, 120 μM), G3 (60, 90, 120, 150 μM) and G4 (150, 180, 210, 240μM; Fig. 3a). In the last gradient (G4) all the concentrations tested were lethal, which is necessary to observe ER events. Three independent cultures were used for each sulfide concentration in each gradient; in total, the experiment comprised 108 populations starting at the lowest sulfide level (G1 gradient). Each population of *M. aeruginosa* was established at a cell density of 1.5×10^5 cells · mL^{-1} and was grown in 15 mL tubes (Falcon™) in 4 mL of BG11-50% buffered with 20 mM HEPES, pH 7.2, with the corresponding sulfide addition.

To simulate the three different environmental deterioration modes, cultures were transferred to the next sulfide gradient at different rates. Populations were transferred to the next gradient every four or every two weeks in the slow and fast environmental deterioration treatments, respectively (Fig. 3a). In the no deterioration treatment, cultures remained at G1 level for 12 weeks before the transfer to G4 level. In all treatments, cultures were kept at G4 level for 4 weeks, after which the number of ER events was computed (Fig. 3a)

To mimic the three dispersal modes all cultures (from all deterioration modes) were transferred each week to new medium in three different ways (Fig. 3b). Control (no dispersal) was achieved by transferring a population aliquot (50 μL) to new medium (Fig. 3b). Local dispersal was achieved by mixing the three cell cultures (three populations) subjected to the same sulfide concentration (Fig. 3b). For this purpose, 75 μL of each cell culture was placed in an Eppendorf tube and mixed. From this mixture, three 50 μL aliquots were transferred to three new tubes with new medium. For global dispersal simulation, all twelve populations from the same sulfide level were mixed (Fig. 3b) as indicated before and twelve 50 μL aliquots were transferred to the corresponding new media. The same procedure was followed

This article is protected by copyright. All rights reserved.

when there was a change of sulfide level, as indicated below.

Then, after two (fast deterioration) or four (slow deterioration) weeks at the same sulfide level, the populations were transferred to the next one. The transfers were different depending on the dispersal mode, as explained before: without any mixture (no dispersal), with mixture among tubes of the same sulfide concentration (local dispersal) or with mixture of all tubes from the same sulfide level (global dispersal). The increase in sulfide concentration from G1 to G2 level was from 0 to 30 µM; from 30 to 60 µM; from 60 to 90 µM and from 90 to 120 µM sulfide. A similar procedure was followed when changing from G2 to G3 and from G3 to G4.

To maintain sulfide concentrations and pH as constant as possible during incubations, all tubes were tightly closed with caps, sulfide additions were made daily and the medium was renewed weekly. To monitor growth rates along the experiment, the absorbance at 750 nm was measured every week in each population to estimate cell density.

An ER event was scored if the growth rate after four weeks at G4 level was similar or higher than the growth rate observed just before the G4 treatment (i.e., an absolute increase in *m* was detected). This is a gross definition because populations may undergo ER (decline and recovery) without exceeding their initial pre-stress density. The complete experiment was performed three times. The ER event data were analyzed using a Model I, two-way ANOVA and a post-hoc Student-Newman-Keuls (SNK) test computed for a percentage of 95%. The statistical analyses were carried out using R Core Team (2013).

Statistical analysis.

The null hypothesis of similar CV values for the number of resistant cells per tube, computed in sets 1 and 2 of the fluctuation analysis, was determined using a one-tailed *Z* test. The comparison of the overall *m* mean values of the LHW-sensitive and LHW-resistant cells of

This article is protected by copyright. All rights reserved.

M. aeruginosa computed for the mutation-selection equilibrium, was performed using a one-tailed Student *t*-test. The ER event data were analyzed using a Model I, two-way ANOVA and a post-hoc Student-Newman-Keuls (SNK) test computed for a percentage of 95%. The homogeneity of variances in both the Student-*t*-test and the ANOVA, were previously checked with the Bartlett test. All the tests were performed in accordance with Zar (1999), using R Core Team (2013).

RESULTS

Toxic effect of sulfide on wild-type strains of Microcystis aeruginosa *and* Oscillatoria *sp.*

In sulfide-free culture medium, the acclimated growth rate (*m*) of *Microcystis aeruginosa* strain was ca. 0.7 doublings · d^{-1}, and it was negatively affected by sulfide, decreasing ca. 70% by the daily addition of 50 µM sulfide and being completely inhibited at concentrations ≥100 µM (Fig. 4). The *m* value of *Oscillatoria* sp. increased linearly from 0.22 ± 0.01 in sulfide-free conditions to 0.31 ± 0.01 doublings · d^{-1} at 100 µM sulfide additions, reaching an overall mean maximum value of 0.30 ± 0.02 doublings · d^{-1}, when sulfide additions were in the range from 100 to 350 µM sulfide. Higher concentrations inhibited *m*, growth being almost undetectable at 900 µM sulfide (Fig. 4).

Fluctuation analysis of the LHW-sensitivity to LHW-resistance transformation in Microcystis aeruginosa.

Cultures of the wild-type *Microcystis aeruginosa* strain were incubated for 6 weeks with LHW. After this time, cell growth was detected in 17 of the 90 cultures of set 1, whereas cell proliferation occurred in all cultures from set 2 (Table 1). Whereas the number of resistant cells proliferating in set 1 LHW-resistant cultures ranged over five orders of magnitude, it showed a very low dispersal in set 2, with CV for set 1 significantly higher (P<0.0001) than

This article is protected by copyright. All rights reserved.

Accepted Article

that of set 2 (Table 1). Consequently, it could be inferred that LHW-resistant cells arose prior to the exposure to LHW by rare spontaneous mutations rather than by specific adaptation during LHW exposure. The spontaneous mutation rate (μ) of LHW-sensitive cells to LHW-resistant cells was estimated as 2.1×10^{-7} mutations per cell per generation (Table 1).

A randomly chosen LHW-resistant culture from set 1 was isolated and maintained in the algal culture collection with daily additions of 0.2 mM sulfide.

Mutation-selection equilibrium.

In the absence of the selective agent (i.e., BG11-50% medium) the *m* value of the LHW-sensitive cells of *Microcystis aeruginosa* was 0.5 ± 0.2 doublings · d^{-1} ($n = 4$), whereas the value for the LHW-resistant cells was 0.4 ± 0.03 doublings · d^{-1} ($n = 4$). That is, *m* significantly decreased ($P<0.01$, Student *t*-test) by 20% in resistant cells. Using the overall mean of both *m* values, a coefficient of selection (*s*) of 0.222 was computed. The estimation of the frequency (*q*) of LHW-resistant alleles in wild type populations of *M. aeruginosa*, in the absence of the selective agent, was 9.4×10^{-7}.

Limits of genetic adaptation to sulfide of Microcystis aeruginosa *and* Oscillatoria *sp.*

The limits of genetic adaptation to sulfide in *Microcystis aeruginosa* and *Oscillatoria* sp. were different, as expected. In the case of *M. aeruginosa*, 11 of the 12 cultures founded at the beginning of the ratchet experiment were able to adapt up to the daily addition of 405 μM sulfide (Fig. 5). That is to say, the resistance to sulfide in *M. aeruginosa* increased 4-fold (from 100 to 405 μM sulfide; see Fig. 4). In the ratchet experiment performed with *Oscillatoria* sp, maximum adaptation values were observed at 1.5 mM, 1.8 mM and 2 mM sulfide additions when the initial sulfide concentrations were 0.3, 0.4 and 0.5 mM, respectively (Fig. 6). In this case, sulfide resistance increased more than 2-fold (from 0.9 to 2

This article is protected by copyright. All rights reserved.

Accepted Article

mM sulfide; see Fig. 4). However, in the ratchet experiment with *Oscillatoria* sp., we detected sulphur (S^0) precipitation in cultures tubes when total sulfide concentration was above 0.4 mM. This phenomenon occurs as well in natural high-sulfide environments (Camacho et al. 2005). However, because the change in turbidity could affect growth rate, we did not test concentrations above 2 mM sulfide in this ratchet experiment.

Because each replicate evolved as an independent population, within-strain differences can be analyzed. Thus, the four culture replicates founded required different time periods (d) to grow to the same cell concentration as control cultures at the next higher sulfide concentration, especially at the highest concentrations. In the case of *Microcystis aeruginosa*, there were no differences (except in one of the replicates) in the time needed to reach the next higher concentration until the concentration was >90 µM sulfide (Fig. 5). In the *Oscillatoria* sp. ratchet experiment, this was observed at concentrations around 1 mM (Fig. 6). That is, in both cases, at concentrations above the sulfide levels at which no growth was detected for each strain (Fig. 4). On the other hand, there were no differences in the highest capacity of adaptation in *M. aeruginosa*, regardless of the initial concentrations tested (10, 30 or 90 µM sulfide; Fig. 5). However, there were differences in the case of *Oscillatoria* sp., because the highest capacity of adaptation (up to 2 mM) was reached by those cultures exposed to the initial higher sulfide concentrations (0.5 mM sulfide; Fig 6).

Evolutionary rescue in Microcystis aeruginosa *under sulfide selection: effect of dispersal and deterioration rate.*

Evolutionary rescue occurred under all combinations of population dispersal modes and rate of enhancement of sulfide concentration, ranging from 2 to 12 events (Fig. 7). Whereas both environmental deterioration rate and dispersal mode significantly affected the ER dynamics

This article is protected by copyright. All rights reserved.

Accepted Article

of *M. aeruginosa* under sulfide selective conditions, the interaction of both factors did not (Table 2).

According to the ANOVA *P*-values, the contribution of the dispersal mode to the variance in ER was higher than the contribution from environmental deterioration (Table 2) and, for this reason, we represented the number of ER events as a function of dispersal mode (Fig. 7). The occurrence of ER events, when populations were globally dispersed (and independently of the deterioration rate), was significantly higher than under local dispersal, or without dispersal, with the exception of no deterioration (SNK test). On the other hand, the fast deterioration rate hinders the achievement of ER events under local and no dispersal modes (SNK test). When the genetic interchange was made only among populations submitted to the same sulfide concentration (local dispersal), the speed of the environmental deterioration determined the number of ER events, being significantly higher in slow than in fast deterioration (SNK test); however, no significant differences between slow and constant deterioration were observed (SNK test). Only when the whole genetic pool was mixed (global dispersal), were there no differences between environmental deterioration speed treatments (SNK test).

DISCUSSION

Neodarwinism postulates that selection pressure and genetic variability are involved in the appearance of new genotypes better adapted than previous ones to new natural conditions. This assumption has been widely accepted by the scientific community, but experimental demonstrations are very difficult and hence very scant (Sniegowski 2005). In this study, we tried to simulate the evolution of two cyanobacteria taxa under selection by sulfide performing for the first time, to our knowledge, three different ‘evolution in action’ experiments. Several studies have addressed the different mechanisms allowing the

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

proliferation of cyanobacteria found today in sulfidic environments, however, very few studies have analyzed the process of sulfide adaptation from an evolutionary point of view (Miller and Bebout 2004, Fernández-Arjona et al. 2013). In this way, the tolerance to sulfide, a selective agent for most photosynthetic microorganisms, could be genetic or physiological and the limit of adaptation to this ion could be different depending on the initial sulfide tolerance.

As expected, *Microcystis aeruginosa* and *Oscillatoria* sp. wild type strains showed different tolerances to the presence of sulfide. Growth of *M. aeruginosa* was not detected at ≥0.1 mM sulfide exposure, whereas the O1LH *Oscillatoria* strain maintained the ability to grow even when submitted to 0.9 mM sulfide additions, in agreement with sulfide levels in the environment from which each strain was isolated (Miller and Bebout 2004). *Microcystis aeruginosa* was isolated from sulfide-free waters, so inhibition of oxygenic photosynthesis, and then growth rate, at low sulfide concentrations (<0.1 mM) is expected (Castenholz, 1977, Cohen et al. 1986, García-Pichel and Castenholz, 1990). On the contrary, *Oscillatoria* sp. was isolated from La Hedionda spa, where sulfide concentration ranged from 0.1-0.2 mM. In fact, this strain showed maximum growth rates when submitted to 0.1-0.35 mM sulfide additions, suggesting that it could exhibit sulfide-resistant oxygenic photosynthesis or even sulfide-dependent anoxygenic photosynthesis (Cohen et al. 1986, Camacho et al. 2000, 2005).

Despite the high sensitivity of *Microcystis aeruginosa* to sulfide, we unequivocally demonstrated that this organism could genetically adapt to the lethal sulfide levels of LHW. The results observed in the fluctuation analysis experiment indicated that LHW-resistant cells arose prior to the exposure to LHW (ca. 150 µM sulfide) by rare spontaneous mutations rather than by acclimation. This qualitative result is similar to that found in the Ma1G *M. aeruginosa* strain exposed to the sulfureous waters from Los Baños de Vilo (ca. 240 µM sulfide, Fernández-Arjona et al. 2013). Moreover, the spontaneous mutation rates in both

This article is protected by copyright. All rights reserved.

cases were of the same order of magnitude (2.1×10^{-7} in this study, and 7.1×10^{-7} in Los Baños de Vilo; units in mutants per cell per generation). Moreover, a similar value of mutation-selection equilibrium of ca. 1 sulfide-resistant mutant cell per 10^6 wild-type cells was found in both strains. The *m* value of LHW-resistant cells in sulfide-free medium decreased significantly by 20% compared to that of Ma1Vc wild strain, which could be an indication of the physiological cost of the mutation conferring sulfide tolerance. However, this reduction was low compared with that obtained in the Ma1G strain mentioned above, as the growth rate decreased by 70% in the sulfide resistant cells (Fernández-Arjona et al. 2013). In these cells, an increase in size and a disrupted morphology were observed, as well as a 50% decrease in photosynthetic rates and photosynthetic efficiency (Bañares-España et al. 2016), which explained the large decrease in *m*. These changes on cell physiology could be not so relevant in the LHW-resistant cells. However, in both cases, the sulfide-resistant genotype would not proliferate in situations without selective agent, since it would be displaced by the wild type in a sulfide-free environment.

Applying the ratchet protocol, adaptation to sulfide in the Ma1Vc strain of *Microcystis aeruginosa* increased up to twice the LHW sulfide concentration. Thus, almost every experimental culture could survive under 0.4 mM sulfide additions, a concentration four times higher than the maximal tolerance of the wild type strain. No differences were found in the level of sulfide adaptation regardless of the initial sulfide concentration used, suggesting that the role of previous sulfide exposure did not influence ultimate sulfide resistance. On the other hand, the O1LH strain of *Oscillatoria*, as expected, developed a greater tolerance to sulfide. Nevertheless, only a few derived experimental populations were able to grow at ca. 2 mM sulfide exposure, the highest concentration tested. This result is in agreement with the fact that several species of the genus *Oscillatoria* proliferate in natural environments where sulfide levels can achieve concentrations ≥1.5 mM (Camacho et al. 1996, 2000) because they

This article is protected by copyright. All rights reserved.

exhibit sulfide-driven anoxygenic photosynthesis (Belkin and Padan 1983, Arieli et al. 1991), or maintain oxygenic photosynthesis relatively insensitive to sulfide (Camacho et al. 1996, 2000, 2005). Interestingly, previous culture conditions modulate the highest adaptation capacity in *Oscillatoria* but not in *M. aeruginosa*. This could be explained by the fact that *M. aeruginosa* is very sensitive to sulfide and, as a consequence, sub-lethal sulfide levels do not modulate the end-point of its maximum adaptation. On the contrary, the presence of moderate-high levels of sulfide (0.5 mM) allowed the adaptation to highest levels of this ion in *Oscillatoria* sp. This could be related to the fact that the presence of high sulfide concentrations induces changes in the photosynthetic characteristics of sulfide-resistant cyanobacteria (Cohen et al. 1986, Camacho et al. 2005). The photosynthetic and morphological characteristics of the sulfide-resistant strains obtained in this study will be further analyzed to shed more light on the process of adaptation to sulfide.

Although some sulfide-rich environments maintain a relatively constant sulfide concentration, like sulfur springs, in others concentrations change with time, like the hypolimnia of stratified lakes or marine and freshwater sediments, where the growth of microorganisms may induce fluctuating vertical gradients of sulfide (Camacho et al. 2005). In the case of La Hedionda spa waters, sulfide content changes along the year depending on the stream flow. So, we wanted to analyze how the population dispersal (a proxy of genetic variation) and the speed of environmental (sulfide concentration) change could affect to population survival at a lethal level of sulfide (evolutionary rescue). Although genetic variation is one of the premises of natural selection, experimental demonstrations are scant (Bell and González, 2009, 2011, Lindsey et al. 2013, Orr and Uncless 2014, Carja and Plotkin 2019, Liu et al. 2019). Our results indicated that the occurrence of ER events was modulated by both genetic variation and deterioration rate, but the effect of the former is greater than the later. In particular, the highest ER events scores were recorded under global dispersal,

This article is protected by copyright. All rights reserved.

regardless of the environmental deterioration rate. This suggests that a high genetic variation in *M. aeruginosa* populations is crucial for genetic adaptation and it could be enough to overcome sulfide stress in the range we examined under any speed of environmental change. However, when the genetic variation is lower or even minimal (as was simulated under the local or without dispersal, respectively) a fast deterioration rate does not favour the ER events. Moreover, at the lowest genetic variation, the ER event reduction occurs under any studied deterioration rate. The results found under local or without dispersal are in agreement with the hypothesis that the rate of environmental deterioration can modulate the effect of beneficial mutations, with rapid change favouring a few mutations of large effect, whereas adaptation to slower deterioration is based on more mutations of smaller effect (Collins and de Meaux, 2009). In fact, Lindsey et al. (2013) obtained similar experimental results in *E. coli*.

Taking into account all these results, we can hypothesize that even when the likelihood of adaptation to lethal levels of sulfide by Ma1Vc is not constrained by the initial concentration to which the cultures are subjected (as showed in the ratchet results), the genetic variability of the population and the speed of deterioration are decisive for the achievement of sulfide resistance, ergo, the population dynamics will influence the evolutionary trajectory. Even so, this assertion must be made carefully and restricted to the Ma1Vc strain of *Microcystis aeruginosa*, since micro-evolutionary routes cannot be extrapolated to explain macro-evolutionary processes (Stanley 1979, Eldredge and Cracraft 1980, Arnold et al. 2001).

From an ecological point of view, it could be hypothesized that the adaptation to sulfide in natural populations of *Microcystis aeruginosa* may easily occur. The reason is that in this cyanobacterium a high percentage of phenotypic variation in several traits is a consequence of genetic variation, making the non-genetic contribution less significant

This article is protected by copyright. All rights reserved.

(Bañares-España et al. 2006, 2007, López-Rodas et al. 2006a,b). Consequently, a high genetic variation in natural populations of *M. aeruginosa* could even overcome a sudden change in sulfide levels above normally lethal conditions.

The experimental evolution approaches addressed in this study allowed us to explore adaptation mechanisms to sulfide and the constraints to this process in two representative taxa of cyanobacteria. Further studies, involving more lineages of cyanobacteria could clarify how the sulfide tolerance could evolve in these microorganisms.

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

This work was financially supported by the projects CGL2014-53862-P and CGL2017-87314-P (Ministerio de Economía y Competitividad, Spain). EM-C was founded by BES-2015-072984 grant (Ministerio de Economía y Competitividad, Spain). Dr. Eric C. Henry kindly revised the English style and usage.

This article is protected by copyright. All rights reserved.

Accepted Article

Arieli, B., Padan, E. & Shahak, Y. 1991. Sulfide-induced sulfide-quinone reductase activity in thylakoids of *Oscillatoria limnetica*. *J. Biol. Chem.* 266:104-11.

Arnold, S. J., Pfender, M. E. & Jones, A. G. 2001.The adaptive landscape as a conceptual bridge between micro and macroevolution. *Genetica.* 112–113:9–32.

Bagarinao, T. 1992. Sulphide as an environmental factor and toxicant: tolerance and adaptations in aquatic organisms. *Aquat. Toxicol.* 24:21–62.

Bañares-España, E., Fernández-Arjona, M. M., García-Sánchez, M. J., Hernández-López, M., Reul, A., Mariné, M. H. & Flores-Moya, A. 2016. Sulphide resistance in the cyanobacterium *Microcystis aeruginosa*: a comparative study of morphology and photosynthetic performance between the sulphide-resistant mutant and the wild-type strain. *Microb. Ecol.* 71:860-72.

Bañares-España, E., López-Rodas, V., Costas, E., Salgado, C. & Flores-Moya, A. 2007. Genetic variability associated with photosynthetic pigment concentration, and photochemical and nonphotochemical quenching, in strains of the cyanobacterium *Microcystis aeruginosa*. *FEMS Microb. Ecol.* 60:449-55.

Bañares-España, E., López-Rodas, V., Salgado, C., Costas, E. & Flores-Moya, A. 2006. Inter-strain variability in the photosynthetic use of inorganic carbon, exemplified by the pH compensation point, in the cyanobacterium *Microcystis aeruginosa*. *Aquat. Bot.* 85:159-62.

Belkin, S. & Padan, E. 1983. Low redox potential promotes sulphide- and light- dependent hydrogen evolution in *Oscillatoria limnetica*. *Microbiol*. 129:3091-8.

Bell, G. & Gonzalez, A. 2009. Evolutionary rescue can prevent extinction following environmental change. *Ecol. Lett.* 12:942-8.

Bell, G. & Gonzalez, A. 2011. Adaptation and evolutionary rescue in metapopulations experiencing environmental deterioration. *Science* 332:1327-30.

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

Blount, Z. D., Borland, C. Z. & Lenski, R. E. 2008. Historical contingency and the evolution of a key innovation in an experimental population of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 105:7899-906.

Camacho, A., Garcia-Pichel, F., Vicente, E. & Castenholz, R. W. 1996. Adaptation to sulfide and to the underwater light field in three cyanobacterial isolates from Lake Arcas (Spain). *FEMS Microbi. Ecol.* 21:293-301.

Camacho, A., Rochera, C., Silvestre, J. J., Vicente, E. & Martin, W. H. 2005. Spatial dominance of inorganic carbon assimilation by conspicuous autotrophic biofilms in a physical and chemical gradient of a cold sulphurous spring: the role of different ecological strategies. *Microb. Ecol.* 50:172-84

Camacho, A.,Vicente, E. & Miracle, M. R. 2000. Ecology of a deep-living *Oscillatoria* (=*Planktothrix*) population in the sulphide-rich waters of a Spanish karstic lake. *Arch. Hydrobiol.* 148:333-55.

Carja, O. & Plotkin, J. B. 2019. Evolutionary rescue through partly heritable phenotypic variability. *Genetics* 211:977-88.

Castenholz, R. W. 1977. The effect of sulfide on the blue-green algae of hot springs II. Yellowstone National Park. *Microb. Ecol.* 3:79–105.

Cohen, Y., Jørgensen, B. B., Paden, E. & Shilo, M. 1975a. Sulfide dependent anoxygenic photosynthesis in the cyanobacterium *Oscillatoria limnetica*. *Nature* 257:489–92

Cohen, Y., Jørgensen, B. B., Revsbech, N. P. & Poplawski, R. 1986. Adaptation to hydrogen sulfide of oxygenic and anoxygenic photosynthesis among cyanobacteria. *Appl. Environ. Microbiol.* 51:398–407.

Cohen, Y., Padan E. & Shilo M. 1975b. Facultative anoxygenic photosynthesis in the cyanobacterium *Oscillatoria limnetica. J. Bacteriol.* 123:855–61.

Collins, S. & De Meaux, J. 2009. Adaptation to different rates of environmental change in

This article is protected by copyright. All rights reserved.

Accepted Article

Chlamydomonas. *Evolution* 63:2952-65.

Costas, E., Carrillo, E., Ferrero, L. M., Agrelo, M., García-Villada, L., Juste, J. & López-Rodas, V. 2001. Mutation of algae from sensitivity to resistance against environmental selective agents: the ecological genetics of *Dictyosphaerium chlorelloides* (Chlorophyceae) under lethal doses of 3-(3,4-dichlorophenyl)-1,1-dimethylurea herbicide. *Phycologia* 40:391-8.

Costas, E., Flores-Moya, A. & López-Rodas, V. 2008. Rapid adaptation of phytoplankters to geothermal waters is achieved by single mutations: were extreme environments 'Noah's Arks' for photosynthesizers during the Neoproterozoic 'snowball Earth'? *New Phytol*. 180:922-32.

Costas, E., Flores-Moya, A., Perdigones, N., Maneiro, E., Blanco, J. L., García, M. E. & López-Rodas, V. 2007. How eukaryotic algae can adapt to the Spain's Rio Tinto: a neo-Darwinian proposal for rapid adaptation to an extremely hostile ecosystem. *New Phytol.* 175:334-9.

Crow, J. F. & Kimura, M. 1970. *An introduction to population genetics theory*. Harper, New York, USA, 612 pp.

Czyzewski, B. K. & Wang, D. N. 2012. Identification and characterization of a bacterial hydrosulphide ion channel. *Nature* 483:494–7.

de Wit, R. & van Gemerden, H. 1987. Oxidation of sulfide to thiosulfate by *Microcoleus chtonoplastes*. *FEMS Microbiol. Ecol.* 45:7–13.

Dodds, W. K. & Castenholz, R. W. 1990. Sulfide and pH effects on variable fluorescence of photosystem II in two strains of the cyanobacterium *Oscillatoria amphigranulata*. *Photosynth. Res.* 24:265–71.

Eldredge, N. & Cracraft, J. 1980. *Phylogenetic Patterns and the Evolutionary Process*. Columbia University Press, New York, USA, 349 pp.

This article is protected by copyright. All rights reserved.

Elena, S. F. & Lenski, R. E. 2003. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nat. Rev. Genet.* 4:457–69

Fernández-Arjona, M. M., Bañares-España, E., García-Sánchez, M. J., Hernández-López, M., López-Rodas, V., Costas, E. & Flores-Moya, A. 2013. Disentangling mechanisms involved in the adaptation of photosynthetic microorganisms to the extreme sulphureous water from Los Baños de Vilo (S Spain). *Microb. Ecol.* 66:742–51.

Flores-Moya, A., Costas, E. & López-Rodas, V. 2008. Roles of adaptation, chance and history in the evolution of the dinoflagellate *Prorocentrum triestinum*. *Naturwissenschaften.* 95:697-703.

Flores-Moya, A., Costas, E., Bañares-España, E., García-Villada, L., Altamirano, M. & López-Rodas, V. 2005. Adaptation of *Spirogyra insignis* (Chlorophyta) to an extreme natural habitat environment (sulphureous waters) through preselective mutations. *New. Phytolog.* 166:655-61.

Flores-Moya, A., Rouco, M., García-Sánchez, M. J., García-Balboa, C., González, R., Costas, E. & López-Rodas, V. 2012. Effects of adaptation, chance and history on the evolution of the toxic dinoflagellate *Alexandrium minutum* under selection of increased temperature and acidification. *Ecol. Evol.* 2:1251-9.

García-Pichel, F. & Castenholz, R. W. 1990. Comparative anoxygenic photosynthetic capacity in 7 strains of a thermophilic cyanobacterium. *Arch. Microbiol.* 153:344–51.

García-Villada, L., López-Rodas, V., Bañares-España, E., Flores-Moya, A., Agrelo, M., Martín-Otero, L. & Costas, E. 2002. Evolution of microalgae in highly stressing environments: an experimental model analyzing the rapid adaptation of *Dictyosphaerium chlorelloides* (chlorophyceae) from sensitivity to resistance against 2, 4, 6-Trinitrotoluene by rare preselective mutations. *J. Phycol.* 38:1074-81.

García-Villada, L., Rico, M., Altamirano, M., Sánchez-Martín, L., López-Rodas, V. & Costas,

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

E. 2004. Occurrence of copper resistant mutants in the toxic cyanobacteria *Microcystis aeruginosa*: characterisation and future implications in the use of copper sulphate as algaecide. *Water Res.* 38:2207-13.

Garland, T., Jr. & M. Rose, M. R. 2009. *Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments.* University of California Press, Berkeley, USA, 730 pp.

Gonzalez, A., Ronce, O., Ferriere, R., & Hochberg, M. E. 2013. Evolutionary rescue: an emerging focus at the intersection between ecology and evolution. *Phil. Trans. R. Soc. B.* 368:20120404.

Gould, J. L. & Gould, C.G. 1989. *Sexual selection*. Scientific American Library, New York, USA.

Hamilton, T. L., Bryant, D. A. & Macalady, J. L. 2016. The role of biology in planetary evolution: cyanobacterial primary production in low-oxygen Proterozoic oceans. *Environ. Microbiol.* 18:325-40.

Howsley, R. & Pearson, H. W. 1979. pH dependent sulphide toxicity to oxygenic photosynthesis in cyanobacteria. *FEMS Microbiol. Lett*. 6:287-92

Huertas, I. E., Rouco, M., López-Rodas, V. & Costas, E. 2010. Estimating the capability of different phytoplankton groups to adapt to contamination: herbicides will affect phytoplankton species differently. *New. Phytol.* 188:478-87.

Huertas, I. E., Rouco, M., López-Rodas, V. & Costas, E. 2011. Warming will affect phytoplankton differently: evidence through a mechanistic approach. *P. Roy. Soc. B-Biol. Sci.* 278:3534-43.

Hughes, A. L. 1999. *Adaptive evolution of genes and genomes*. Oxford University Press, Oxford, UK, 288 pp.

This article is protected by copyright. All rights reserved.

Accepted Article

Kimura, M. & Maruyama, T. 1966. The mutational load with epistatic gene interactions in fitness. *Genetics* 54:1337.

Klatt, J. M., Al-Najjar, M. A., Yilmaz, P., Lavik, G., de Beer, D. & Polerecky, L. 2015. Anoxygenic photosynthesis controls oxygenic photosynthesis in a cyanobacterium from a sulfidic spring. *Appl. Environ. Microbiol.* 81:2025–31.

Leister, D. 2017. Experimental evolution in photoautotrophic microorganisms as a means of enhancing chloroplast functions. *Essays Biochem.* 62:77-84.

Lindsey, H. A., Gallie, J., Taylor, S. & Kerr, B. 2013. Evolutionary rescue from extinction is contingent on a lower rate of environmental change. *Nature* 494:463.

Liu, M., Li, J., Wang, W. & Jiang, X. 2019. Immigration shapes evolutionary tolerance to toxic cyanobacteria in two cladoceran grazers. *Hydrobiologia* 826:397-409.

López-Rodas, V., Agrelo, M., Carrillo, E., Ferrero, L. M., Larrauri, A., Martín-Otero, L. & Costas, E. 2001. Resistance of microalgae to modern water contaminants as the result of rare spontaneous mutations. *Eur. J. Phycol.* 36:179–90.

López-Rodas, V., Costas, E., Bañares-España, E., García-Villada, L., Altamirano, M., Rico, M., Salgado, C. & Flores-Moya, A. 2006a. Analysis of polygenic traits of *Microcystis aeruginosa* (Cyanobacteria) strains by Restricted Maximum Likelihood (REML) procedures: 2. Microcystin net production, photosynthesis and respiration. *Phycologia* 45:243-8.

López-Rodas, V., Costas, E., García-Villada, L. & Flores-Moya, A. 2006b. Phenotypic evolution in microalgae: a dramatic morphological shift in *Dictyosphaerium chlorelloides* (Chlorophyta) after exposure to TNT. *Acta Botanica Malacitana* 31:141-7.

López-Rodas, V., Costas, E., Maneiro, E., Marvá, F., Rouco, M., Delgado, A. & Flores-Moya, A. 2009. Living in Vulcan's forge: Algal adaptation to stressful geothermal ponds on

This article is protected by copyright. All rights reserved.

Accepted Article

Vulcano Island (southern Italy) as a result of pre-selective mutations. *Phycol. Res.* 57:111-7.

López-Rodas, V., Flores-Moya, A., Maneiro, E., Perdigones, N., Marvá, F., García, M. E. & Costas, E. 2007. Resistance to glyphosate in the cyanobacterium *Microcystis aeruginosa* as result of pre-selective mutations. *Evol. Ecol.* 21:535-57.

López-Rodas, V., Marvá, F., Costas, E. & Flores-Moya, A. 2008a. Microalgal adaptation to a stressful environment (acidic, metal-rich mine waters) could be due to selection of pre-selective mutants originating in non-extreme environments. *Environ. Exp. Bot.* 64:43-8.

López-Rodas, V., Marvá, F., Rouco, M., Costas, E. & Flores-Moya, A. 2008b. Adaptation of the chlorophycean *Dictyosphaerium chlorelloides* to stressful acidic, mine metal-rich waters as result of pre-selective mutations. *Chemosphere* 72:703-7.

López-Rodas, V., Rouco, M., Sánchez-Fortún, S., Flores-Moya, A. & Costas, E. 2011. Genetic adaptation and acclimation of phytoplankton along a stress gradient in the extreme waters of the Agrio River– Caviahue Lake (Argentina). *J. Phycol.* 47:1036-43.

Luria, S. E. & Delbrück, M. 1943. Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 28:491.

Marvá, F., López-Rodas, V., Rouco, M., Navarro, M., Toro, F. J., Costas, E. & Flores-Moya, A. 2010. Adaptation of green microalgae to the herbicides simazine and diquat as result of pre-selective mutations. *Aquat. Toxicol.* 96:130-4.

Melero-Jiménez, I. J, Martín-Clemente, E., García-Sánchez, M. J., Flores-Moya, A. & Bañares-España, E. 2019. Adaptation of the toxic freshwater cyanobacterium Microcystis aeruginosa to salinity is achieved by the selection of spontaneous mutants. *Phycol. Res.* 67:192-201.

Meyer, K. M. & Kump, L. R. 2008. Oceanic euxinia in Earth history: causes and consequences. *Annu. Rev. Earth. Pl. Sc.* 36:251-88.

This article is protected by copyright. All rights reserved.

Miller, S. R. & Bebout, B. M. 2004. Variation in sulfide tolerance of photosystem II in phylogenetically diverse cyanobacteria from sulfidic habitats. *Appl. Environ. Microb.* 70:736–44.

Olson, K. R. & Straub, K. D. 2016. The role of hydrogen sulfide in evolution and the evolution of hydrogen sulphide in metabolism and signaling. *Physiology* 31:60-72.

Orr, H. A. & Unckless, R. L. 2014. The population genetics of evolutionary rescue. *PLoS Genet.* 10:e1004551.

Padan E. 1979. Facultative anoxygenic photosynthesis in cyanobacteria. *Ann. Rev. Plant. Physio.* 30:27–40.

Poulton, S. W. & Canfield, D. E. 2011. Ferruginous conditions: a dominant feature of the ocean through Earth's history. *Elements.* 7:107–12.

Poulton, S. W., Fralick, P. W. & Canfield, D. E. 2010. Spatial variability in oceanic redox structure 1.8 billion years ago. *Nat. Geosci.* 3:486–90.

R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. http://www.R-project.org/. Accessed 1-7 September 2018.

Rouco, M., López-Rodas, V., González, R., Huertas, I. E., García-Sánchez, M. J. & Flores-Moya A., Costas, E. 2014. The limit of the genetic adaptation to copper in freshwater phytoplankton. *Oecologia.* 175:1179-88.

Scott, C., Lyons, T. W., Bekker, A., Shen, Y., Poulton, S. W., Chu, X. & Anbar, A. D. 2008. Tracing the stepwise oxygenation of the Proterzoic ocean. *Nature.* 452:456–9.

Sniegowski P. D. 2005. Linking mutation to adaptation: overcoming stress at the spa. *New Phytol.* 166:330-62

Stal, L. J. 1995. Physiological ecology of cyanobacteria in microbial mats and other communities. *New Phytol.* 131:1–32.

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Stal, L. J. 2012. Cyanobacterial mats and stromatolites. *In* Whitton, B.A. [Eds.] *Ecology of Cyanobacteria II: Their Diversity in Space and Time.* Springer, Dordrecht, The Netherlands, pp. 65-125.

Stanley, S.M. 1979. *Macroevolution: Pattern and Process*. W. H. Freeman and Co., San Francisco, USA, 370 pp.

Travisano, M., Mongold, J. A., Bennett, A. F. & Lenski, R. E. 1995. Experimental tests of the roles of adaptation, chance, and history in evolution. *Science* 267:87-90.

Walter, J. M., Coutinho, F. H., Dutilh, B. E., Swings, J., Thompson, F. L., & Thompson, C. C. 2017. Ecogenomics and taxonomy of Cyanobacteria phylum. *Front. Microbiol.* 8:2132.

Wellburn, A. R. 1994. The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *J. Plant. Physiol.* 144:307-13.

Zar, J. H. 1999. *Biostatistical Analysis.* 4th Ed. Prentice Hall, Upper Saddle River, NJ, USA, 960 pp.

This article is protected by copyright. All rights reserved.

Accepted Article

TABLE 1. Fluctuation analysis of LHW-sensitivity to LHW-resistance transformation in the Ma1Vc *Microcystis aeruginosa* strain

		Set 1	Set 2
No. of replicate cultures:		90	40
No. of cultures with cells in the rank:			
	0	73	0
	$1\text{-}10^4$	1	0
	$10^4\text{-}10^5$	1	0
	$10^5\text{-}10^6$	6	0
	$> 10^6$	9	40
CV of the no. of LHW-resistant cells per culture:		2.351	0.214
One-tailed Z-test for comparison of CVs:		10.891[a]	
μ (mutants cells · generation^{-1})		2.1×10^{-7}	

[a] $P<0.0001$ (significant)

This article is protected by copyright. All rights reserved.

TABLE 2**.** Two-way ANOVA for the comparison of the ER events of *Microcystis aeruginosa* at sulphide lethal conditions. Modes of population dispersal and rates of environmental deterioration are indicated in Figure 3

Source of variation	df	SS	MS	*F*	*P*-value
Dispersal	2	86.518	43.259	16.69	7.65×10^{-5}
Deterioration	2	50.074	25.037	9.65	0.001
Dispersal × deterioration	4	14.592	3.648	1.40	0.27
Error	18	46.666	2.592		

This article is protected by copyright. All rights reserved.

Accepted Article

FIGURE LEGENDS

FIG. 1. Fluctuation analysis schematic diagram and possible results. In Set 1, different cultures of *Microcystis aeruginosa* (started from a small inoculum) were propagated under non-selective conditions (BG11-50% medium) until a very high cell density (indicated by dots) was reached and then transferred to the selective agent (La Hedionda water, LHW). If resistant cells arose during the exposure to LHW (acclimation), the number of resistant cells in all cultures would be similar. If resistant cells arose by rare mutations occurring during culture propagation before exposure to sulfide waters (i.e., genetic adaptation), the number of resistant cells in all cultures would be different. The diagram also shows that no mutational events occurred in most of the cultures (clear cultures) and that rare mutational events could appear early (high cell density) or late (low cell density) during propagation. Set 2 replicates were founded from the same parental culture, so the variance of the parental population is used as an experimental control. In this case, the number of resistant cells in all cultures must be similar.

FIG. 2. Ratchet protocol scheme. During the initial phase, 4 replicates of *Microcystis aeruginosa* strain cultures containing control growth medium (BG11-50%, 20 mM HEPES, pH 7.2), and 4 replicates of three treatments containing the same medium with increasing sulfide concentrations (10, 30 and 90 μM) were prepared. All cultures started with the same low cell density (white cultures) and were maintained 7 d in each treatment. Cultures showing the same final cell concentration (indicated by dots) as control ones were transferred to the next sulfide level or ratchet cycle (30, 90 and 270 μM sulfide) Cultures not reaching a similar biomass to that found in control cultures were not transferred (asterisk) and were

This article is protected by copyright. All rights reserved.

Accepted Article

maintained at the same sulfide concentration until they reached the control biomass. The transference to the next sulfide level (ratchet cycle) is not shown (see Material and Methods for a detailed description of the concentrations tested for *M. aeruginosa* and *Oscillatoria* sp., as they were different). A population (replicate) was considered to have reached the maximum capacity of adaptation when no further cell growth was observed after 90 d of exposure to the same sulfide concentration (not shown).

FIG. 3. Schematic diagram of the evolutionary rescue experiment in *Microcystis aeruginosa*. (a) Each sulfide gradient (from G1 to G4) comprised four increasing sulfide concentrations (represented by different intensities of gray) and these concentrations were increased in each new sulfide gradient from G1 (0, 30, 60 and 90 μM) to G2 (30, 60, 90 and 120 μM), G3 (60, 90, 120 and 150 μM) and G4 (150, 180, 210 and 240 μM) as indicated. Each concentration was assayed in three different populations, but only one is shown. In the no deterioration treatment, populations remained on the initial sulfide gradient for 12 weeks, until the transfer to the G4 gradient (where all sulfide concentrations assayed are supposed to be lethal). Slow and fast deterioration treatments went through G2 and G3, but the transfer to the next gradient was performed after 4 or 2 weeks, on slow or fast deterioration treatments, respectively. Populations remained 4 weeks in G4 in all treatments. (b) All cultures from the three deterioration conditions were transferred every week to fresh medium in a different way depending on the dispersal condition applied. In the no dispersal condition, the transferences were made directly. On the local dispersal condition, the 3 populations or replicates at the same sulfide concentration (only one shown) were mixed, and the new 3 populations were founded with the mixed culture. On global dispersal, all tubes from the 4 different sulfide concentrations, 12 tubes in total, were mixed, and the 12 new populations (4 concentrations with 3 replicates each) were founded with this mixed culture. In the scheme only the

This article is protected by copyright. All rights reserved.

transferences from G1 to G2 are shown as an example, but the process was the same regardless the sulfide concentrations tested.

FIG. 4. Effect of sulfide exposure on the acclimated growth rate (*m*) of the wild-type *Microcystis aeruginosa* and *Oscillatoria* sp. strains. Growth rate was estimated by the increment of the number of cells (*M. aeruginosa*, gray circles, $n = 5$) or Chl *a* concentration (*Oscillatoria* sp., black circles, $n = 4$) after 5 d of culture at increasing sulfide concentrations in the growth medium.

FIG. 5. Time required for cultures under increasing sulfide concentrations to grow as dense as control cultures (without sulfide) during the ratchet experiment cycles performed with *Microcystis aeruginosa* Ma1Vc strain. The experiment started with the exposure of cells, previously grown in the absence of sulfide, to three different initial levels of sulfide: 10 µM (a), 30 µM (b) and 90 µM (c). The increase in sulfide concentration at each ratchet cycle is indicated in the horizontal axis for each initial concentration. Each of the four replicates founded at the beginning of each ratchet cycle are shown with different colors. The shaded area indicates the ratchet cycle at which the cells were subjected to lethal sulfide concentrations for this strain.

FIG. 6. Time required for cultures under increasing sulfide concentrations to grow as dense as control cultures (with 0.2 mM sulfide) during the ratchet experiment cycles performed with *Oscillatoria* sp. O1LH strain. The experiment started with the exposure of cells, previously grown in the presence of 0.2 mM sulfide, to three different initial levels of sulfide: 0.3 mM (a), 0.4 mM (b) and 0.5 mM (c). The increase in sulfide concentration at each ratchet cycle is indicated in the horizontal axis for each initial concentration. Each of the four replicates

This article is protected by copyright. All rights reserved.

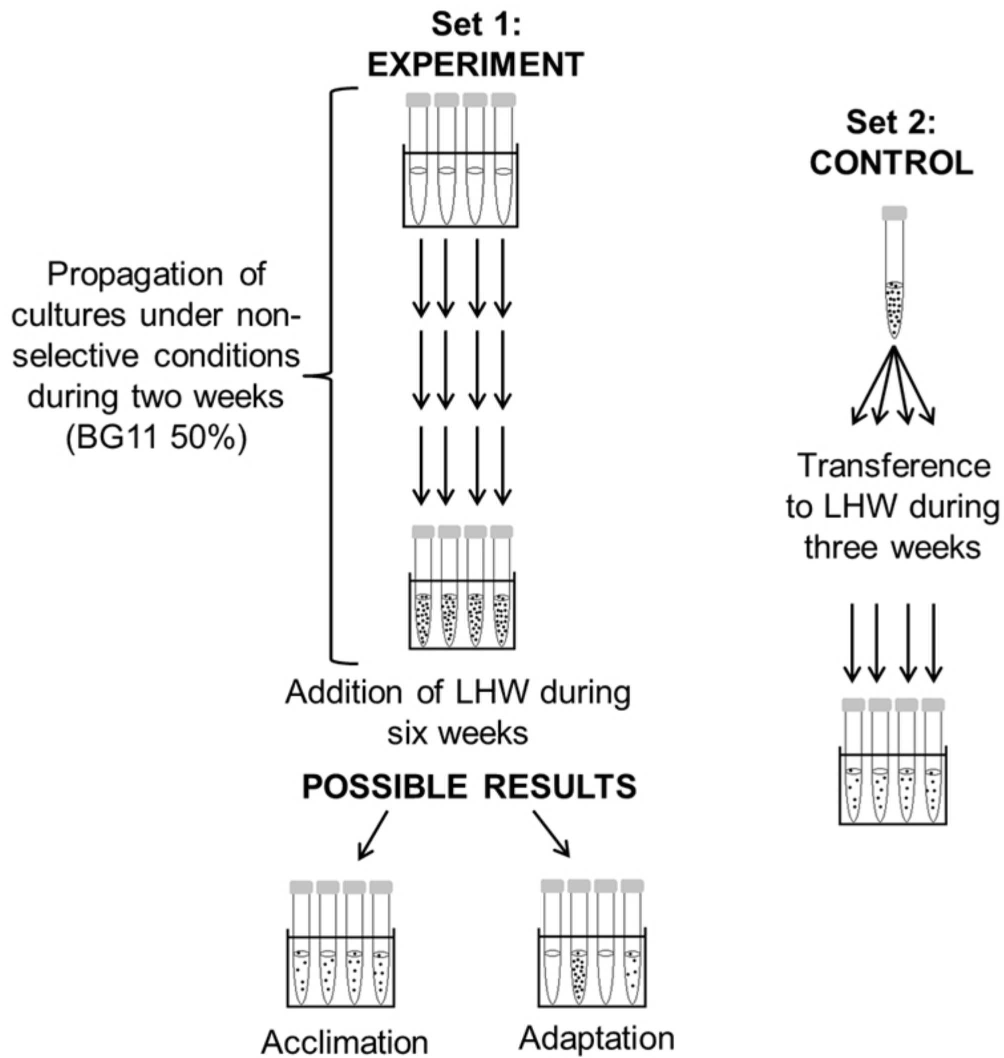
Accepted Article

founded at the beginning of each ratchet cycle are shown with different colors. The shaded area indicates the ratchet cycle at which the cells were subjected to lethal sulfide concentrations for this strain. For the three initial sulfide concentrations, every ratchet till the cycle 0.7 → 0.8 mM took 7 d, so they are not represented in order to simplify the figure.

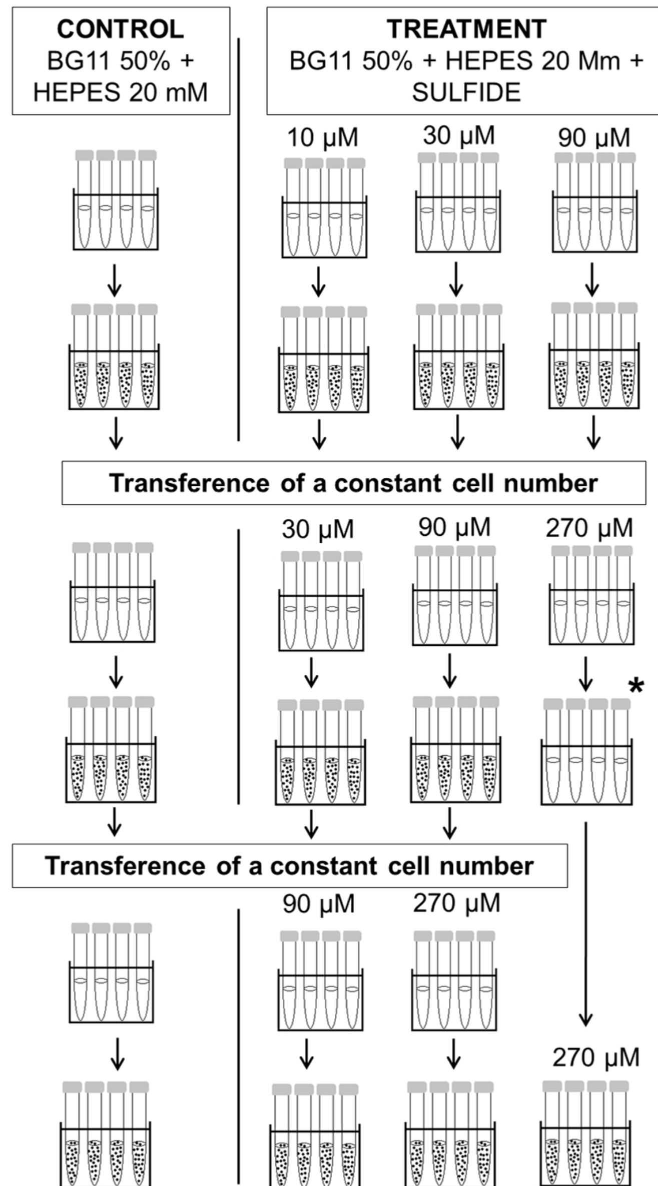
FIG. 7. Effect of environmental deterioration change and dispersal mode on the number of evolutionary rescue (ER) events observed in *Microcystis aeruginosa* populations submitted to lethal sulfide concentrations (maximum 12 ER events). Vertical lines on bars show SD (n=3). Treatments at each dispersal mode are represented by white bars (control-no deterioration), light gray bars (slow deterioration) and dark gray bars (fast deterioration). Different letters indicate significant differences detected by Student-Newman-Keuls (SNK) post-hoc test ($P < 0.05$).

This article is protected by copyright. All rights reserved.

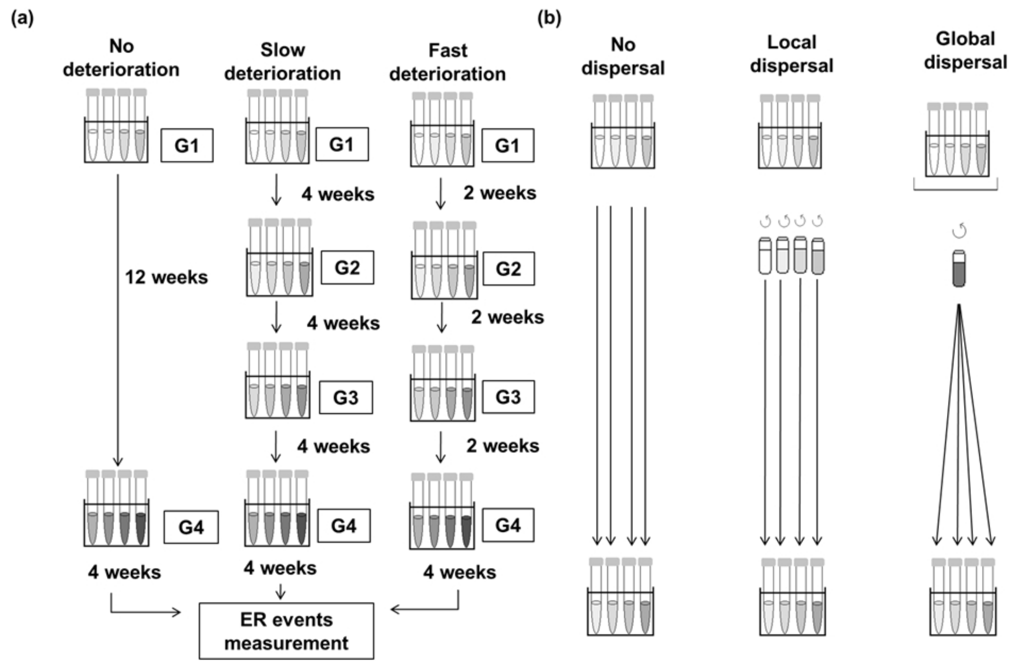
Accepted Article



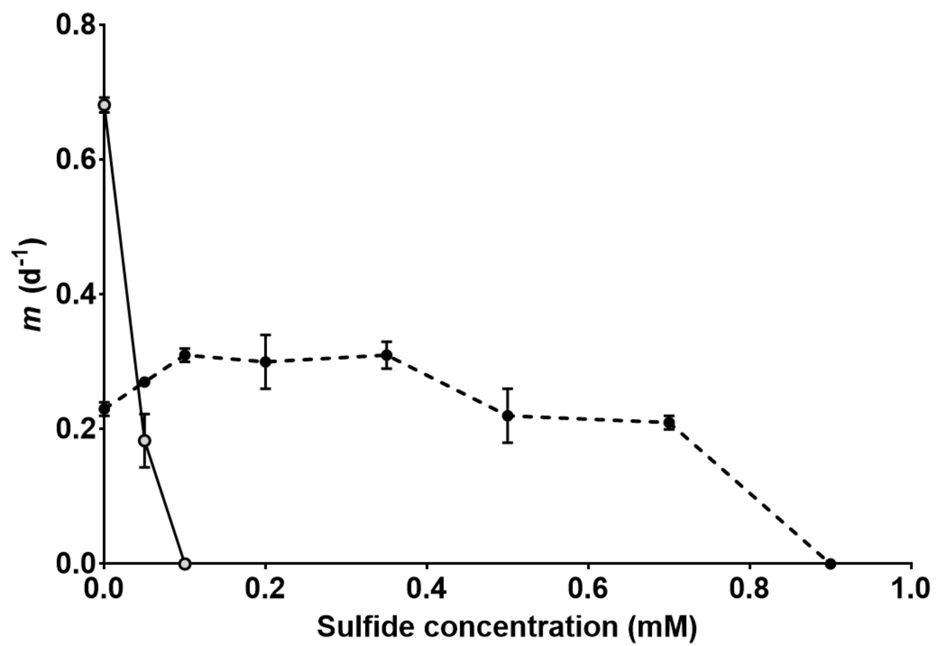
This article is protected by copyright. All rights reserved.



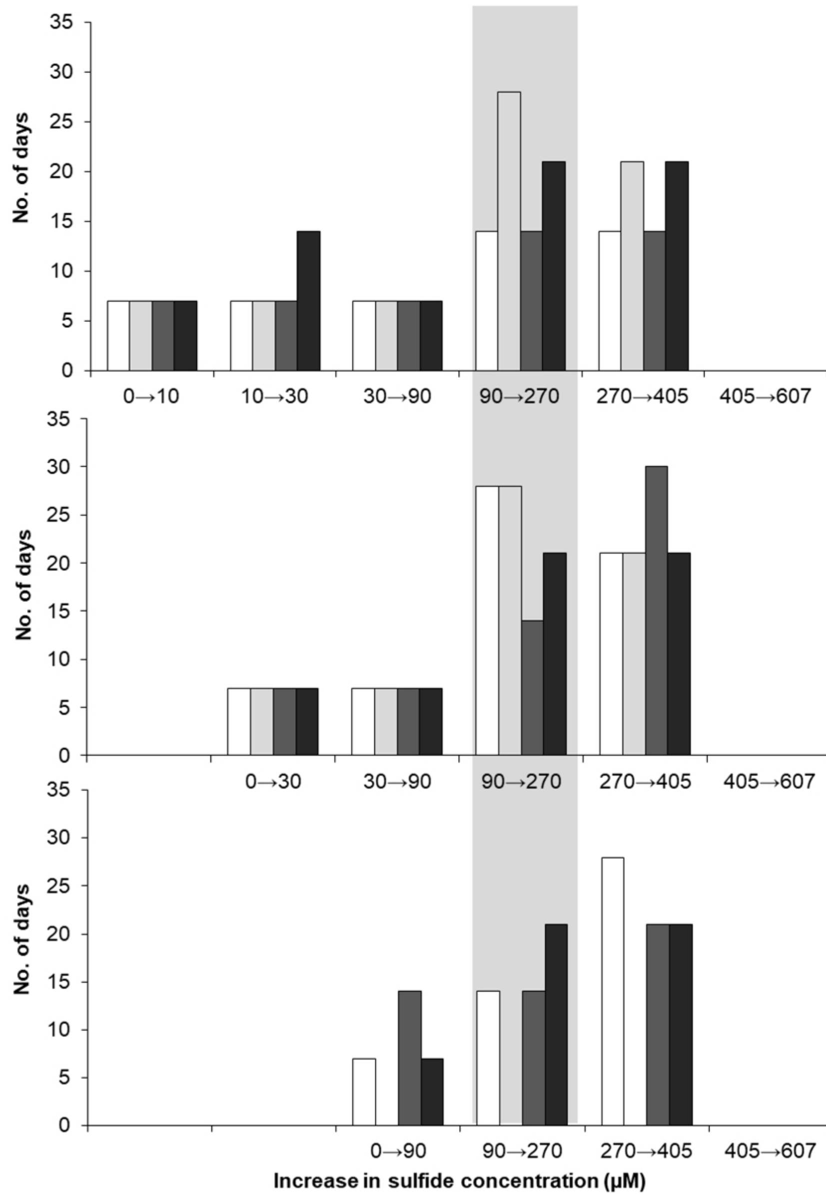
This article is protected by copyright. All rights reserved.



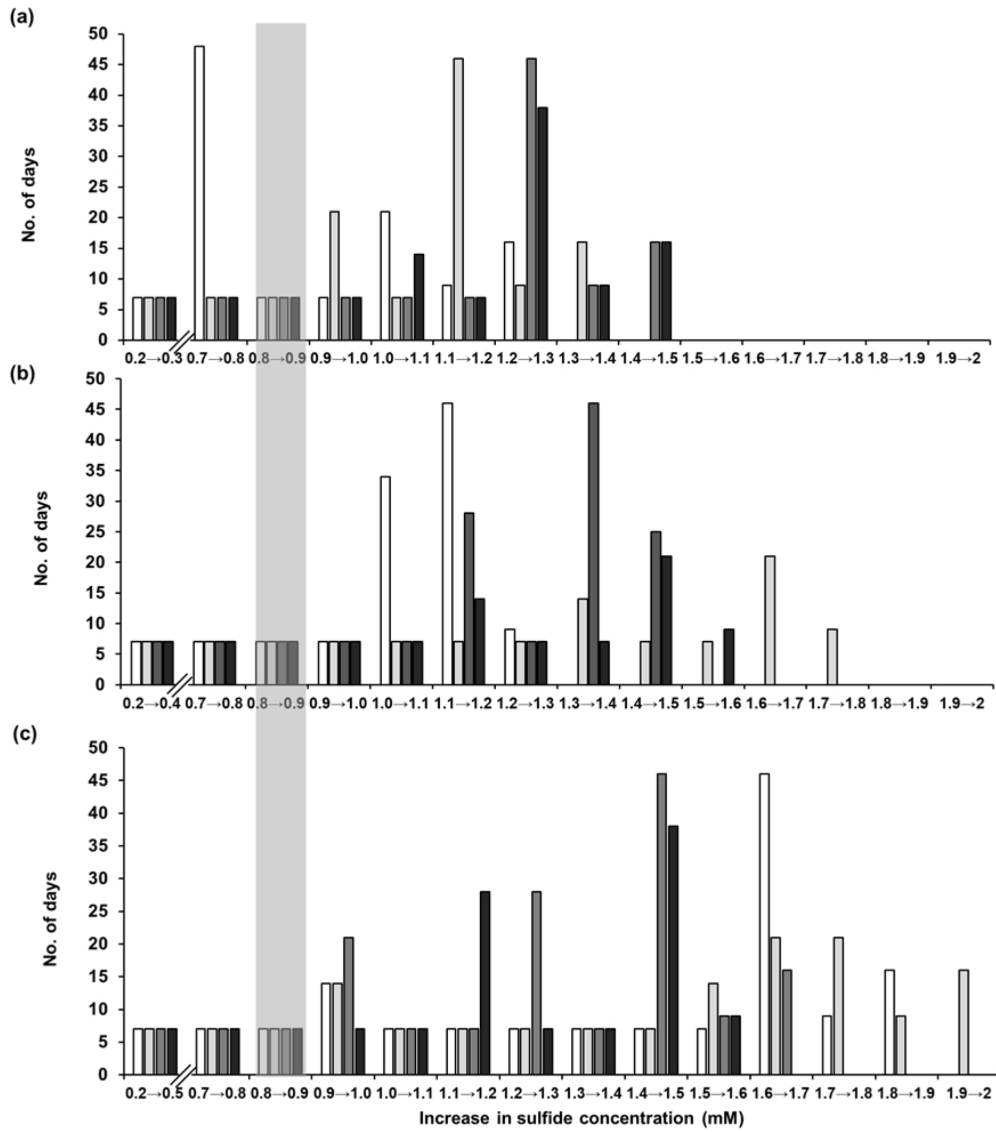
This article is protected by copyright. All rights reserved.



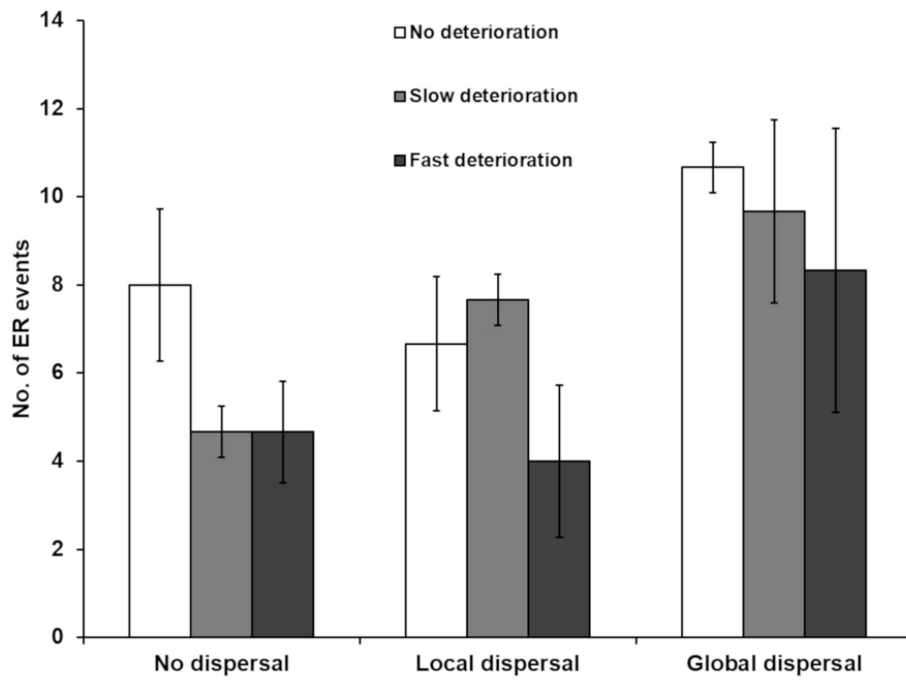
This article is protected by copyright. All rights reserved.



This article is protected by copyright. All rights reserved.



This article is protected by copyright. All rights reserved.



This article is protected by copyright. All rights reserved.