Aquatic Toxicology 240 (2021) 105973

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Detection of the maximum resistance to the herbicides diuron and glyphosate, and evaluation of its phenotypic cost, in freshwater phytoplankton

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ARTICLE INFO

Keywords:
chlorophycean
cyanobacteria
herbicide
ratchet protocol
resistance

ABSTRACT

One of the most important anthropogenic impacts on freshwater aquatic ecosystems close to intensive agriculture areas is the cumulative increase in herbicide concentrations. The threat is especially relevant for phytoplankton organisms because they have the same physiological targets as the plants for which herbicides have been designed. This led us to explore the evolutionary response of three phytoplanktonic species to increasing concentrations of two herbicides and its consequences in terms of growth and photosynthesis performance. Specifically, we used an experimental ratchet protocol to investigate the differential evolution and the limit of resistance of a cyanobacterium (*Microcystis aeruginosa*) and two chlorophyceans (*Chlamydomonas reinhardtii* and *Dictyosphaerium chlorelloides*) to two herbicides in worldwide use: glyphosate and diuron. Initially, the growth rate of *M. aeruginosa* and *D. chlorelloides* was completely inhibited when they were exposed to a dose of 0.23 ppm diuron or 40 ppm glyphosate, whereas a higher concentration of both herbicides (0.46 ppm diuron or 90 ppm glyphosate) was necessary to abolish *C. reinhardtii* growth. However, after running a ratchet protocol, the resistance of the three species to both herbicides increased by an adaptation process. *M. aeruginosa* and *D. chlorelloides* were able to grow at 1.84 ppm diuron and 80 ppm glyphosate and *C. reinhardtii* proliferated at twice these concentrations. Herbicide-resistant strains showed lower growth rates than their wild-type counterparts in the absence of herbicides, as well as changes on morphology and differences on photosynthetic pigment content. Besides, herbicide-resistant cells generally showed a lower photosynthetic performance than wild-type strains in the three species. These results indicate that the introduction of both herbicides in freshwater ecosystems could produce a diminution of primary production due to the selection of herbicide-resistant mutants, that would exhibit lower photosynthetic performance than wild-type populations.

1. Introduction

One of the main environmental threats resulting from the impact of human activities characterizing the Anthropocene is the loss of biological diversity, resulting in extinction rates of species up to a hundred times faster than the value estimated over geological time (Pimm et al., 2014). For this reason, it has been proposed that we are witnessing Anthropocene extinction (Ceballos et al., 2015; Ceballos and Ehrlich, 2018). The biodiversity crisis has been intensively addressed for vertebrates, but little is known about what could happen among microorganisms (Ceballos et al., 2010). Studies on protists and bacteria are clearly necessary because most of life on Earth, as well as biogeochemical cycles, pivot on them (Woodruff, 2001). Given that phytoplankton are the main contributors to primary production in aquatic

Abbreviations: a, chlorophyll-specific optical absorption cross section; A_{750}, absorbance at 750 nm; CD, cell density; Chl *a*, chlorophyll *a*; DMSO, dimethyl sulfoxide; *DR*, dark respiration rate; *ETR*, electron transport rate; ETR_{max}, maximum *ETR*; g, number of generations; I, irradiance; I_c, irradiance compensation point; $I_{0.5}$, half-saturation irradiance; m, growth rate; *NPR*, net photosynthetic rate; NPR_{max}, maximum *NPR*; N_t, final no. of cells; N_0, initial no. of cells; PC, phycocyanin; PSII, photosystem II; TC, total carotenoids; α^{ETR}, Photosynthetic efficiency calculated from *ETR-I* relationship; α^{NPR}, Photosynthetic efficiency calculated from *NPR-I* relationship; F_v/F_m, maximum quantum yield of PSII; β, photoinhibition parameter.

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https://doi.org/10.1016/j.aquatox.2021.105973
Received 23 March 2021; Received in revised form 9 September 2021; Accepted 15 September 2021
Available online 21 September 2021

ecosystems (Kirk, 1994; Raven and Falkowski, 1999), understanding how microalgae and cyanobacteria can evolve in scenarios of intense environmental deterioration is crucial from an eco-evolutionary point of view. One of the stressing future scenarios in freshwater ecosystems is due to the increase in herbicide use in areas of intensive agriculture (Arts and Hanson, 2018; Koenig, 2001; Vila-aiub et al., 2009). Because herbicides are persistent, they exert a strong selection pressure on numerous non-target species (Arts and Hanson, 2018; Belfiore and Anderson, 2001; Mcknight et al., 2015; Palumbi, 2001), including cyanobacteria and microalgae (Cedergreen and Streibig, 2005; Lu et al., 2021; Muturi et al., 2017; Ramakrishnan et al., 2010; Smedbol et al., 2018). Two of the most relevant herbicides impacting freshwater ecosystems are glyphosate ($C_3H_8NO_5P$, *N*-(phosphonomethyl) glycine) and diuron ($C_9H_{10}Cl_2N_2O$, 3-(3,4-dichlorophenyl)-1,1-dimethylurea) (Arts and Hanson, 2018). Glyphosate is a broad-spectrum non-selective herbicide, which has been extensively used to eliminate weeds in agricultural, forest and aquatic systems (Duke and Powles, 2008; Van Bruggen et al., 2018). This herbicide inhibits the shikimate pathway enzyme 5-enolpyruvylshikimate-3-phosphate synthase, EPSPS (Steinrücken and Amrhein, 1980), which is essential for the biosynthesis of aromatic compounds in bacteria, algae, plants and fungi (Healy-Fried et al., 2007). It is known to be toxic to non-target aquatic microorganisms, fishes and amphibians (Arunakumara et al., 2013; Bridi et al., 2017; Edge et al., 2013; López-Rodas et al., 2007; Mensah et al., 2011; Samanta et al., 2014; Wu et al., 2016; Zhang et al., 2018), and recent studies show toxic effects on humans (Van Bruggen et al., 2018). Glyphosate has been detected in water bodies in agricultural areas reaching occasionally 0.3-0.4 ppm (Annett et al., 2014; Fugère et al., 2020; Van Bruggen et al., 2018). Diuron inhibits electron transport in the PSII (van Rensen, 1989) and it is considered a 'Priority Hazardous Substance' by the European Commission (European Water Framework Directive 2000/60/EC). Several studies have reported diuron presence in surface waters in a range from 1×10^{-4} to 1×10^{-2} ppm with peak concentrations up to 2.7×10^{-2} ppm (Blanchoud et al., 2004; Field et al., 2003; Hernández et al., 2008; Huang et al., 2004; Rabiet et al., 2010; Rodriguez-Mozaz et al., 2004).

There is ample evidence that microalgae and cyanobacteria can overcome the challenges of the increased concentrations of herbicides in freshwater ecosystems through rapid evolutionary processes showing a physiological cost (Baselga-Cervera et al., 2016; Cameron et al., 2019; Huertas et al., 2010; López-Rodas et al., 2007, 2008; Marvá et al., 2010; Reboud et al., 2007; Rouco et al., 2014). On the other hand, it has been observed that the toxic effect of herbicides on these organisms could lead to decreased primary productivity and cause significant impacts at different ecosystem levels (Arts and Hanson, 2018; Bester et al., 1995; DeNoyelles et al., 1982; Dorigo and Leboulanger, 2001; Occhipinti-Ambrogi and Sheppard, 2007; Reish et al., 2004; Smedbol et al., 2018). Consequently, it is relevant to address what would be the limit of resistance allowing the survival of freshwater phytoplankton species under herbicide concentrations exceeding the initial lethal levels.

The aim of this work was to explore the differential evolutionary potential of freshwater phytoplankton model species growing under increasing concentrations of glyphosate and diuron: the cosmopolitan cyanobacterium, *Microcystis aeruginosa*, and two species of chlorophyceans, *Chlamydomonas reinhardtti* (also showing a cosmopolitan distribution) and *Dictyosphaerium chlorelloides*. These species show different degree of tolerance to other herbicides (Baselga-Cervera et al. 2016) and could respond differently to the exposure to glyphosate and diuron. We analyzed the limit of resistance to both herbicides in the three phytoplankton species using a ratchet protocol. Moreover, the mechanisms (acclimation *vs*. adaptation) allowing the herbicide resistance processes were tested. We considered that resistance is caused by acclimation when it is based on the modification of the expression of genes already present in the populations (Bradshaw and Hardwick, 1989; Fogg, 2001; Borowitzka, 2018). On the other hand, resistance is caused by adaptation when there is a selection of new genotypes which appear randomly due to spontaneous mutations (Belfiore and Anderson, 2001; Borowitzka, 2018). Finally, in order to estimate the physiological cost of the resistance to these herbicides, the growth rate, morphology, photosynthetic pigment content and photosynthetic performance of the derived strains were compared to those from wild-type cells. Based on our results, we hypothesize that the impact of increased herbicide concentrations in freshwater ecosystems will involve changes in composition and productivity of phytoplankton, due to the selection of herbicide-resistant strains characterized by lower growth rates than their ancestral counterparts.

2. Materials and methods

2.1. Experimental organisms and culture conditions

Three phytoplankton species were used in the experiments: the cyanobacterium *Microcystis aeruginosa* (Kützing) Kützing and the chlorophyceans *Dictyosphaerium chlorelloides* (Nauman) Komárek & Perman and *Chlamydomonas reindhardtii* P. A. Dang. A strain of each species was supplied by the Algal Culture Collection, Veterinary School of the Universidad Complutense (Madrid, Spain): the strain MaAVc of *M. aeruginosa* (isolated from Valmayor reservoir, Community of Madrid, Spain); the *D. chlorelloides* Dc1M strain (isolated from a pristine mountain lake in Sierra Nevada, S Spain) and the *C. reindhardtii* ChlaA strain (isolated from a freshwater pond in Doñana National Park, SW Spain). Details of isolation procedures were described in Carrillo et al. (2003) and culture conditions were the same as in our previous studies (Martín-Clemente et al., 2019; Melero-Jiménez et al., 2019, 2020). In short, culture medium was BG-11 (Sigma Aldrich Chemie, Taufkirchen, Germany) diluted 50% (BG-11-50%) and cultures were maintained in mid-log exponential growth at 20 °C and an irradiance of 60 μmol m^{-2} s^{-1}. It must be highlighted that under these culture conditions the MaAVc strain grows as single cells and does not form colonies (as indicated by periodically checking with an inverted microscope).

2.2. Toxicity test: effect of herbicides on growth rate

The toxic effect of glyphosate and diuron (both compounds supplied by Sigma Aldrich Chemie, Taufkirchen, Germany) on growth rate of the phytoplankton cells was assessed as follows. A stock solution of glyphosate was prepared in BG11-50% medium and serial dilutions of 10, 25, 50, 100 and 150 ppm glyphosate were obtained. A stock solution of diuron was prepared in dimethyl sulfoxide (DMSO) (99.9%, supplied by Panreac Química, Spain) and aliquots were added to BG11-50% medium to obtain final concentrations of 2.3,11, 23, 34 and 46 $\times10^{-2}$ ppm diuron. The concentration of DMSO in cultures was always less than 0.05% v/v. We initiated experimental populations inoculating 1.5×10^5 cells from mid-log exponentially growing populations. Four replicates were prepared of each herbicide concentration, unexposed controls, and controls containing DMSO 0.05% (v/v) in the case of the diuron assays and were grown at the same temperature and irradiance indicated before. The effect of each herbicide was assessed by calculating growth rate (m) in mid-log exponentially growing cells (Crow and Kimura, 1970; Spiess and Florian, 1989). Specifically, m was computed as $\log_e(N_t/N_0)/t$, where $t = 6$ days, and N_t and N_0 were the cell numbers at the end (sixth day) and at the start of the experiment, respectively. The number of cells was estimated by linear regression fit between cell density (CD; units in cells•mL^{-1}) and the absorbance at $\lambda = 750$ nm (A_{750}):

CD (cells mL^{-1}) = $1.10\times10^7 \times A_{750}$ ($r^2 = 0.980$, $n = 18$) for *M. aeruginosa*
CD (cells mL^{-1}) = $4.35\times10^6\times A_{750}$ ($r^2 = 0.981$, $n = 28$) for *C. reinhardtii*
CD (cells mL^{-1}) = $7.87\times10^6 \times A_{750}$ ($r^2 = 0.978$, $n = 28$) for *D. chlorelloides*

2.3. Ratchet protocol to detect the limit of resistance to herbicides

The experiment consists of exposing large populations to increasing concentrations of the selective agent (herbicides), in order to ensure a population size large enough to assure the occurrence of new mutations that confer resistance. When populations reach a given cellular concentration, they are then exposed to a higher level of herbicide. The experiment is terminated when cell growth is not detected. This experimental approach has been used previously to detect the limits of resistance of phytoplankton to anthropogenic and natural selective agents (Huertas et al., 2011, 2010; Martín-Clemente et al., 2019; Melero-Jiménez et al., 2019, 2020; Reboud et al., 2007; Rouco et al., 2014). First, we made serial dilutions to obtain a single cell of each species, in order to minimize the probability of including any resistant cell that might be present in the population (Andersen and Kawachi, 2005). The number of replicates of experimental cultures (containing glyphosate or diuron) and control cultures (without herbicides) was four. The initial herbicide doses were identical for all species, being 10 ppm glyphosate or 11 $\times 10^{-2}$ ppm diuron. Cultures were grown separately in 24-well plates at the same temperature and irradiance as stock cultures. Replicates were prepared in 2 mL culture volume, inoculated with 1×10^5 cells per well and were kept under the experimental herbicide level for 7 d prior to observation to avoid populations from reaching their carrying capacity. At this point, we compared cell concentrations of control and experimental cultures. Experimental populations were only ratcheted to the next cycle of herbicide concentration when their cell concentration were the same or higher than those observed on control populations. At the starting point of each ratchet cycle, both control and experimental cultures were inoculated with the same cell concentrations than those used at the beginning of the ratchet experiment (1×10^5 cells per well). The experiment finished when no further cell growth was observed after 100 d of culture at the same herbicide concentration (Fig. 1). Finally, we determined the maximum level of resistance as the highest herbicide concentration that allowed the occurrence and growth of a resistant variant. The number of generations (g) during the ratchet experiment was calculated following Novick and Szilard (1950).

2.4. Disentangling acclimation vs adaptation

The ratchet protocol is not designed to disentangle the resistance mechanism (acclimation *vs* adaptation) supporting the highest resistance capacity. For this reason, to test whether the limit of resistance to herbicides resulted from acclimation or by selection of new genetic variants arising by mutations, the approach designed by Rouco et al. (2014) was applied. This complementary experiment is based on the fact that, at least for bacteria, acclimation effects processes, including unstable epigenetic events affecting gene expression, can span two-three generations (Bennett and Lenski, 1997), so a change in growth rate could be expected if derived cells from the ratchet experiment are returned back for a few generations to a medium without herbicides.

In this complementary experiment (Fig. 2), resistant populations, derived from the ratchet experiment, were incubated in free-herbicide medium during ~4 generations and growth rate was computed (m_1; Fig. 2). Then, cells were exposed to the highest herbicide level that they could tolerate for another ~4 generations and growth rate was calculated again (m_2; Fig 2). Specifically, adaptation can be recognized in herbicide-resistant cells if the values of m_1 and m_2 are similar, i. e. before and after the addition of herbicide. Alternatively, if acclimation were involved in the resistance process (although genetic changes cannot be totally rejected), m_2 must be significantly lower than m_1.

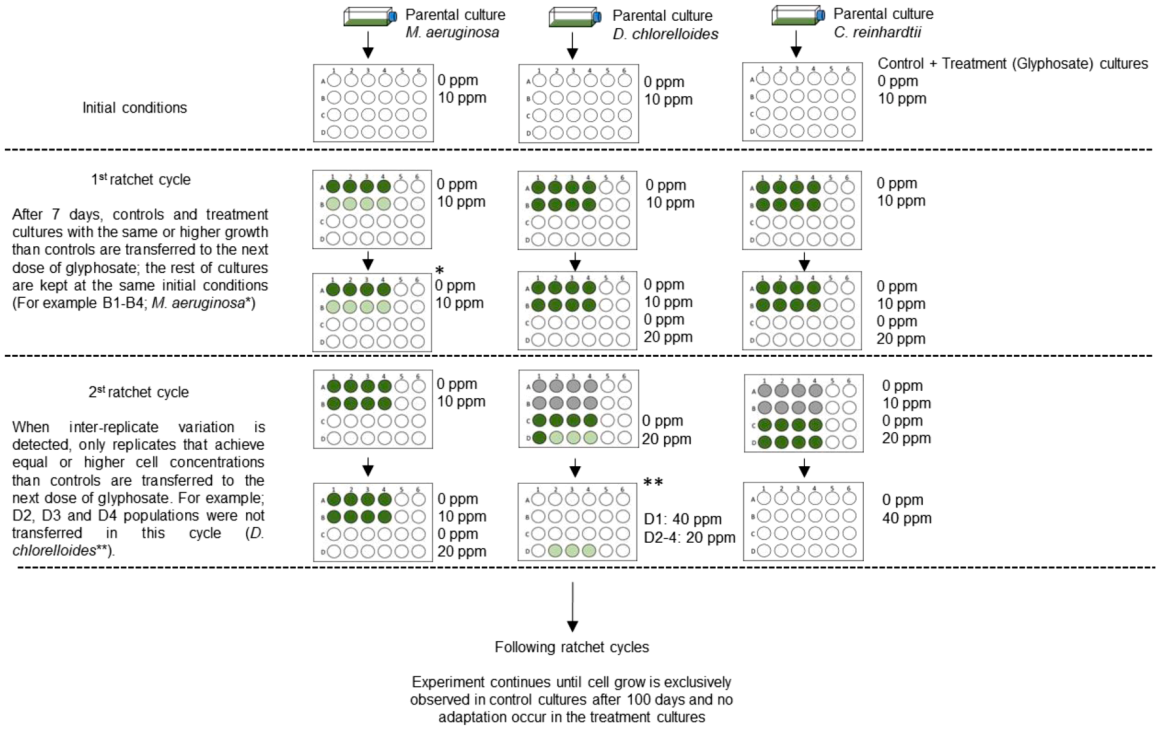

Fig. 1. Ratchet experimental design scheme. In each species analysed, 4 replicates (columns) of the control cultures (row A wells) and 4 replicates of treatment cultures at the initial dose of glyphosate (row B wells) or diuron (not shown) were incubated for 7 d at each ratchet cycle. Each culture was transferred to the next, 2-fold higher herbicide concentration when similar growth rates were observed in control and treatment cultures. Populations that did not grow as much as control cultures were maintained another 7 d at the same herbicide concentration (marked with asterisks in the figure). A new ratchet cycle began when the treatment cultures were transferred to the next, higher herbicide concentration. The limit of tolerance of a species corresponds to the maximum herbicide concentration at which cell growth was detected. The concentrations tested for both herbicides are shown in Fig. 3.

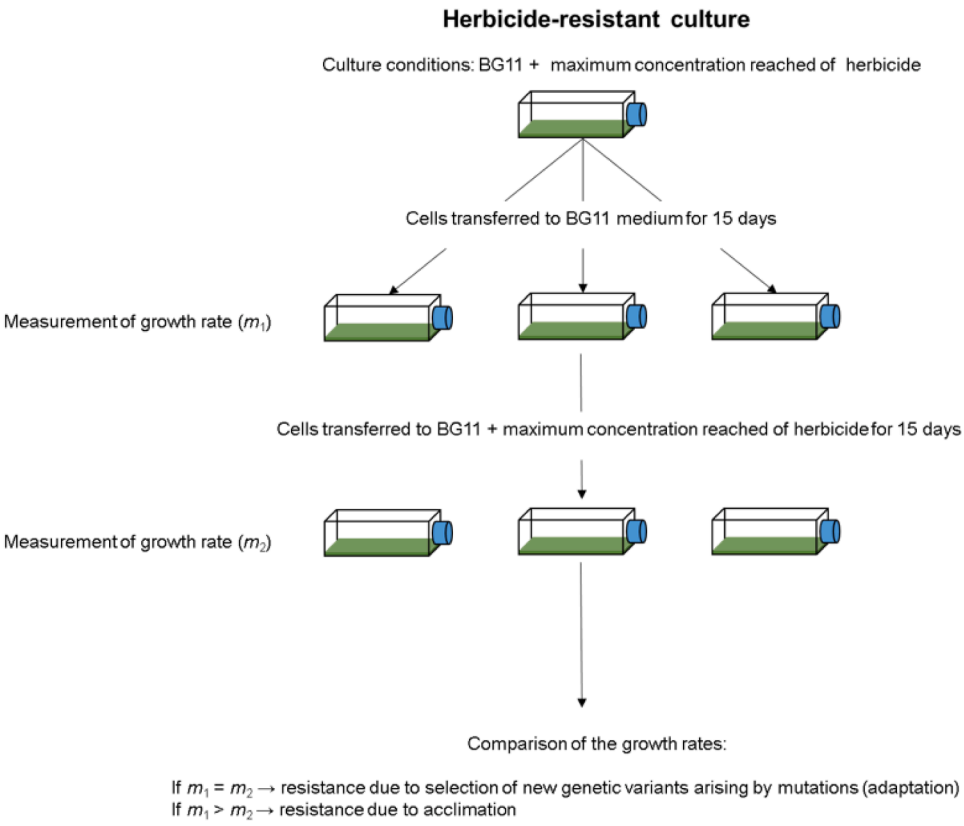

Fig. 2. Experimental design to disentangle the mechanism, adaptation or acclimation, involved in the herbicide resistance of cells derived from the ratchet protocol. In the first step, herbicide resistant cells were cultured for 15 d in BG11-50% medium (without herbicide) and the growth rate (m_1) was computed. Secondly, herbicide resistant cells were exposed for another 15 d to BG11-50% supplemented with the highest herbicide concentration reached in the ratchet experiment, and the growth rates (m_2) were monitored again. Similar m_1 and m_2 values suggest that resistance is mainly due to the selection of new genetic variants, whereas if m_1 is significantly higher than m_2, it can be inferred that resistance is mainly caused by acclimation.

2.5. Cell volume and photosynthetic pigment concentration

Wild-type and herbicide-resistant (to diuron or to glyphosate) cells, were analyzed with a periodically calibrated FlowCAM® VS Benchtop model and VisualSpreadsheet® software (Fluid Imaging Technologies, Scarborough, ME, USA). Samples were analyzed in autoimage mode (25 frames per second) using a 50-µm Flow Cell FC50 at ×200 magnification. For statistical comparison of the cell size among the treatments, one hundred fifty cells were selected randomly from the analyzed cell pool. Pigment concentrations were determined spectrophotometrically following the protocols previously detailed in Bañares-España *et al.* (2016).

2.6. Photosynthetic performance of the ancestral and the derived herbicide-resistant cells

Net photosynthetic rate (*NPR*) as a function of irradiance (*I*) was estimated from O_2 production by using an Oxygraph system DW1/AD (Hansatech Instruments Ltd, Norfolk, UK) connected to a controlled temperature circulating bath (20 ± 1 °C). Samples were incubated in a temperature-controlled magnetic gentle stirring chamber illuminated with cool-white fluorescent lamps (Sylvania Grolux 36W, Erlangen, Germany). Samples of a cell density of 5×10^6 cells mL^{-1} in 2 ml BG-50% medium were incubated for 15 min in darkness and then exposed to eight irradiance (*I*) levels (from 5 to 300 µmol photons m^{-2} s^{-1}) for 5 min each. Irradiance was measured with a spherical quantum sensor (US-SQS/L, Walz, Germany), connected to a radiometer (Model Li-250 Light Meter, Lincoln, NE, USA). Four replicates of *NPR-I* curves were carried out with the ancestral and derived strains of the three species. Data of the *NPR-I* relationship were fitted to the Edwards and Walker (1983) model (eq. 1):

$$NPR = NPR_{max} \times (I - I_c)/(I + I_{0.5}) \quad (1)$$

where NPR_{max} is irradiance-saturated *NPR*, I_c is the irradiance compensation point, and $I_{0.5}$ is the half-saturation irradiance. The photosynthetic efficiency (α^{NPR}) was computed as the initial slope of the linear fit of the four initial values of the *NPR-I* relationship. The dark respiration rate (*DR*) was measured in darkness.

The electron transport rate (*ETR*) was computed from photochemical efficiency of PSII ($\Delta F/F'_m$) measurements at a given *I* value, following Genty et al. (1989)(eq. 2):

$$ETR = I \times \Delta F/F'_m \times a \times \text{fraction PSII} \quad (2)$$

where *a* is the chlorophyll-specific optical absorption cross section calculated as in Figueroa et al. (2003) and fraction PSII accounts for the fraction of irradiance arriving to PSII (0.5 for chlorophyceans and 0.36 for cyanobacteria; Johnsen and Sakshaug, 2007). The maximum quantum yield of PSII (F_v/F_m) was measured in cells exposed to darkness for 30 min. Data of the *ETR-I* relationships were fitted to the Platt et al. (1980) model (eq. 3):

$$ETR = ETR_{max} \times \left[1 - \exp\left(-\alpha^{ETR} \times I / ETR_{max}\right)\right] \times \exp(-\beta \times I / ETR_{max}) \quad (3)$$

where ETR_{max} is the maximum irradiance-saturated *ETR*, α^{ETR} is the photosynthetic efficiency and β is the photoinhibition parameter.

2.7. Statistical analysis

The m_1 *and* m_2 values of herbicide-resistant cells, as well as the *m* values of the wild-type cells of the same species were compared using the non-parametric Kruskal–Wallis *H* test. We applied a Mann–Whitney test (with Bonferroni correction) when significant differences were detected. Cell volume, photosynthetic pigment concentrations and photosynthetic parameters from all strains were compared by ANOVA. When significant differences were found, the Tukey test was performed. The homogeneity of variances from data analyzed by ANOVA was

previously checked by Levene test. All the statistical analyses were performed using the free software R Core Team (2020)

3. Results

3.1. Ratchet experiment

The growth rate of *M. aeruginosa* and *D. chlorelloides* was completely inhibited when cells of these species were exposed for 6 d to 0.23 ppm diuron or 40 ppm glyphosate, whereas a higher concentration of both herbicides (0.46 ppm diuron or 90 ppm glyphosate) was necessary to abolish *C. reinhardtii* growth (Fig. 3). However, after running the ratchet experiment, several populations of the three species showed an increment of the maximum level of both herbicides that they could tolerate. While *M. aeruginosa* and *D. chlorelloides* were able to survive up to 1.84 ppm diuron and 80 ppm glyphosate after the experiment, *C. reinhardtii* was able to proliferate in up to 3.68 ppm diuron and 160 ppm glyphosate (Fig. 4). It must be highlighted that because each replicate per species evolved as an independent population, different numbers of generations were required for each species to reach to the same cell concentration as control cultures, especially at the highest herbicide concentrations (Fig. 4). For example, three isolates of *M. aeruginosa*, were able to grow at 40 ppm glyphosate after different numbers of generations, whereas another isolate of the same strain was unable to survive at this concentration (Fig. 4).

3.2. Disentangling acclimation vs adaptation

Since the ratchet protocol is not specifically designed to determine the mechanism involved in the survival at the highest selection pressure (i.e., to discriminate between acclimation and adaptation), we used an indirect approach to analyse this issue. In the three phytoplankton species, no significant differences between the *m* values of the herbicide-resistant cells cultured in the absence or the presence of herbicide were detected (Fig. 5), suggesting that the maximum resistance to each herbicide was due to adaptation rather than acclimation. On the other hand, the *m* values of the ancestral, wild-type cells (Fig. 3) were two to three times higher than those found in the herbicide-resistant cells in the three species.

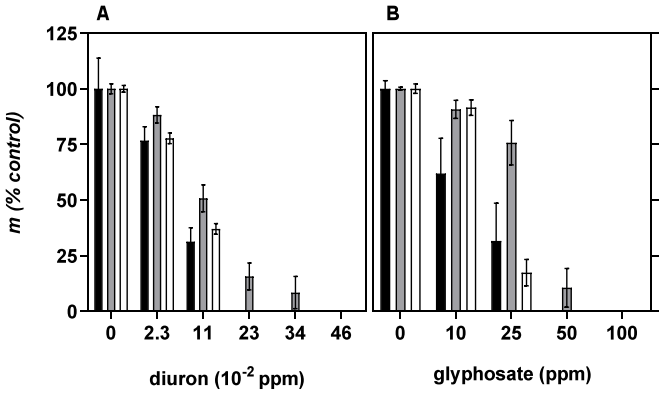

Fig. 3. Effect of diuron (A) and glyphosate (B) on the growth rate (*m*, percentage with respect to controls without herbicide) of the different freshwater phytoplankton species used in the ratchet experiment. The values of *m* were computed after 6 d of exposure to the different herbicide concentrations, in mid-log exponentially growing cells. Mean ± SD ($n = 4$). The 100% *m* values were 0.42 ± 0.05 (doublings d^{-1}) for *M. aeruginosa*, 0.63 ± 0.01 (doublings d^{-1}) for *C. reinhardtii* and 0.51 ± 0.15 (doublings d^{-1}) for *D. chlorelloides*.

3.3. Cell volume and pigment content of ancestral and derived, herbicide-resistant cells

The wild-type cells of the three species showed a greater volume than herbicide-resistant cells (Table 1), except for the diuron-resistant *M. aeruginosa* cells, which showed no change of volume. Cell volume of both resistant strains were similar in microalgae species, however glyphosate-resistant cells were statistically smaller than diuron-resistant cells of *M. aeruginosa* (Table 1).

In relation with photosynthetic pigment content, we only detected significant differences on cellular Chl *a* content in diuron-resistant cells with respect to wild type cells in *M. aeruginosa* (Table 1). With respect to TC concentration, the pattern was the inverse: wild-type cells showed a higher cell TC content than diuron-resistant cells in *M. aeruginosa*. However, in *C. reinhardtii* strains, the result was the opposite for diuron-resistant cells and no differences were found in glyphosate-resistant cells. The PC content of wild-type cells was higher than those of both herbicide-resistant strains of *M. aeruginosa* (Table 1). Differences on pigment content where not detectable between wild-type and herbicide-resistant cells of *D. chlorelloides*.

3.4. Photosynthetic performance and respiration of ancestral and derived, herbicide-resistant cells

The physiological cost of the resistance to herbicides was explored by analyzing the main metabolic process in phytoplankton cells, i.e., photosynthesis. The NPR_{max} of the wild-type, sensitive cells were approximately two-three times higher than those of herbicide-resistant cells in microalgae species (Fig. 6; Table 1). However, there was only a slight decrement in NPR_{max} of diuron-resistant cells of *M. aeruginosa* cells, and no significant differences were observed in glyphosate-resistant cells (Fig. 6; Table 1). Additionally, all herbicide-resistant cells showed a higher I_c than wild-type cells (Table 1). The increment on I_c values was more than double in *M. aeruginosa* resistant cells, whereas I_c values increased in a range of 1.1-1.6 on microalgae resistant cells (Table 1). The cost of resistance on $I_{0.5}$ values differed between the cyanobacterium and the two microalgae: in the case of *M. aeruginosa,* diuron resistance did not affect $I_{0.5}$ values whereas glyphosate resistance produced a slightly increase of this value. In the case of the green microalgae, diuron resistance caused a higher diminution on $I_{0.5}$ values than glyphosate resistance in *C. reinhardtii*; however, this difference was not observed for *D. chlorelloides* resistant cells, with a similar reduction of the $I_{0.5}$ values for both herbicides. We did not find differences among α^{NPR} values in the ancestral *vs* derived cells of the three species (Table 1).

The maximum quantum efficiency of PSII (F_v/F_m) of the glyphosate-resistant *M. aeruginosa* strain was significantly higher than those of the wild-type or diuron-resistant strains (Table 1). A different pattern was found in the two chlorophyceans, where the highest F_v/F_m figures were observed in wild-type cells, followed by the glyphosate-resistant strains and, the lowest values were detected in diuron-resistant strains (Table 1).

In relation with ETR_{max} and α^{ETR}, the values were always higher in wild-type cells of the three species than in herbicide-resistant, derived counterparts, with the exception of α^{ETR} of glyphosate-resistant *D. chlorelloides* that did not change (Table 1). The β values were not significantly different from 0 for all species, indicating that resistance to both herbicides did not imply a photoinhibition process at least for the highest irradiance tested in the experiments.

The effect of herbicide-resistance on respiration differed among species and herbicides. We observed that the *DR* increased in both diuron- and glyphosate-resistant *M. aeruginosa* cells, being twice the value observed in wild-type cells. (Table 1). However, an increment on *DR* was only detected in diuron-resistant cells of *D. chlorelloides* (Table 1).

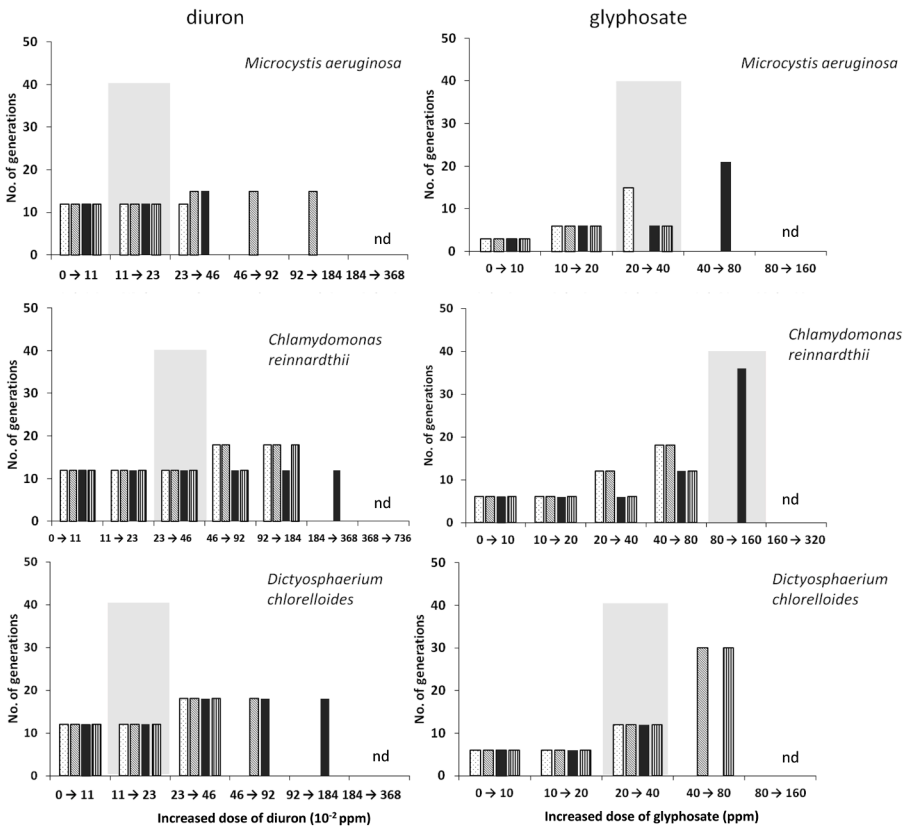

Fig 4. Number of generations required to grow under increasing doses of diuron and glyphosate during the ratchet experiment. Four independent cultures (represented by different column patterns) were tested per each herbicide and species. The ratchet experiment was finished when growth was not observed in any of the four replicates tested (designated 'nd', non-detectable growth). Grey shading over a ratchet cycle indicates that the concentration tested equalled or surpassed the initial lethal dose for each herbicide and species (see Fig. 3).

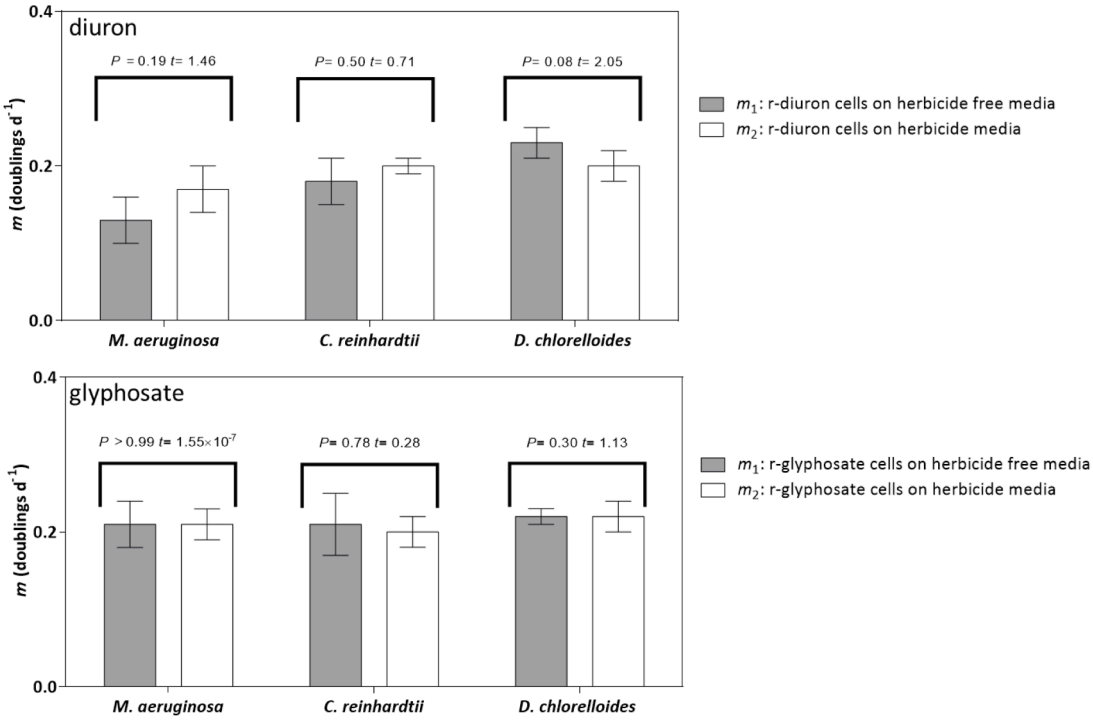

Fig. 5. Growth rate (m) of herbicide-resistant cells (r-diuron and r-glyphosate) obtained in the experiment of acclimation *vs* adaptation (see Fig.2). Columns show mean ± SD ($n = 4$). No significant differences were found between m_1 and m_2 (Student's t-test, $df = 6$)

4. Discussion

It is known that the tolerance to herbicides of phytoplankton species vary depending on lineages and species (Beaulieu et al., 2020; Fugère et al., 2020; Huertas et al., 2010; Knauert et al., 2009; Kumar et al., 2014; Lam et al., 2020; Lu et al., 2021; Marvá et al., 2010; Muturi et al., 2017; Rouco et al., 2014; Vendrell et al., 2009), as it has been observed in our results. Specifically, we found that glyphosate and diuron doses

Table 1
Cell volume, photosynthetic pigment concentrations, photosynthetic parameters derived from the *NPR-I* and *ETR-I* relationships and dark respiration rate of wild-type and herbicide-resistant strains (r-diuron and r-glyphosate) of *M. aeruginosa, C. reinhardtii* and *D. chlorelloides*. Significant differences among parameters, detected by ANOVA (columns of *F* and *P*-values are included) and post hoc Tukey test, are indicated by different letters. Data are mean ± SD ($n = 150$ for cell volume and $n = 4$ for photosynthetic pigment concentrations, photosynthetic parameters and respiration rate). Units: cell volume, μm^3; Chl *a*, TC and PC, fg $cell^{-1}$; NPR_{max}, nmol O_2 10^6 $cell^{-1}$ h^{-1}; α^{NPR} nmol O_2 10^6 $cell^{-1}$ h^{-1} [µmol photons m^{-2} s^{-1}]$^{-1}$; I_c and $I_{0.5}$, µmol photons m^{-2} s^{-1}; ETR_{max}, µmol photons m^{-2} s^{-1}; α^{ETR}, mol e− [mol photons]$^{-1}$; *DR*, nmol O_2 10^6 $cell^{-1}$ h^{-1}

M. aeruginosa Phenotypic trait	wild-type	r-diuron	r-glyphosate	*F*	*P*
Cell volume	54 ± 3^a	53 ± 3^a	49 ± 3^b	82.47	<0.01
Chl *a*	79 ± 8^a	132 ± 25^b	87 ± 12^a	8.34	<0.01
TC	21 ± 4^a	9 ± 7^b	13 ± 1^{ab}	5.28	0.03
PC	8 ± 1^a	4 ± 1^b	4 ± 1^b	24.90	<0.01
NPR_{max}	85 ± 3^a	71 ± 4^b	79 ± 4^a	15.32	<0.01
α^{NPR}	2.7 ± 0.1	2.7 ± 0.2	2.7 ± 0.1	0.66	0.53
I_c	18 ± 1^a	54 ± 1^b	52 ±5^b	197.55	<0.01
$I_{0.5}$	28 ± 2^a	29 ± 2^a	36 ± 2^b	16.90	<0.01
F_v/F_m	0.58 ± 0.03^a	0.57 ± 0.02^a	0.62 ± 0.01^b	10.79	<0.01
ETR_{max}	3.7 ± 0.2^a	1.5 ± 0.1^b	3.7 ± 0.3^a	130.95	<0.01
α^{ETR}	0.04 ± 0.00^a	0.02 ± 0.00^b	0.04 ± 0.00^a	189.71	<0.01
DR	53 ± 3^a	122 ± 6^b	112 ± 9^b	139.77	<0.01
C. reinhardtii Phenotypic trait	wild-type	r-diuron	r-glyphosate	*F*	*P*
Cell volume	158 ± 65^a	137 ± 62^b	129 ± 58^b	9.00	<0.01
Chl *a*	1211 ± 448	1510 ± 331	927 ± 178	3.07	0.09
TC	227 ± 8^a	387 ± 53^b	235 ± 44^a	14.03	<0.01
NPR_{max}	223 ± 15^a	126 ± 4^b	171 ± 5^c	100.87	<0.01
α^{NPR}	4.5 ± 0.3	4.5 ± 0.4	4.7 ± 0.2	0.797	0.43
I_c	18 ± 1^a	21 ± 1^b	24 ± 1^c	41.69	<0.01
$I_{0.5}$	44 ± 5^a	22 ± 3^b	31 ± 0^c	40.18	<0.01
F_v/F_m	0.73 ± 0.01^a	0.57 ± 0.02^b	0.62 ± 0.01^c	190.61	<0.01
ETR_{max}	7.7 ± 0.4^a	5.2 ± 0.2^b	6.1 ± 0.3^c	61.49	<0.01
α^{ETR}	0.1 ± 0.00^a	0.08 ± 0.00^b	0.09 ± 0.00^c	146.47	<0.01
DR	102 ± 7	120 ± 13	116 ± 4	4.43	0.46
D. chlorelloides Phenotypic trait	wild-type	r-diuron	r-glyphosate	*F*	*P*
Cell volume	67 ± 33^a	54 ± 29^b	55 ± 34^b	8.24	<0.01
Chl a	2455 ± 550	2843 ± 475	2379 ± 90	0.90	0.44
TC	450 ± 141	589 ± 158	507 ± 16	0.98	0.41
NPR_{max}	110 ± 11^a	35 ± 3^b	36 ± 4^b	162.25	<0.01
α^{NPR}	3.0 ± 0.2^a	2.8 ± 0.5^a	2.1 ± 0.3^b	8.92	<0.01
I_c	22 ± 1^a	36 ± 4^b	34 ± 5^b	14.92	<0.01
$I_{0.5}$	36 ± 7^a	13 ± 3^b	18 ± 5^b	21.17	<0.01
F_v/F_m	0.72 ± 0.01^a	0.41 ± 0.01^b	0.67 ± 0.04^c	190.31	<0.01
ETR_{max}	2.3 ± 0.2^a	0.9 ± 0.1^b	1.6 ± 0.3^c	38.99	<0.01
α^{ETR}	0.1 ± 0.00^a	0.03 ± 0.00^c	0.1 ± 0.01^a	367.58	<0.01
DR	77 ± 7^a	101 ± 11^b	75 ± 5^a	4.43	<0.01

(40 ppm and 0.23 ppm, respectively) inhibiting the growth of the ancestral wild-type strains of *M. aeruginosa* and *D. chlorelloides* were half of those needed for *C. reinhardtii*. These lethal concentrations were in the range of those reported previously for *M. aeruginosa* (Costas and López-Rodas, 2006; Swain et al., 1994; Zhang et al., 2018), *C. reinhardtii* (Bruggeman et al., 2014; Galloway and Mets, 1984) and *D. chlorelloides* (Costas et al., 2001). Although the resistance to herbicides seems to be higher in chlorophyceans than in cyanobacteria (Hadjoudja et al., 2009; Ma, 2005; Takamura et al., 1990, 1989; Zeng et al., 2010), it must be highlighted that we did not observe any differences between *D. chlorelloides* and *M. aeruginosa* for both glyphosate and diuron.

When the three species were exposed to step-by-step increased concentrations of glyphosate and diuron, eventually surpassing their initial lethal value, we found that *C. reinhardtii* was able to grow in up to 160 ppm glyphosate and 3.68 ppm diuron, whereas *D. chlorelloides* and *M. aeruginosa* could only adapt up to 80 ppm glyphosate and 1.34 ppm diuron. These results demonstrated that the cells could grow at a concentration eight and two times higher than the initial lethal doses of diuron and glyphosate, respectively, i. e. at herbicide levels much higher than those observed at present in polluted areas. These results also indicate that *C. reinhardtii* would outnumber the other two species if herbicide concentrations increase in the environment. Although this result could be different in a phosphorus-deficient environment, where it has been reported that *M. aeruginosa* acquires a strong competitive advantage in the presence of glyphosate (Jones, 2021; Ren et al., 2017). However, these results confirm that the increase of herbicides in freshwater ecosystems will cause changes in the community's structure, as it has been observed recently (Lu et al., 2020).

The increase in glyphosate resistance of the two chlorophyceans was not very high compared with other herbicides; for example, derived populations of *D. chlorelloides* can tolerate 90 and 22 times the initial lethal dose of simazine and copper, respectively (Huertas et al., 2010; Rouco et al., 2014). In the case of *M. aeruginosa*, the increase in tolerance level to glyphosate (2 times) was also less to that found with simazine or copper (9 and 4-12 times the initial lethal dose, respectively; Baselga-Cervera et al., 2016). Furthermore, the increased tolerance to glyphosate was much lower than that reached with diuron. This is consistent with the results of a previous study, where it was observed that the rate of spontaneous mutations that allow adaptation to glyphosate was lower compared to other herbicides (Baselga-Cervera et al., 2016).

The mechanisms underneath the increase in resistance to herbicides could be acclimation (changes in gene expression) or adaptation (selection of new mutants' variants). It could be hypothesized that the former mechanism occurs at doses lower than the lethal concentration, while the latter is the mechanism operating above the initial lethal dose. However, it must be considered that the ratchet experiment cannot strictly discriminate between acclimation *vs* adaptation and, for this reason, we performed a complementary experiment following Rouco et al. (2014). We observed that the cells resistant to the highest herbicide level had a similar growth rate under non-stressing (in free-herbicide medium) or in the presence of herbicides (Fig. 5). This is an indication of selection of spontaneous mutants being involved in the increase of resistance of the three species analyzed, although other mechanisms cannot be excluded. In fact, heritable epigenetic changes have been recently proposed to contribute, together with DNA sequence changes, to evolutionary adaptation in microalgae (Kronholm et al. 2017), and these stable epigenetic changes have also been observed in bacteria (Maier et al. 2017). On the other hand, the growth rate of resistant cells of the three species were lower than those of their ancestors under non-herbicide environment (Fig. 3). This fact could be related with the physiological cost of the genetic change conferring herbicide resistance (Darmency et al., 2015), and it has been reported previously on herbicide resistant populations of *C. reinhardtii, D. chlorelloides* and *M. aeruginosa* (Lagator et al., 2013; Rouco et al., 2014; Vogwill et al., 2012). For this reason, we suggest that the mechanism that permits survival after the ratchet experiment is adaptation. Moreover, since each replicate at the ratchet experiment evolved as an independent population with a different evolutionary trajectory, the differences among replicates can also give us information about the resistance mechanism (acclimation or adaptation) allowing the survival of the population. The reasoning is that, if acclimation is the main process involved in resistance, all cells must have the same probability to develop resistance and, consequently, no differences among replicates should occur. On the contrary, if adaptation is the main component supporting resistance, differences among replicates can be detected depending on the timing of

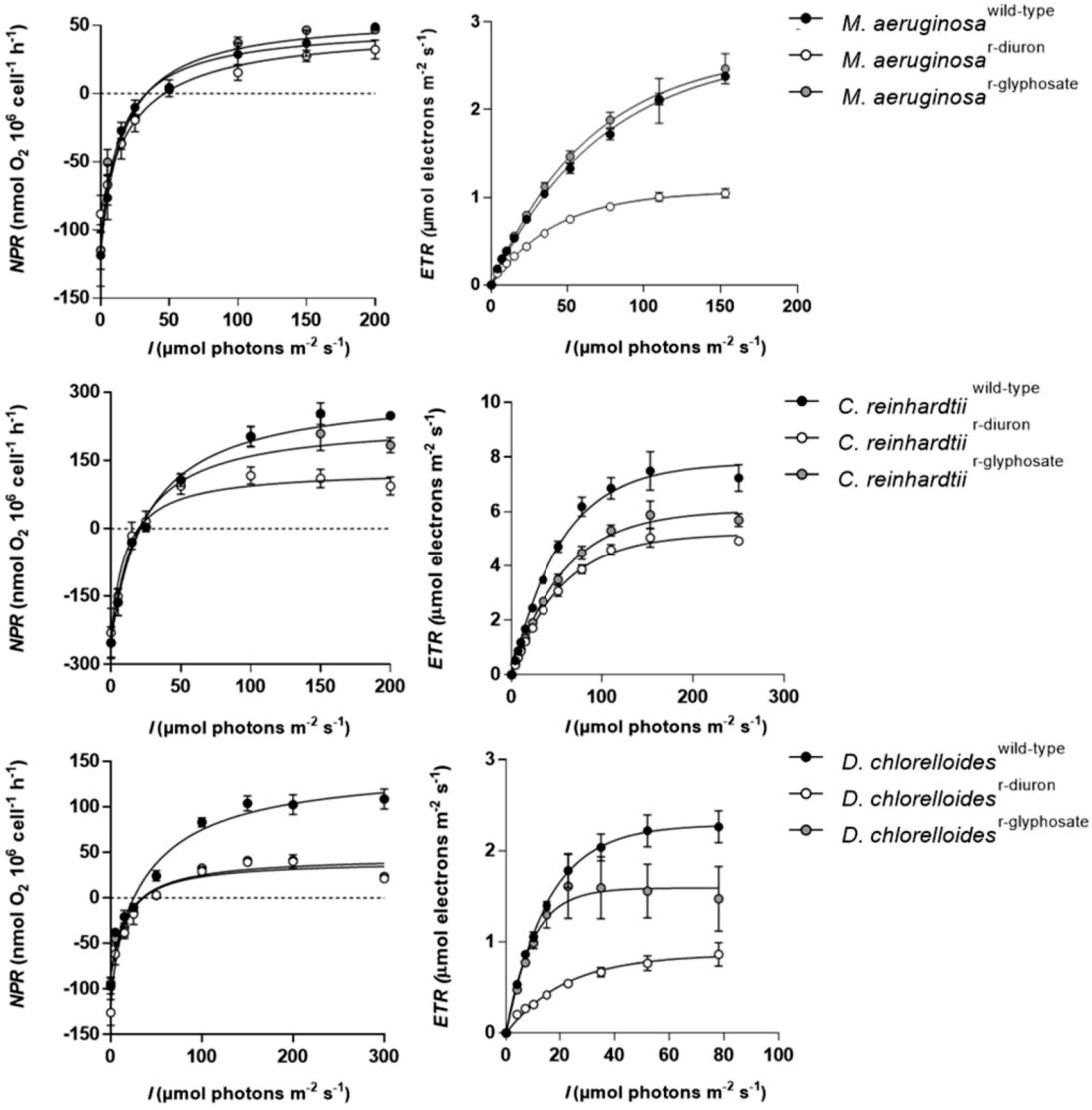

Fig. 6. Net photosynthetic rate (*NPR*) and electron transport rate (*ETR*) at increasing irradiances (*I*) of the wild-type and herbicide-resistant strains of each species. Data are mean ± SD (n=4). Lines indicate curve fitting of the values to the Edwards & Walker (1983) equation for *NPR-I* and Platt et al. (1980) equation for *ETR-I* relationships. The photosynthetic parameters derived of the fitting are shown in Table 1.

mutations (or other heritable changes) allowing resistance and the number of descendant mutant cells. Since a high variability among replicates occurred (Fig. 4), this result also supports the conclusion that the maximum resistance to herbicides was achieved by the selection of new genetic variants appearing during the experiment.

We detected that the resistance to both herbicides resulted in modifications on morphology and pigment content. These observations are in accordance with previous experimental evolution studies in these species in which changes on morphology and pigment content were detected during the process of adaptation to other selective agents (Bañares-España et al., 2016; Costas and López-Rodas, 2006; López-Rodas et al., 2007). In this sense, we could hypothesize that the presence of different pesticides on freshwater ecosystems would cause the appearance of new morphological populations with different pigment contents.

Herbicide-resistant cells showed drastic alterations of photosynthetic performance. Thus, photosynthetic capacity (computed as both O_2 production and electrons flowing in the electron transport chain) was significantly lower in the herbicide-resistant than in the wild-type cells, for both herbicides and in the three species (Table 1; Fig. 6), although the inhibition of NPR_{max} was higher in chlorophyceans than in *M. aeruginosa*. These results are consistent with the lower growth rates of herbicide-resistant cells compared to wild-type cells, and with the lower reduction of growth rate in *M. aeruginosa* compared to chlorophyceans. This clearly shows that the selection of new genetic variants that provide resistance to herbicides have a physiological cost, as was suggested for the adaptation to other stressors (Bañares-España et al., 2016; Fernández-Arjona et al., 2013; García-Villada et al., 2004). It is known that herbicide resistance can be caused by rapid adaptation events (Baselga-Cervera et al., 2016; Fugère et al., 2020; Kreiner et al., 2018). For example, it has been observed that a single-site mutation in the EPSPS enzyme is the basis of glyphosate resistance both in several microorganisms (Funke et al., 2006) and weeds (Healy-Fried et al., 2007, Sammons and Gaines, 2014), although more complex genetic changes have been recently observed in resistant plants (Chen et al., 2016; Malone et al., 2016; Patterson et al., 2018). In this sense, we can hypothesize that the resistance of the phytoplanktonic species analyzed to high levels of glyphosate could be also related with a mutation of the EPSPS gene. Regarding diuron, resistance could be possibly due to mutations on *psbA* gene. Previous studies have shown that the resistance of microalgae to herbicides which inhibit PSII is due to a mutation that alters the binding coding between protein D1 and diuron (Dupraz et al.,

2016; Erickson et al., 1989, 1985; Galloway and Mets, 1984; Oettmeier, 1999). The reduction on ETR_{max} in diuron-resistant cells of all species was always higher than the decrement observed in glyphosate-resistant cells, which could be explained because diuron-resistance could rely on the alteration of the PSII structure, as suggested previously. In fact, the same result was observed with F_v/F_m, the maximum efficiency of PSII, in chlorophyceans but, surprisingly, we did not observe a reduction in F_v/F_m in *M. aeruginosa* in diuron-resistant cells. We observed a reduction in NPR_{max} and ETR_{max} in glyphosate-resistant cells of the two green microalgae but not in *M. aeruginosa*. Consequently, glyphosate resistance of *M. aeruginosa* could be linked to the maintaining of the operation of PSII and the electron transport chain, as it has been observed that glyphosate affects these processes negatively (Smedbol et al., 2017). However, non-target site mutations could also be involved in herbicide resistance (Kreiner et al., 2018) as, among others, those related with the function of efflux pumps, present in both procaryotic and eucaryotic organisms, that have been invoked for the resistance to different chemical stressors (Blanco et al., 2016). This could lead to cross-herbicide resistance, as observed in *C. reinhardtii* (Lagator et al., 2013; Vogwill et al., 2012).

5. Conclusions

The present study has demonstrated that phytoplankton organisms could adapt and survive in environments contaminated by tested herbicides (depending on the rate of deterioration and concentration) by rapid evolutionary processes. It must be taken into account that this study was performed with a single strain per species but, in nature, many strains coexist, that differ genetically and, consequently, increasing the probability that natural selection occurs. Moreover, it is known that the high phenotypic variation observed in several traits in *M. aeruginosa* is mostly explained by genetics (Bañares-España et al., 2007, 2006; López-Rodas et al., 2007; Rico et al., 2006), that is, this organism shows a high broad-sense heritability for some traits (Falconer and Mackay, 1996). The same fact could be expected for microalgae, which also present sexual reproduction. However, resistant cells will suffer a physiological cost and, consequently, phytoplankton community structure could change, and primary production in aquatic ecosystems could be altered affecting the whole trophic food-web by cascade effects (Reul et al., 2014).

Author contribution

IJM-J performed the experiments, analyzed the data, wrote the paper, prepared figures and tables, and reviewed drafts of the paper; AR and EB-E conceived and designed the experiments, analyzed the data and reviewed drafts of the paper; AF-M and MJG-S conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper and reviewed drafts of the paper.

Formatting of funding sources

This work was financially supported by the project CGL2017-87314-P (Ministerio de Economía, Industria y Competitividad, Spain).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was financially supported by the project CGL2017-87314-P (Ministerio de Economía, Industria y Competitividad, Spain). The acquisition of the FlowCAM by the University of Málaga was co-financed by the 2008-2011 FEDER program for scientific-technique infrastructure (Proposal number: UNMA08-1E005). Funding for open access charge: Universidad de Málaga/CBUA. Dr. Eric C. Henry kindly revised the English style and usage.

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