

Atrazine induced changes in elemental and biochemical composition and nitrate reductase activity in *Chlamydomonas reinhardtii*

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Abstract

Herbicides play an important role in agricultural practices but the introduction of these compounds into the aquatic environment can have severe consequences for non-target organisms such as microalgae. The ubiquitous green freshwater microalga *Chlamydomonas reinhardtii*, a model species in all aspects of microalgal physiology, was used to assess the toxicity of atrazine, one of the most widely used herbicides throughout the world. Atrazine acts on photosynthesis and therefore can affect non-target primary producers, such as microalgae.

Growth, dry weight, elemental composition, photosynthetic pigments and protein contents and nitrate reductase activity were studied. After 96 h of exposure to different atrazine concentrations all the parameters studied were affected, but different sensitivities to the herbicide were shown. Nitrate reductase (NR) activity was strongly affected even at an atrazine concentration that did not affect

growth (0.1 μM); the lowest concentrations of atrazine assayed (0.1 and 0.25 μM) provoked a > 40% decrease in NR activity and NR decreased > 80% with atrazine concentrations of 0.5 μM . C/N ratio was also affected by all the atrazine concentrations assayed. Nitrate reductase activity and C/N ratio were better indicators of the cellular stress state than data on other biochemical components or growth rate. Among cell parameters assayed, the NR activity stood out as a sensitive cytotoxicity endpoint and the activity of this enzyme can be suggested as a sensitive biomarker of stress induced by atrazine in *C. reinhardtii*.

Key words: Atrazine, *Chlamydomonas reinhardtii*; Nitrate reductase; Toxicity

INTRODUCTION

Anthropogenic activities have resulted in the increasing release of toxic contaminants into aquatic ecosystems, including herbicides. Herbicides play an important role in agricultural practices but they can enter aquatic ecosystems from agricultural runoff or leaching, and consequently have become some of the most frequent organic contaminants in these environments (Villeneuve *et al.*, 2011). Increased herbicide use has elicited extensive research into their effects on non-target organisms such as microalgae. Their potential effect on aquatic primary producers is particularly important and has been studied in ecotoxicological experiments (Cairns & Niederlehner, 1995).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is one of the most widely used herbicides throughout the world, although it is banned in the European Union and in other European countries. Atrazine is also one of the most frequently detected herbicides in surface waters, even in the countries where it is banned (Na *et al.*, 2006; Battaglin *et al.*, 2009; Loos *et al.*, 2009). Atrazine acts on photosynthesis, displacing plastoquinone (QB) from its binding site in the D1 protein of photosystem II (PS II), i.e. its primary target is the inhibition of photosynthetic electron transport (Cremllyn, 1991; Rea *et al.*, 2009). This mode of action makes atrazine potentially toxic for a wide variety of non-target species of primary producers, including microalgae. In this way, atrazine has demonstrated the capacity to reduce primary productivity in aquatic ecosystems (Huber, 1993; Solomon *et al.*, 1996; DeLorenzo *et al.*, 2001). The action of toxic substances on microalgae is therefore not only important for the organisms themselves, but also for other levels in the food chain (DeLorenzo *et al.*, 2002; Rioboo *et al.*, 2007). Because of their short generation times, microalgae respond rapidly to environmental changes and, thus, may act as an indicator of impacts on higher organisms, which generally respond on longer time scales (McCormick & Cairns, 1994). Furthermore, microalgal tests are generally sensitive, rapid and low-cost effective (Sosak-Swidarska *et al.*, 1998);

indeed some standardized tests, focused on growth inhibition, are mandatory for the registration and notification of new chemicals, according to USEPA (2002) and OECD (2011) guidelines.

Algal responses to atrazine vary widely, depending on concentrations used, time of exposure, species tested and endpoints measured (Huber, 1993; Solomon et al., 1996; DeLorenzo et al., 2001). Sensitivity of algal cells to atrazine may be attributed to a number of factors, such as the uptake of atrazine, changes in pigment profile and photosynthetic capacity or algal cell size (Tang et al., 1998; DeLorenzo et al., 2004; Weiner et al., 2004). Among freshwater microalgae, chlorophytes seem to be the most sensitive to atrazine (Tang et al., 1997; Bérard et al., 2003; DeLorenzo et al., 2004; Weiner et al., 2004).

Besides growth, pigment content is the most commonly monitored parameter to assess the effects of toxicants on microalgae. However a very limited number of studies have reported on the effects of herbicides on other biochemical components, such as proteins which are fundamental cellular components. Nitrogen assimilation in phytoplankton plays a direct role in protein synthesis, and nitrate reductase (NR) is a crucial rate-limiting enzyme in nitrogen assimilation (Solomonson&Barber, 1990; Allen et al., 2006). The effect of atrazine on NR activity has been studied in land plants but there is not much literature dealing with the effect of atrazine on NR activity in microalgae. Nitrogen metabolism is not only one of the basic processes of plant and microalgal physiology but also part of the global chemical cycles.

Chlamydomonas reinhardtii is a freshwater green microalga (chlorophyte), ubiquitous and amenable to culture, which for decades served as a model in molecular biology and photosynthesis research (Harris, 2001). It has recently gained importance in ecotoxicology where it is used to analyse the effects of stressors, including various herbicides (Reboud, 2002; Fischer et al., 2010; Jamers & De Coen, 2010; Nestler et al., 2012).

In the present study, atrazine phytotoxicity assays were conducted in the laboratory on *C. reinhardtii*. Growth parameters and photosynthetic pigment content, frequently used in microalgal toxicity assays, were examined. Furthermore, other parameters related to nitrogen metabolism were also considered such as protein content, nitrate reductase activity and C/N ratio.

MATERIALS AND METHODS

Chemicals

All the chemicals used in the culture medium and in the analyses were purchased from Sigma Aldrich.

Microalgal cultures

Chlamydomonas reinhardtii Dangeard was obtained from the Culture Collection of Algae and Protozoa of Dunstaffnage Marine Laboratory (Scotland, UK) (strain CCAP 11/32A mt+) and was grown on sterile TAP (Tris-acetate phosphate) medium (Gorman & Levine, 1965; Harris, 2009) with 4mM NaNO₃ as the sole N source. Atrazine concentrations assayed were 0.1, 0.25, 0.5, 1 and 2 µM; cultures without atrazine were included as a control. Atrazine stock solution (5 mM) was prepared by dissolving the granulated herbicide (Sigma; MW: 215.68) in methanol. Final methanol concentrations for each treatment did not exceed 0.05% (v/v), and no measurable effects on growth or on the other parameters assayed were observed when this concentration of methanol was tested in the absence of herbicide. The exact concentration of atrazine in each culture was confirmed by gas chromatography/mass spectrometry analysis (Thermo Finnigan Polaris Q). The chromatographic column used was J&W, DB-XLB 60 m × 0.25 mm, with helium as carrier gas at a flow of 1 ml min⁻¹. The column temperature was programmed from 40°C (5 min) rising at 40°C min⁻¹ to 200°C, rising at 30°C min⁻¹ to 300°C (10 min). The instrument was operated in SIR mode. No significant differences between nominal and effective concentration were found. The same method was applied to confirm the stability of the atrazine concentrations over the course of a 96 h experiment without presence of the microalga.

All cultures were carried out in Pyrex glass bottles containing 450 ml of medium. Initial cell density for each experiment was 4 × 10⁵ cells ml⁻¹. Microalgal cells in the early exponential growth phase were used as inoculum for the assays. Microalgal cultures were maintained at 18 ± 1°C, with an incident irradiance of 68.25 µmol photon m² s⁻¹, with a dark:light cycle of 12:12 h, and continuously aerated at a rate of 10 l min⁻¹. All cultures were carried out in triplicate for 96 h.

Growth measurements

Growth of microalgal cultures was measured daily by microscopic counting of culture aliquots in a Neubauer hemocytometer using an Eclipse E400 microscope (Nikon, Japan). Growth rates, (μ), expressed in day⁻¹, were calculated using the following formula:

$$\mu = [\ln(N_t) - \ln(N_0)] / \ln 2 (t - t_0)$$

where N_t is the cell density (cells ml⁻¹) at time t (expressed in days) and N_0 is the cell density at time 0.

One of the most common parameters used in toxicity assays is the IC₅₀ (Leboulanger et al., 2001), i.e. the concentration of the tested substance that decreases the growth by 50%. IC₅₀ values at 48 h and 96 h were calculated using non-linear regression analysis (four parameters sigmoidal) of transformed herbicide concentration as natural logarithm data versus percentage of growth inhibition.

The regression analysis was performed using the regression Wizard software (Sigma-Plot 12.5, SPSS Inc.).

Dry weight and elemental composition

The dry weight was determined in triplicate according to Utting (1985) Culture aliquots (10 ml) were filtered through oven dried (80°C for 72 h) and weighed Whatman GF/C filters.

Samples for elemental composition were taken by centrifugation of aliquots of the cultures at 3500 *g* at 4°C for 15 min in an Heraeus Multifuge centrifuge and frozen at -70°C prior to lyophilization for 24 h. Carbon and nitrogen content were obtained with an elemental analyser (Carlo Erba CHNS-O 1108).

Biochemical composition

Samples for biochemical analysis were always collected at the same time in the light period because biochemical composition of microalgal cells can vary depending on the moment of the light period in which sample is taken (Ganf *et al.*,1986). All determinations were made in triplicate. *Chlamydomonas reinhardtii* were collected by centrifugation (3500 *g* at 4°C for 15 min in an Heraeus Multifuge centrifuge) and photosynthetic pigments were determined spectrophotometrically from 90% acetone extracts using the formula from Jeffrey & Humphrey (1975) for chlorophylls *a* and *b* and the equation of Parsons & Strickland (1965) for total carotenoids.

Hydrosoluble protein content was measured in the crude extracts obtained after breaking microalgal cells in an ultrasonic disintegrator. After sonication at 4°C, the extracts were centrifuged, the pellets discarded and protein content was measured in the supernatant by the Coomassie dye-binding method (Bradford, 1976) using BSA as standard.

Nitrate reductase activity

Nitrate reductase activity was determined using an *in situ* assay protocol based on the colorimetric determination of nitrite formed after enzymatic reduction of nitrate with NADH as electron donor for the nitrate reductase activity (Barea & Cárdenas, 1975; Berges & Harrison, 1995). Colorimetric determination of nitrite was carried out according to Snell & Snell (1949). Enzymatic activity was expressed as specific activity, in units of enzymatic activity per milligram of protein, taking into account that one unit of enzymatic activity catalyses the conversion of 1 μmol of substrate to product per minute.

Data analysis

Analyses were replicated 3 times for each culture, and mean and standard deviation (SD) values were calculated. To determine significant differences among test concentrations, data were statistically analysed by an analysis of variance (ANOVA) using SPSS 21.0 software. A p -value < 0.05 was considered statistically significant. When significant differences were observed, means were compared using the multiple comparison Tukey test at a level of significance of 0.05 ($p < 0.05$).

RESULTS

Growth

Atrazine affected the growth of the freshwater microalga *C. reinhardtii* and this negative effect on growth was concentration-dependent (Fig. 1, Table 1). All atrazine concentrations assayed, except for the lowest (0.1 μM), provoked a significant ($p < 0.05$) decrease in the growth rate of this microalga, in comparison with control cultures. The highest concentration assayed (2 μM) drastically inhibited the microalgal growth, with growth rates of 0 day^{-1} after 48 h and 96 h of exposure.

The concentration-response curves and their mathematical functions are shown in Fig. 2. The IC_{50} of atrazine for growth was 0.20 μM after 48 h of herbicide exposure and 0.27 μM after 96 h of atrazine exposure.

Table 1. Growth rate (GR) after 48 h and 96 h, and dry weight and carbon/nitrogen (C/N) ratio obtained in cultures of *C. reinhardtii* exposed to different atrazine concentrations after 96 h. Significant differences with respect to control ($p < 0.05$) are represented by an asterisk (*).

Atrazine μM	48 h GR (μ) (day^{-1})	96 h GR (μ) (day^{-1})	96 h Dry weight (pg cell^{-1})	96 h C/N
Control	1.51 \pm 0.17	1.13 \pm 0.05	29.32 \pm 0.71	5.01 \pm 0.04
0.1	1.67 \pm 0.01	1.19 \pm 0.01	28.21 \pm 2.12	4.87 \pm 0.03*
0.25	1.06 \pm 0.01*	1.01 \pm 0.01*	29.25 \pm 1.47	4.79 \pm 0.01*
0.5	0.46 \pm 0.14*	0.70 \pm 0.06*	37.75 \pm 1.29*	4.47 \pm 0.07*
1	0.04 \pm 0.15*	0.23 \pm 0.07*	68.55 \pm 1.59*	4.49 \pm 0.01*
2	0 \pm 0.02*	0 \pm 0.04*	73.30 \pm 2.06*	4.65 \pm 0.04*

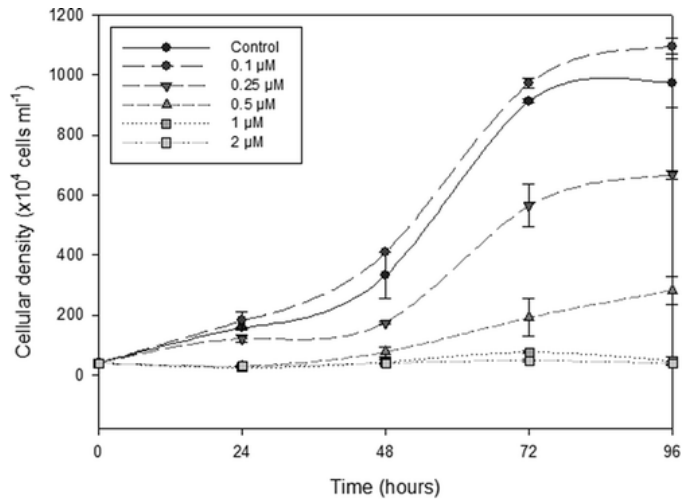


Fig. 1. Growth curves of cultures of *C. reinhardtii* exposed to different atrazine concentrations (μM). Data are given as mean values ($n = 3$) \pm standard deviation.

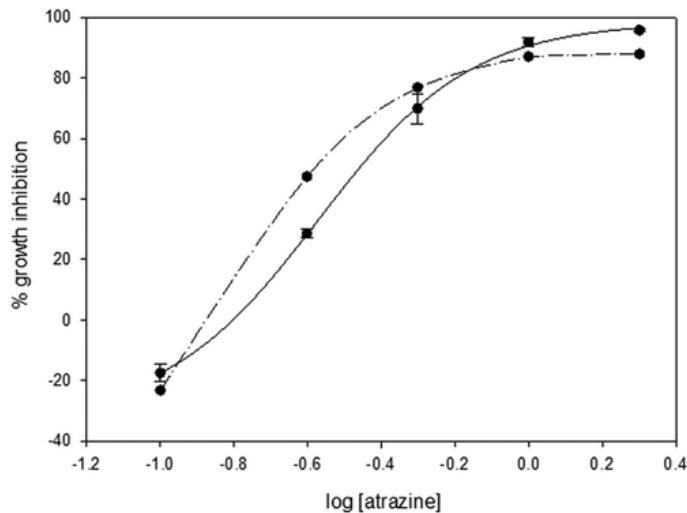


Fig. 2. Concentration–response curves represented by % growth inhibition vs. logarithm of different atrazine concentrations (μM) after 48 h (dashed line) and 96 h (continuous line) of herbicide exposure. 48 h: $y = 77.207/(1+\exp(-(x+0.6860)/0.0921))$ ($r = 0.9869$) $\text{IC}_{50} = 0.20$; 96 h: $y = 98.46/(1+\exp(-(x+0.5708)/0.2074))$ ($r = 0.99$) $\text{IC}_{50} = 0.26$.

Dry weight and elemental composition

Cellular dry weight (DW) after 96 h of atrazine exposure showed a significant ($p < 0.05$) increase in cultures exposed to atrazine concentrations of $0.5 \mu\text{M}$ or higher, with respect to the control cultures (Table 1), reaching a maximum value ($73.30 \pm 2.06 \text{ pg cell}^{-1}$) in those exposed to the highest atrazine concentration ($2 \mu\text{M}$); in these cultures, dry weight was 2.5 times the value obtained for control cells ($29.32 \pm 0.71 \text{ pg cell}^{-1}$).

Carbon and nitrogen percentages in dry biomass, determined after 96 h of culture, showed that the C/N ratio decreased significantly ($p < 0.05$) in all cultures exposed to atrazine with respect to the control (Table 1).

Biochemical composition

After 96 h of atrazine exposure, the photosynthetic pigment content in cells (chlorophyll *a*, chlorophyll *b* and total carotenoids) increased as the herbicide concentration increased (Fig. 3A), but differences were significant only in cells exposed to atrazine concentrations of 1 μM and 2 μM . However, when pigment content was expressed as μg per ml of culture (Fig. 3B), values decreased significantly when atrazine concentration increased in all the concentrations assayed.

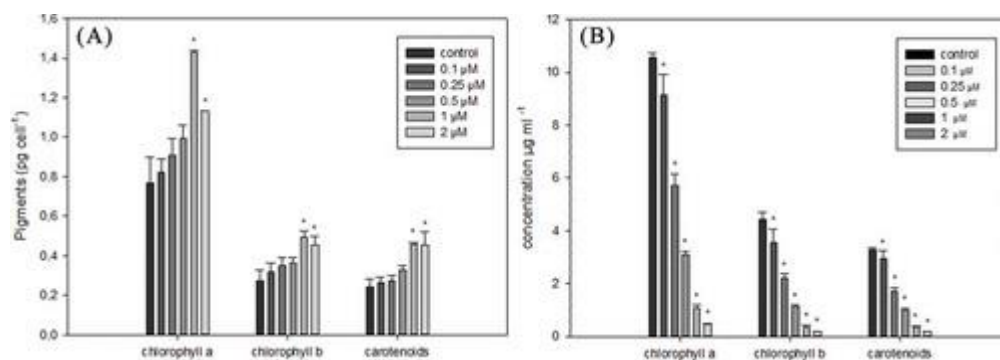


Fig. 3. Photosynthetic pigments (chlorophylls *a* and *b*, and total carotenoids), per cell (A) and per ml of culture (B), obtained from cultures of *C. reinhardtii* exposed to different atrazine concentrations (μM) during 96 h. Data are given as mean values ($n = 3$) \pm standard deviation. Significant differences with respect to control at a level of significance of 0.05 ($p < 0.05$) are represented by an asterisk (*).

Atrazine concentrations of 0.5 μM or higher produced a significant increase ($p < 0.05$) in the protein content of *C. reinhardtii* cells after 96 h of exposure (Fig. 4A). The maximum increase was at 2 μM atrazine with a protein content of $35.77 \pm 2.06 \text{ pg cell}^{-1}$, 2.2 times higher than that obtained in control ($16.22 \pm 1.00 \text{ pg cell}^{-1}$). Conversely, cultures exposed to atrazine concentrations $\geq 0.25 \mu\text{M}$ presented a significant decrease in their protein content expressed as μg per ml of culture (Fig. 4B).

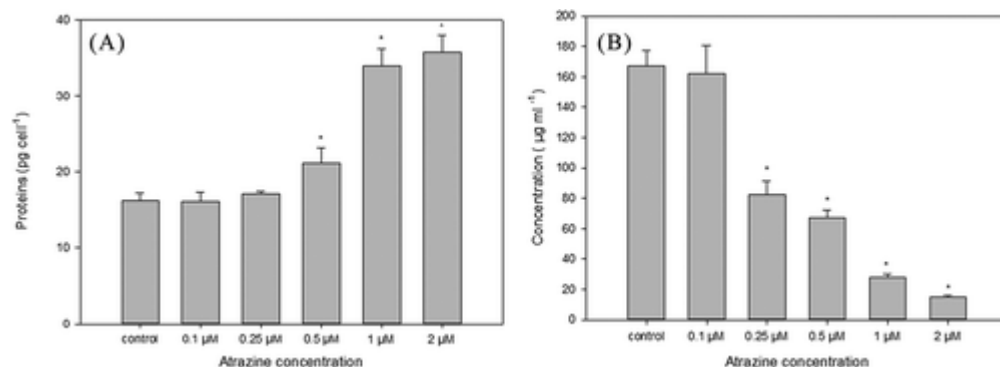


Fig. 4. Protein content, per cell (A) and per ml of culture (B), obtained from cultures of *C. reinhardtii* exposed to different atrazine concentrations (μM) during 96 h. Data are given as mean values ($n = 3$) \pm standard deviation. Significant differences with respect to control at a level of significance of 0.05 ($p < 0.05$) are represented by an asterisk (*).

Nitrate reductase activity

Nitrate reductase activity was significantly reduced in all cultures after 96 h of exposure to atrazine, in comparison with the control cultures which presented a NR activity of $65.06 \pm 0.25 \text{ U mg}^{-1} \text{ protein}$ (Fig. 5). The lowest concentrations of atrazine assayed (0.1 and 0.25 μM) provoked a decrease in NR activity $> 40\%$ and NR dropped $> 80\%$ with atrazine concentrations of 0.5 μM ($10.47 \pm 3.81 \text{ U mg}^{-1} \text{ protein}$). Nitrate reductase could not be detected in cultures exposed to the highest atrazine concentrations (1 and 2 μM).

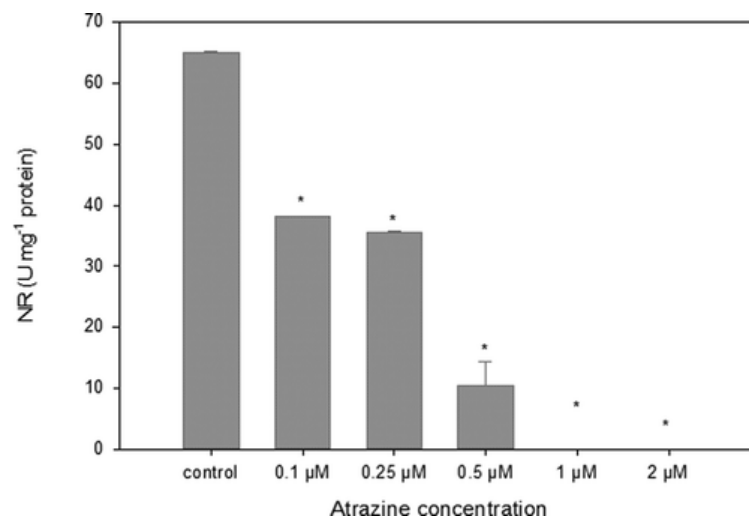


Fig. 5. Nitrate reductase activity ($\text{U mg}^{-1} \text{ protein}$) obtained from cultures of *C. reinhardtii* exposed to different atrazine concentrations, after 96 h of exposure. Data are given as mean values ($n = 3$) \pm standard deviation. Significant differences with respect to control at a level of significance of 0.05 ($p < 0.05$) are represented by an asterisk (*).

DISCUSSION

Chlamydomonas reinhardtii growth was affected by atrazine in a dose-dependent manner (Table 1, Fig. 1) as has been reported for other microalgal species (Solomon et al., 1996; Tang et al., 1997; Bérard et al., 2003; DeLorenzo et al., 2004; Weiner et al., 2004). Data reported about the effect of atrazine on *C. reinhardtii* have varied depending on time of exposure and the endpoint considered, although most IC_{50} values were determined using growth rate as endpoint (Schafer et al., 1994; Solomon et al., 1996; DeLorenzo et al., 2001). The IC_{50} value obtained for *C. reinhardtii* in the present work (0.27 μM) is similar to reported values and close to those for other freshwater green microalgae after the same duration of atrazine exposure: 0.33 μM for *Chlorella fusca* (Kotrikla et al., 1999); 0.26

μM for *Scenedesmus acutus* (Bérard *et al.*, 2003; 0.22 μM for *Pseudokirchneriella subcapitata* (previously *Selenastrum capricornutum*) (Weiner *et al.*, 2004). However, higher values have been reported for *Pseudokirchneriella subcapitata* (also known as *Raphidocelis subcapitata*): 0.53 μM (Bérard *et al.*, 2003) or 0.51 μM (Ma *et al.*, 2006). This species is used as a test organism in the OECD Guidelines for Testing Chemicals (2001).

Although it is highly relevant ecotoxicologically, inhibition of growth is a somewhat unspecific endpoint, because it can be caused by toxicant interference with a wide range of cellular processes (Nestler *et al.*, 2012). This limitation may be overcome by measuring other biochemical and physiological parameters. Therefore, parameters such as cellular dry weight, elemental composition, pigments and protein content, as well as nitrate reductase activity were considered.

The increase in cellular dry weight may be related to the growth inhibition observed in cultures exposed to high atrazine concentrations. Photosynthesis-inhibiting herbicides may alter the overall bioenergetics status of the organisms (Wilson *et al.*, 2000), leading to the uncoupling of cell growth and reproductive processes, and this is reflected in the increase in dry weight. This effect is in accordance with other results showing increases in cell volume or dry weight in different microalgae exposed to high concentrations of different contaminants (Cid *et al.*, 1996; Tang *et al.*, 1998; Prado *et al.*, 2009). This increase in cellular dry weight influences the remaining cellular parameters, i.e. pigment and protein content (Figs 3A, 4A). It has been suggested that due to the toxicity of pollutants microalgal cells are not able to complete cell division, but are still able to synthesize new cellular components (Gonzalez-Barreiro *et al.*, 2004; Geoffroy *et al.*, 2007).

The C/N ratio decreased as atrazine concentration increased (Table 1). The decrease in the C/N ratio in cultures exposed to high atrazine concentrations was fundamentally due to a strong decrease in C content in the dry biomass whereas N decrease was lower. Similar results were reported for *Chlorella vulgaris* exposed to the herbicides terbutryn and isoproturon (Rioboo *et al.*, 2002). As a result of the photosynthetic inhibition process, microalgal cells do not have enough energy for CO₂ fixation, this explains the C content decrease in the cultures after 96 h of atrazine exposure. Nitrogen metabolism is also affected by the overall bioenergetics status which affects NR activity. Thus, the C/N ratio is affected and can be considered as an indicator of the cellular stress state.

Photosystem II is the primary biochemical target of atrazine, which can explain the significant reduction of the photosynthetic pigments per ml of culture in *C. reinhardtii* exposed to the atrazine concentrations assayed (Fig. 3B). It is well known that photosynthetic pigments are affected by many common classes of herbicides (Moreland, 1980; Mayer *et al.*, 1997; Wakabayashi & Böger, 2004), provoking their loss (Rioboo *et al.*, 2002; Gonzalez-Barreiro *et al.*, 2004; Prado *et al.*, 2009; Nestler *et al.*, 2012). However, concentrations of herbicide that provoke an inhibition of microalgal growth

produced an increase in chlorophylls and carotenoids content per cell (Fig. 3A) which can be explained by the increase in the cellular dry weight, as previously mentioned.

Changes in protein content induced by exposure to high herbicide concentrations have been observed (DeLorenzo *et al.*, 2004; Gonzalez-Barreiro *et al.*, 2004; Prado *et al.*, 2009; Romero *et al.*, 2011). Glyphosate provoked an increase in protein content in *Chlorella kessleri* (Romero *et al.*, 2011), as occurred in *C. reinhardtii* exposed to atrazine; an increase in protein concentration per cell as a function of atrazine concentration was also reported in four estuarine microalgal species (DeLorenzo *et al.*, 2004). Likewise, the decrease in protein content expressed as a function of culture volume (Fig. 4B) is in accordance with other reports (Gonzalez-Barreiro *et al.*, 2004; Prado *et al.*, 2009) and with the impairment in NR activity (Fig. 5).

Enzyme inhibition measurements in microalgae are becoming increasingly popular indicators of environmental stress because they offer rapid and sensitive endpoints. Nitrate reductase is the main enzyme controlling nitrogen assimilation in plants and algae. It catalyses the first step in the assimilation of nitrate, namely reduction of nitrate to nitrite, by electron transfer from NAD(P)H (Kato *et al.*, 2006; Zalugin and Pick, 2014). The expression, stability and activity of NR are subject to complex regulations. The activation level of NR is influenced by environmental factors, including the presence of different pollutants (Mosulen *et al.*, 2003; Lillo *et al.*, 2004; Fayez & Abd-Elfattah, 2007; Prado *et al.*, 2009; Xie *et al.*, 2014). Results obtained with *C. reinhardtii* show that the NR activity is extremely sensitive to atrazine (Fig. 5). Although the effect of herbicides on NR has been studied in plants, there are not many data on this subject in microalgae, but decreases in NR due to exposure to different herbicides have been reported (Fayez & Abd-Elfattah, 2007; Prado *et al.*, 2009). However, NR activity was not significantly affected by the herbicide glufosinate in the marine diatom *Phaeodactylum tricorutum* (Xie *et al.*, 2014). Transcriptomic analyses carried out by RNA-Seq technique in *C. reinhardtii* cells exposed to atrazine have also shown a downregulation of NIT1 gene which encodes a nitrate reductase (Esperanza *et al.*, 2015).

Taking into account the mode of action of atrazine (Cremlyn, 1991; Rea *et al.*, 2009), blocking electron transport is expected to inhibit NR activity. This inhibition must be due to the lower availability of photosynthetically supplied electrons for the reduction of nitrate to nitrite. Moreover, nitrogen assimilation and carbon fixation are highly coordinated in unicellular algae (Giordano *et al.*, 2005); the expression of nitrate reductase is coupled to photosynthesis via a sensor related to the redox poise of the plastoquinone pool. When the pool is oxidized, carbon fixation is low and nitrate reductase is downregulated; conversely, when the pool is reduced, carbon fixation is high and the gene and enzyme activity are upregulated (Giordano *et al.*, 2005). Therefore, atrazine, a herbicide that interferes in the photosynthetic electron transport, decreases the photosynthetic carbon fixation and also interferes in enzymatic activities involved in nitrogen assimilation.

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AUTHOR CONTRIBUTIONS

A. Fernández-Naveira: performed the experiments; analysed the data; wrote the paper; revised the final version of the manuscript C. Rioboo: analysis of data; revised the final version of the manuscript A. Cid: original concept; revised the final version of the manuscript C. Herrero: original concept; analysis of data; revised the final version of the manuscript.

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the author(s).

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