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Genetic Adaptation of Phytoplankters to Herbicides

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1. Introduction

One of the most important unwanted effects of herbicides is the arising of resistant non-target organisms. In fact, weeds which are resistant to almost all kinds of herbicides have been documented and the number of resistant variants is continuously increasing (http://www.weedscience.org/In.asp). Since phytoplankters (including cyanobacteria and eukaryotic microalgae) are responsible of the highest fraction of primary production in aquatic ecosystems (Falkowski & Raven, 1997), they must be highlighted among non-target herbicide organisms (Koenig, 2001). Unrelenting application of herbicides during recent decades has resulted in water pollution, with serious environmental implications and evolutionary consequences due to strong selection pressure on numerous species (Belfiore & Anderson, 2001). The problem is especially relevant in those freshwater habitats which are close to agricultural areas; these habitats are usually sinks for a large array of herbicides, so that phytoplankters are exposed to a multitude of these toxic compounds (Junghans et al., 2006). In fact, it is considered that herbicides are among the most significant human-synthesized pollutants in aquatic ecosystems (Koenig, 2001). Moreover, has been proposed that the emergence of unpredictable novelties could be a distinctive feature of the future biosphere (Tilman, 1999; Myers & Knoll 2001; Palumbi, 2001) and, consequently, the arising of resistant-herbicide phytoplankters could be considered as one of the relevant examples of human-driven selection.

The majority of studies on the effects of herbicides on phytoplankters have been focused on the degree of tolerance to the herbicides (Shehata et al., 1997; Berard et al., 1999; Kasai, 1999; Nelson et al., 1999; revised by Koenig 2001; Pinckney et al., 2002). However, adaptation includes different processes which are not usually discriminated; in particular, adaptation conferring resistance to herbicides can be achieved by three processes differing in some particular aspects (Fig. 1).

Under toxic but sub-lethal doses of herbicides, adaptation could be supported by modification of gene expression occurring in a short time (days to weeks) and within one organism's lifetime (i.e. physiological adaptation, also called acclimatization; Bradshaw & Hardwick, 1989); however, some evolutionary studies in bacteria (Cairns et al., 1988; Foster, 2000; Roth et al., 2006) and yeasts (Heidenreich, 2007) have suggested that adaptive mutations could be a process resembling Lamarckism which, in the absence of lethal selection, produces mutations that relieve selective pressure. Finally, under lethal doses of

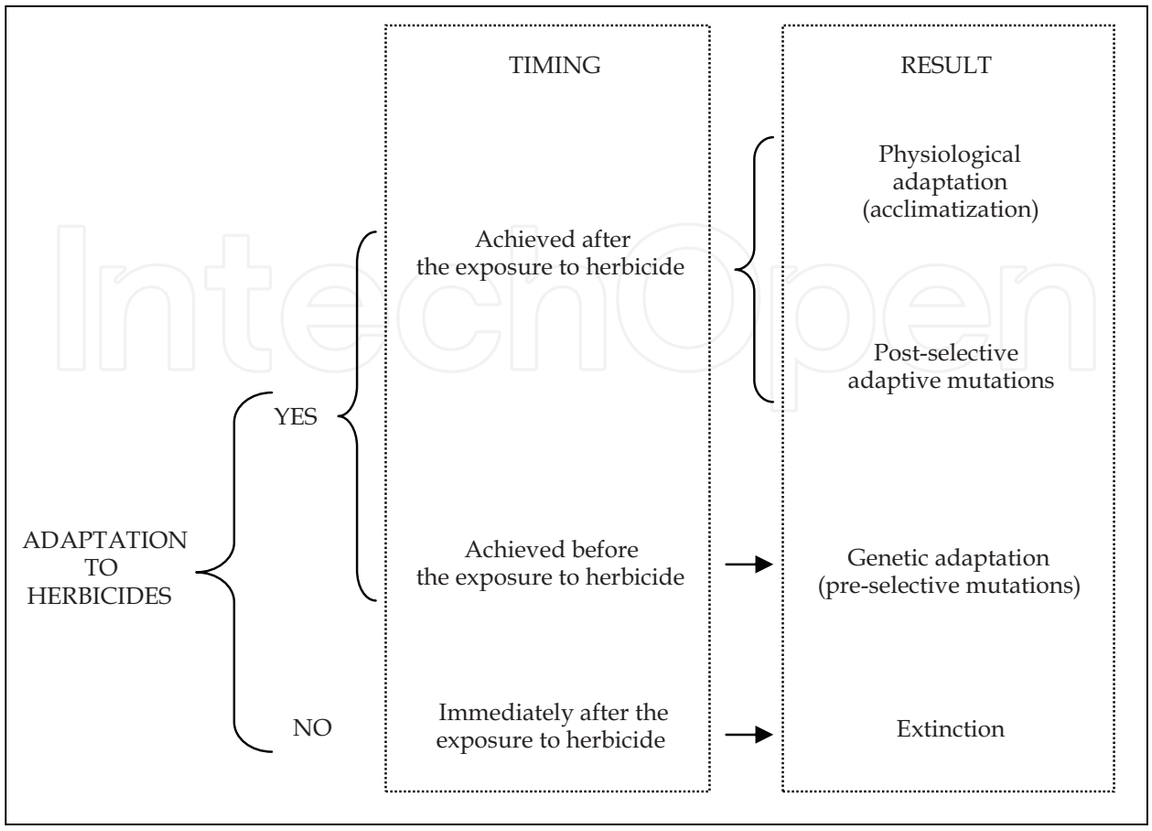

Fig. 1. Adaptation strategies of phytoplankton in herbicide-polluted waters (adapted from Marvá et al., 2010)

herbicides, genetic adaptation supported by selection of new genetic variants originated by spontaneous mutation (Sniegowski & Lenski, 1995; Belfiore & Anderson 2001; Orr, 2005) is the only possibility. Although it is assumed that this last mechanism is responsible for the arising of herbicide-resistant organisms, the empirical evidences are scarce. Thus, the discrimination of mechanisms involved in adaptation of phytoplankters to herbicides was addressed in our framework which is focused on evolutionary genetic of adaptation to anthropogenic pollution. For this purpose, we applied rigorous experimental techniques as an approach to evolutionary ecotoxicology of phytoplankters.

Here we review the studies which demonstrate the arising of new genetic-variants in phytoplankters under lethal doses of herbicides, as well as the highest concentrations of herbicides that allow genetic adaptation (an estimator of the limits of adaptation). It must be taken into account that the arising of herbicide-resistant phytoplankters is an example of adaptive evolution (Sniegowski & Lenski, 1995; Palumbi, 2001) and this allows us to hypothesize about future performance of phytoplankton communities in aquatic ecosystems.

2. Demonstrating genetic adaptation to herbicides: fluctuation analysis

2.1 Fluctuation analysis of the transformation herbicide-sensitive → herbicide-resistance

The experimental procedure called fluctuation analysis (Luria & Delbrück, 1943) is the best way to demonstrate if the adaptation to lethal doses of herbicides could take place in wild-

strains of phytoplankters and, secondly, to discriminate between acquired adaptations in response to the herbicides (by acclimatization or putative adaptive mutations) and resistant cells arising from rare spontaneous mutations that appear prior to the herbicide exposure (Fig. 1). A modified fluctuation analysis for application to liquid cultures with phytoplankters (Costas et al., 2001; López-Rodas et al., 2001) has been used to investigate the origin of herbicide-resistant cells. The modification of the analysis involves the use of liquid medium containing the selective agent (different kind of herbicides) rather than plating on a solid medium, as was done by Luria & Delbrück (1943) with bacterial cultures.

Two different sets of experimental cultures are prepared. In the first set (set 1), ca. 100 culture flasks, containing non-selective culture medium, are inoculated with $N_0 = 10^1$-10^2 cells (a number small enough to reasonably ensure the absence of pre-existing mutants in the strain). When each culture reaches $N_t = 10^5$-10^6 cells, it is supplemented with a lethal dose of herbicide. The dose is previously calculated from a dose-growth rate relationship, and it is selected a dose 2-4 times higher than that originating the 100% inhibition on growth rate. For set 2 (set control), 25-50 aliquots of 10^6-10^8 cells from the same parental population are separately transferred to culture flasks containing fresh liquid medium with the herbicide at the same concentration as set 1 cultures.

In order to maintain lethal doses of herbicides in the cultures, they are centrifuged to form a pellet of cells in the tube, the medium is decanted and fresh liquid medium with the herbicide is added each 5 d. All cultures are kept under selective conditions and observed after 90 d, a period of time long enough to allow resistant cells to grow. At the end of the experiments, the number of resistant cells in both sets is counted.

Two different results can be found in the set 1 experiment when conducting a fluctuation analysis, each result being interpreted as the independent consequence of different phenomena of adaptation (Luria & Delbrück, 1943; Jones et al., 1994). In the first case, if resistant cells arose during the exposure to the herbicides (i.e. by acclimatization or putative adaptive mutations), the variance in the number of cells per culture would be low because every cell is likely to have the same chance of developing resistance (Fig. 2, set 1A). Consequently, inter-culture (flask-to-flask) variation would be consistent with the Poisson model (i.e. variance/mean ≈1). By contrast, if resistant cells arose before the exposure to the herbicides (i.e. genetic adaptation by rare spontaneous mutation occurring during the time in which the cultures grew to N_t from N_0 cells before the exposure to herbicides), a high variation in the inter-culture number of resistant cells per culture would be found (Fig. 2, set 1B). Consequently, the flask-to-flask variation would not be consistent with the Poisson model (i.e. variance/mean >1). Obviously, another result (0 resistant cells in each culture) could also be found, indicating that neither selection on spontaneous mutations that occur prior to herbicides exposure, nor specific adaptation during the exposure to the herbicides, took place.

The set 2 cultures are the experimental controls of the fluctuation analysis (Fig. 2). Variance is expected to be low, because set 2 samples the variance of the parental population. If the variance/mean ratio of set 1 is significantly greater than the variance/mean ratio of set 2 (fluctuation), this confirms that resistant cells arose by rare mutations that occurred before exposure to the herbicide. If a similar variance/mean ratio between set 1 and set 2 is found, it confirms that resistant cells arose during the exposure to the herbicide.

In addition, the fluctuation analysis allows estimation of the rate of appearance of resistant cells. There are different approaches for accomplishing this estimation (Rosche & Foster,

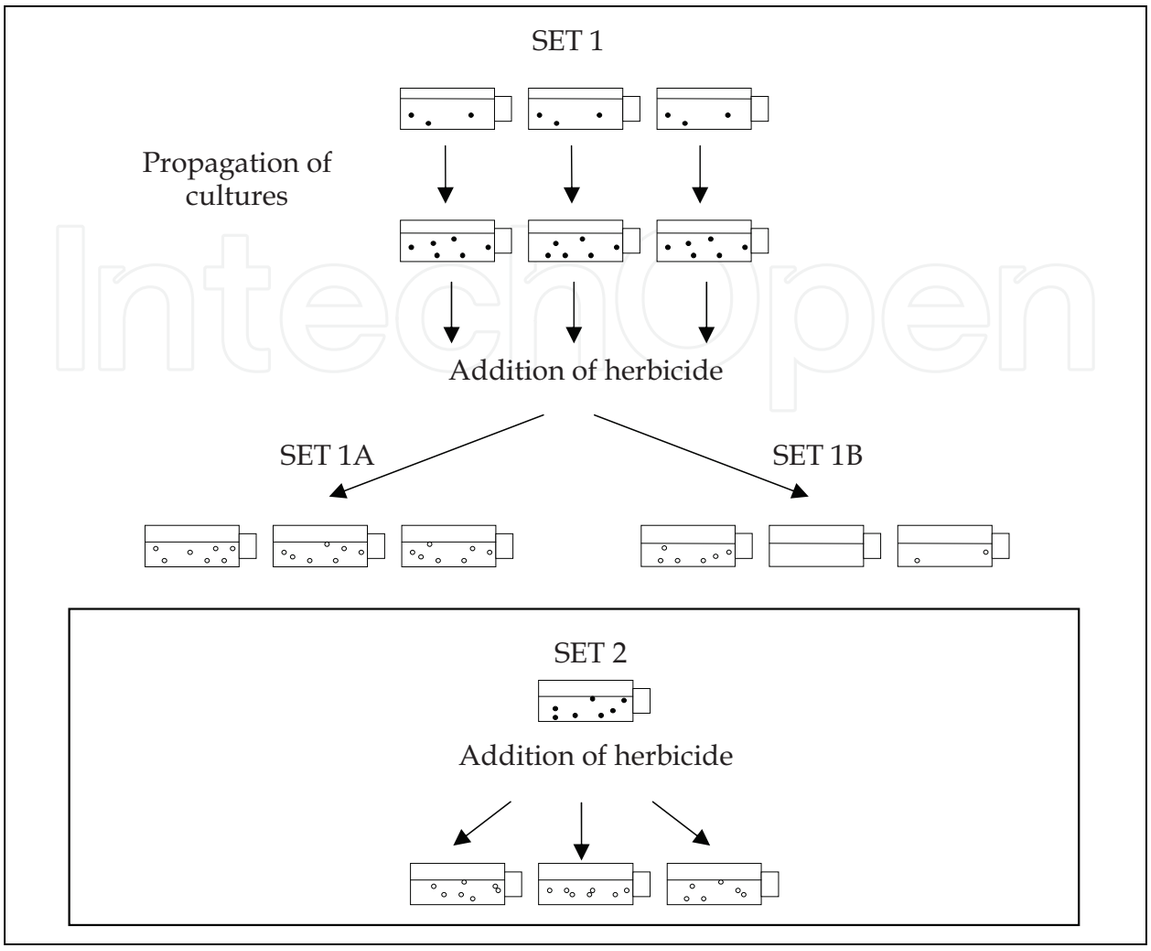

Fig. 2. Schematic diagram of the modified Luria & Delbrück (1943) fluctuation analysis. In the set 1, several cultures each inoculated with small inoculums were propagated until a high cell density was reached, and then a lethal dose of herbicide was added. If resistant cells arose by acclimatization or post-adaptive mutations, the number of resistant cells in all the cultures must be similar (set 1A). If adaptation is achieved by rare mutations (see Fig. 1) occurring in the period of the propagation of cultures the difference of the number of resistant cells in each culture must be huge (set 1B). Set 2 samples the variance of parental populations as an experimental control (adapted from Marvá et al. 2010)

2000). Due to methodological limitations imposed by a fluctuation analysis using liquid cultures, the proportion of cultures from set 1 showing no resistant cells (P_0 estimator; Luria & Delbrück, 1943) can be used to calculate the mutation rate (μ) by using the equation:

$$\mu = -\text{Log}_e P_0 / (N_t - N_0) \quad (1)$$

2.2 Mutation-selection equilibrium

If the mutation from wild-type, herbicide-sensitive allele to herbicide-resistant allele is recurrent and, in addition, the herbicide-resistant allele is detrimental in growth rate in the absence of herbicides, most of these mutants are eliminated sooner or later by natural selection, if not by chance. At any one time, there will be a certain number of cells that are

not yet eliminated. The average number of such mutants (q, frequency of the herbicide resistant allele) will be determined by the balance between μ and the rate of selective elimination, in accordance with the equation from Kimura & Maruyama (1966):

$$q = \mu / (\mu + s) \quad (2)$$

where s is the coefficient of selection, calculated as:

$$s = 1 - (m_S{}^r / m_S{}^s) \quad (3)$$

where m^r and m^s are the acclimated maximal growth rates of herbicide-resistance and herbicide-sensitive cells measured in non-selective conditions, respectively.

3. Example cases of genetic adaptation of phytoplankters to herbicides

Numerous resistant mutants to herbicides have been generated or selected in cyanobacteria and microalgae (Astier et al., 1979; Erikson et al., 1984; Golden & Haselkorn, 1985; Johanningmeier & Hallick, 1987; Chamovitz et al., 1991; Trebst et al., 1993; Singh & Singh, 1997; revised by Koenig, 2001). However, the empirical evidence of the arising of herbicide resistant-mutants in phytoplankters from wild, sensitive-cells by spontaneous mutations has only been addressed recently by using fluctuation analysis (Table 1).

In particular, when phytoplankton cultures were exposed to herbicides, they became clear after some days due to total growth inhibition and subsequent massive destruction of the cells by the lethal effect of herbicides. But after being further incubated a few cultures became green again, due to the growth of variants that were resistant to the herbicides. By using the fluctuation analysis, we demonstrated that resistance in different species of phytoplankters was due to the arising of new genetic variants caused by rare spontaneous mutation occurring randomly during propagation under non-selective conditions for the herbicides DCMU, glyphosate, simazine and diquat (Costas et al., 2001; López-Rodas et al., 2001, 2007; Marvá et al., 2010; see Table 1). Studies on adaptation to other types of herbicides are being carried out at the present.

Species	Herbicide	μ (mutants per cell per division)	q (no. of resistant cells per wild-type cell)	Ref.
Cyanobacteria				
Pseudanabaena sp.	DCMU	2.4×10^{-6}	6×10^{-4}	1
Microcystis aeruginosa	Glyphostate	3.6×10^{-7}	65×10^{-4}	2
Chlorophyta				
Dictyosphaerium chlorelloides	DCMU	2.1×10^{-6}	21×10^{-4}	3
Dunaliella tertiolecta	DCMU	3.6×10^{-6}	21×10^{-4}	1
Scenedesmus intermedius	Simazine	3.0 to 9.2×10^{-6}	11 to 30×10^{-6}	4
S. intermedius	Diquat	1.8×10^{-5}	83×10^{-6}	4

Ref., reference : 1, López-Rodas et al. (2001); 2, López-Rodas et al. (2007); 3, Costas et al. (2001); 4, Marvá et al. (2010)

Table 1. Mutation rate (μ) and mutation-selection balance (q) of phytoplankters in the genetic adaptation to different herbicides

Table 1 shows the figures of mutation rate from herbicide-sensitivity to herbicide-resistance in different species of phytoplankters (ranging from 0.4 to 17.9 mutants per 10^6 cells per generation, depending of the species and the herbicide). Since mutation is recurrent in each generation, new mutant cells are arising continuously. One of the main characteristics of the herbicide-resistant mutants, in comparison to the wild, herbicide-sensitive organisms is that the former have significantly lower growth rate than the latter, as reflected by the *q* figures in Table 1. It must be taking into account that mutations usually imply an energetic cost that may affect the survival of adapting populations (Coustau et al., 2000; Vila-Auib et al., 2009). Consequently, most of resistant-mutants are eventually eliminated by natural selection (Crow & Kimura, 1970). At any given time, the balance between the continuous appearance of mutants and their selective elimination determines the number of remaining herbicide-resistant mutants in algal populations growing in the absence of herbicides. This may be the case in wild-type populations developing in non-polluted waters. Consequently, the population would be predominantly a clone line of herbicide-sensitive genotypes, accompanied by, as a very small fraction, clone lines of herbicide-resistant mutants.
Summarizing, rare spontaneous mutations conferring resistance against herbicide seems to be enough to assure survival of phytoplankters in herbicide-polluted waters. Moreover, in a hypothetical future scenario with herbicide-polluted waters, the primary production supported by phytoplankters could be significantly lower that in the present, as a consequence of the diminished growth rate of resistant mutants in comparison to wild-type cells.

4. Testing the limits of genetic adaptation: ratchet protocol

4.1 Theoretical and experimental setup

When genetic adaptation is found via fluctuation analysis, the adaptation is referred to a given lethal dose of herbicide (usually, 2-4 times higher than that originating the 100% inhibition on growth rate). However, the potential limit of adaptation to the highest concentration of the toxic is difficult to estimate via fluctuation analysis, since stronger selection pressures drastically reduce population size. This constraint can be overcome by performing experiments that include several values of selection pressure. To this end, Reboud et al. (2007) developed an experimental model aimed at evaluating the maximal potential for herbicide resistance evolution in the green microalga *Chlamydomonas*. This experiment was based on the use of different herbicide concentrations, which thereby constituted selection pressures. Furthermore, Orellana et al. (2008) provided a modified procedure that allowed for maximizing the occurrence of mutants in microalgae and their selection by applying variable selection pressures. A significant enhancement of this experimental procedure was achieved by using different replicates of each strain under each selection condition. This assures repeatability and it is referred as ratchet assays (Huertas et al. 2010). The protocol aims at reaching equilibrium between strong selection intensity, by means of ratcheting to increase herbicide dose, and at the maintenance of a population size large enough to increase probability of rare spontaneous mutations that confer adaptation. Cultures must be ratcheted only up to a dose that supports population growth. The experimental procedure is then applied in several independent replicates (Fig. 3).
During the initial phase, replicates of the control cultures containing growth medium and replicates of cultures for each of the initial doses of herbicide treatment are prepared. Each culture is transferred to the next concentration when the same net growth of the control

cultures is reached; cultures that do not present net growth are maintained at the same concentration. A new ratchet cycle is concluded each time that the control cultures are transferred. The experiment ends after several cycles with net growth occurring only in the control cultures. At this point the maximal capability of adaptation corresponds to the maximal concentration of the selective agent that presents net growth (Fig. 3).

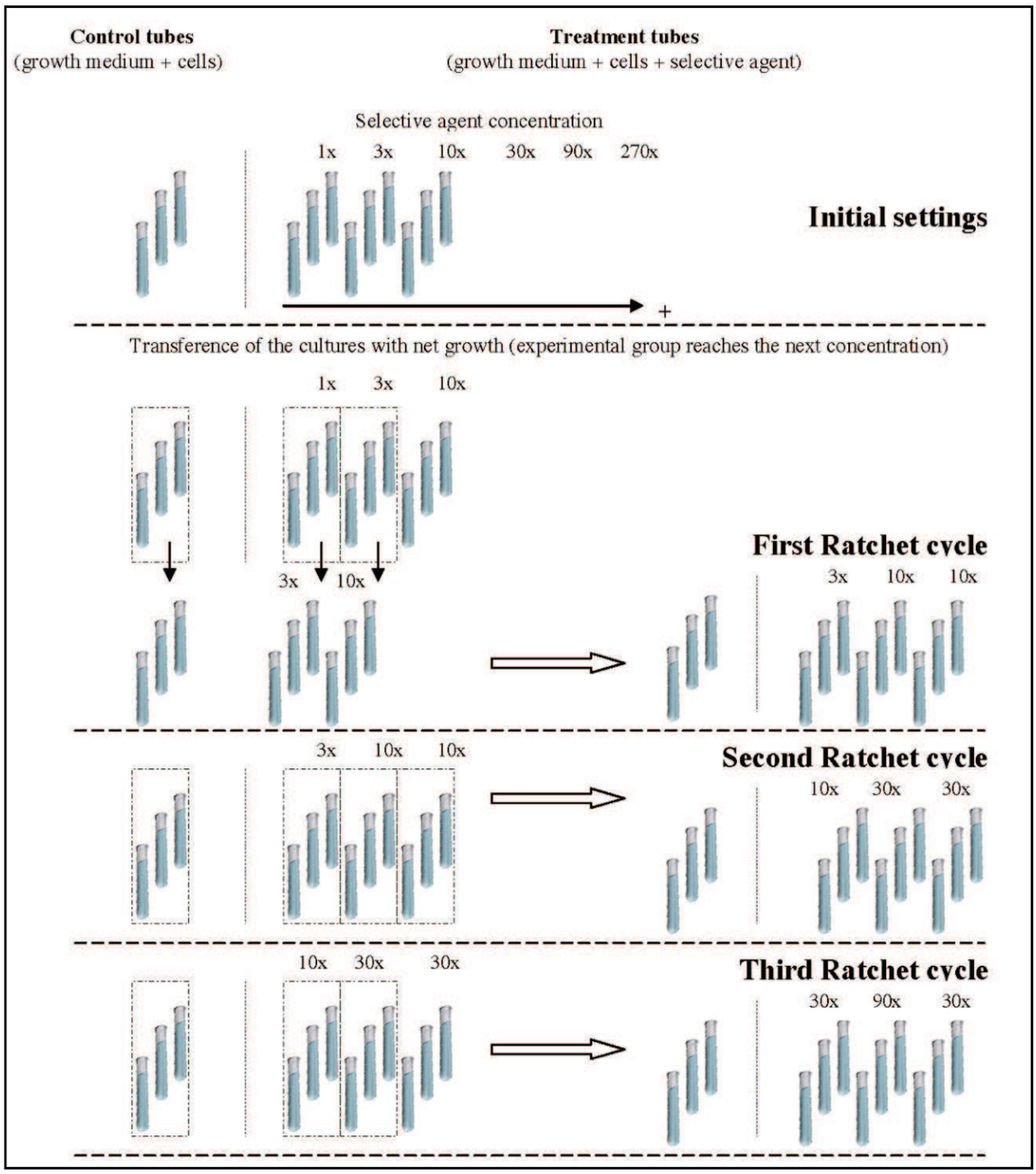

Fig. 3. Schematic representation of the ratchet experimental design (Huertas et al. 2010). Three ratchet cycles are represented but the experiment ends when net growth only occurs in controls after several ratchet cycles

4.2 Increase in adaptation to simazine in relation to concentration causing 100% growth inhibition

Huertas et al. (2010) showed differential maximal adaptation to simazine depending on taxonomic group, ploidy level (haploid and diploid vegetative cells occur in different taxonomical groups of microalgae), growth rate and habitat (Table 2).

In relation to the taxonomic group, Chlorophyta showed the greatest capacity to adapt to simazine, while Cyanobacteria did not adapt so well. It was suggested that the difference could result from the fact that prokaryotic organisms are more adversely affected by simazine than eukaryotic species (Fournadzhieva et al., 1995). Bacillariophyta and Haptophyta showed moderate and scarce ability to adapt, respectively.

Species	Adaptation increase (times)	Ploidy level	Cell division[1]	Habitat
Chlorophyta				
Scenedesmus intermedius	270	Haploid	Rapid	Continental
Dictyosphaerium chlorelloides	90	Haploid	Rapid	Continental
Tetraselmis suecica	10	Haploid	Rapid	Coastal
Cyanobacteria				
Microcystis aeruginosa (3D)	9	Haploid	Moderate	Continental
M. aeruginosa (6D)	9	Haploid	Moderate	Continental
M. aeruginosa (7D)	9	Haploid	Moderate	Continental
Bacillariophyta				
Phaeodactylum tricornutum	4.5	Diploid	Rapid	Coastal
Haptophyta				
Emiliania huxleyi (CCMP373)	3	Haploid	Slow	Oceanic
E. huxleyi (CCMP371)	1.5	Haploid	Slow	Oceanic
E. huxleyi (CCMP372)	1.5	Haploid	Slow	Oceanic
Isochrysis galbana	1.5	Haploid	Moderate	Oceanic
Monochrysis lutheri	1.5	Haploid	Moderate	Oceanic

[1]Cell division: rapid, 1 doubling every 3-4 d; moderate, 1 doubling every 4-5 d; slow, 1 doubling every 5-7 d

Table 2. Characteristics implicated in the capability of different phytoplankters to adapt to simazine. Adaptation increase column shows how much times simazine concentration causing 100% growth inhibition before the ratchet experiments is higher than that measured at the end of the experiment (data from Huertas et al., 2010)

The species with the greatest capacity for adaptation to simazine are haploid populations growing rapidly (Table 2). It is supposed that haploids will respond to selection more quickly than diploids because non-neutral mutations are expressed immediately. Moreover, growth rate is also involved in adaptation since a greater number of generations during a given time allows for more speedy adaptive evolution.

From an ecological point of view, it is very interesting to highlight that clear differences in adaptation to simazine were found depending on the habitat (Table 2). Thus, the greatest ability was found in phytoplankters from epicontinental freshwaters (usually, the sink of an array of herbicides), followed by coastal marine microalgae and, finally, the most sensitive

group was formed by oceanic microalgae (without prior exposure to simazine or other related compounds). Thus, it can be hypothesized that the capability of phytoplankters to adapt to simazine depends on a previous evolutionary history. In this way, a sudden contamination episode could be relieved by freshwater phytoplankters but not by oceanic phytoplankters.

5. Prospective

Genetic adaptation of phytoplankters to herbicides, as well as the limits of genetic adaptation, has been addressed in several species of phytoplankters representing some taxonomical and ecological groups. However, other species must be tested in order to have an extended view on the occurrence of genetic adaptation. Moreover, different herbicides are distinguished on the basis of their site and mode of action; in our framework, we tested the genetic adaptation of phytoplankters to a few of the different type of herbicides but other types will be addressed in the future.

6. Acknowledgments

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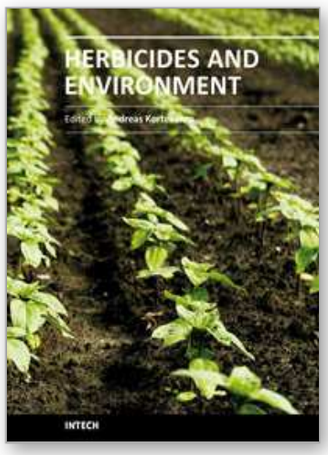

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Herbicides are much more than just weed killers. They may exhibit beneficial or adverse effects on other organisms. Given their toxicological, environmental but also agricultural relevance, herbicides are an interesting field of activity not only for scientists working in the field of agriculture. It seems that the investigation of herbicide-induced effects on weeds, crop plants, ecosystems, microorganisms, and higher organism requires a multidisciplinary approach. Some important aspects regarding the multisided impacts of herbicides on the living world are highlighted in this book. I am sure that the readers will find a lot of helpful information, even if they are only slightly interested in the topic.

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